

# World Journal of *Gastroenterology*

2013 Bound Volume 19 Issue 17-24: 2587-3914

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 May 7; 19(17): 2587-2730



Baishideng Publishing Group  
www.wjgnet.com

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 May 21; 19(19): 2841-2978



Baishideng Publishing Group  
www.wjgnet.com

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 May 28; 19(20): 2979-3172



Baishideng Publishing Group  
www.wjgnet.com

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 June 7; 19(21): 3173-3370



Baishideng Publishing Group  
www.wjgnet.com

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 June 14; 19(22): 3371-3530



Baishideng Publishing Group  
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ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

## World Journal of *Gastroenterology*

World J Gastroenterol 2013 June 28; 19(24): 3713-3914



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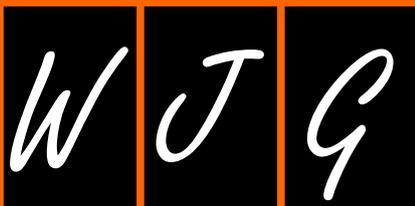
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# World Journal of *Gastroenterology*

*World J Gastroenterol* 2013 May 7; 19(17): 2587-2730



**FIELD OF VISION**

**2587** Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma

*Matsuda Y, Wakai T, Kubota M, Osawa M, Sanpei A, Fujimaki S*

**REVIEW**

**2591** Tumor necrosis factor- $\alpha$  inhibitor therapy and fetal risk: A systematic literature review

*Marchioni RM, Lichtenstein GR*

**2603** CD133: A cancer stem cells marker, is used in colorectal cancers

*Ren F, Sheng WQ, Du X*

**ORIGINAL ARTICLE**

**2612** Prevalence of celiac disease in Germany: A prospective follow-up study

*Kratzer W, Kibele M, Akinli A, Porzner M, Boehm BO, Koenig W, Oeztuerk S, Mason RA, Mao R, Haenle MH*

**2621** CYP24A1 inhibition facilitates the anti-tumor effect of vitamin D3 on colorectal cancer cells

*Kósa JP, Horváth P, Wöfling J, Kovács D, Balla B, Mátyus P, Horváth E, Speer G, Takács I, Nagy Z, Horváth H, Lakatos P*

**2629** Establishment of mouse intestinal myofibroblast cell lines

*Kawasaki H, Ohama T, Hori M, Sato K*

**2638** Expression of interleukin-22/STAT3 signaling pathway in ulcerative colitis and related carcinogenesis

*Yu LZ, Wang HY, Yang SP, Yuan ZP, Xu FY, Sun C, Shi RH*

**2650** Prognostic and survival analysis of 837 Chinese colorectal cancer patients

*Yuan Y, Li MD, Hu HG, Dong CX, Chen JQ, Li XF, Li JJ, Shen H*

**BRIEF ARTICLE**

**2660** Short- and long-term efficacy of endoscopic balloon dilation in Crohn's disease strictures

*de'Angelis N, Carra MC, Borrelli O, Bizzarri B, Vincenzi F, Fornaroli F, De Caro G, de'Angelis GL*

- 2668** Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia  
*Savarino E, Corbo M, Dulbecco P, Gemignani L, Giambruno E, Mastracci L, Grillo F, Savarino V*
- 2676** Clinical effects of adalimumab treatment with concomitant azathioprine in Japanese Crohn's disease patients  
*Ishida K, Inoue T, Fujiwara K, Sakanaka T, Narabayashi K, Nouda S, Okada T, Kakimoto K, Kuramoto T, Kawakami K, Abe Y, Takeuchi T, Murano M, Tokioka S, Umegaki E, Higuchi K*
- 2683** Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study  
*Wang ZJ, Ohnaka K, Morita M, Toyomura K, Kono S, Ueki T, Tanaka M, Kakeji Y, Maehara Y, Okamura T, Ikejiri K, Futami K, Maekawa T, Yasunami Y, Takenaka K, Ichimiya H, Terasaka R*
- 2691** Coinfection with hepatitis C virus and schistosomiasis: Fibrosis and treatment response  
*Abdel-Rahman M, El-Sayed M, El Raziky M, Elsharkawy A, El-Akel W, Ghoneim H, Khattab H, Esmat G*
- 2697** Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma  
*Tang P, Huang H, Chang J, Zhao GF, Lu ML, Wang Y*
- 2704** Effects of medical adhesives in prevention of complications after endoscopic submucosal dissection  
*Zhang Y, Chen Y, Qu CY, Zhou M, Ni QW, Xu LM*
- 2709** Difference in *DRB1*\* gene polymorphisms between Han and Uyghur ulcerative colitis patients in China  
*Aheman A, Gao F, Kuerbanjiang A, Li YX, Abuduhadeer M*
- CASE REPORT**
- 2714** Isolated arterioportal fistula presenting with variceal hemorrhage  
*Nookala A, Saberi B, Ter-Oganesyan R, Kanel G, Duong P, Saito T*
- 2718** Therapeutic efficacy of the Qing Dai in patients with intractable ulcerative colitis  
*Suzuki H, Kaneko T, Mizokami Y, Narasaka T, Endo S, Matsui H, Yanaka A, Hirayama A, Hyodo I*

- 2723** Accurate hemostasis with a new endoscopic overtube for emergency endoscopy  
*Mori H, Kobara H, Fujihara S, Nishiyama N, Oryu M, Rafiq K, Masaki T*
- 2727** Meckel's diverticulum bleeding diagnosed with magnetic resonance enterography: A case report  
*Zhou FR, Huang LY, Xie HZ*

**APPENDIX** I-VI Instructions to authors

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**INDEXING/ABSTRACTING** *World Journal of Gastroenterology* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16 951 (7/74); Current Articles: 677 (1/74); and Eigenfactor® Score: 0.06035 (5/74).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

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Weekly

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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 25/F, Lucky Plaza,  
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Fax: +852-65557188  
Telephone: +852-31779906  
E-mail: [bpgoffice@wjgnet.com](mailto:bpgoffice@wjgnet.com)  
<http://www.wjgnet.com>

**PUBLICATION DATE**  
May 7, 2013

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## Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma

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Received: January 23, 2013 Revised: March 10, 2013

Accepted: March 21, 2013

Published online: May 7, 2013

might be a good biomarker for HCC. In this article, we review recent studies of OTA, and discuss its possible significance as a biomarker of HCC.

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**Key words:** Hepatocellular carcinoma; Mycotoxin; Aflatoxin; Ochratoxin; High-performance liquid chromatography

**Core tip:** Mycotoxins are one of the possible important carcinogens of hepatocellular carcinoma (HCC). Recently, a chromatographic separation technique based on high-performance liquid chromatography (HPLC) has been recognized as a useful method for the quantitative analyses of mycotoxins in the sera of individuals. Using this technique, the serum levels of ochratoxin A, a type of food mycotoxin widely spread in cereals, has been recently reported to be increased in the sera of HCC patients in Egypt. HPLC-based analysis of mycotoxins in the clinical samples would provide some new epidemiological information about non-viral HCC.

### Abstract

Hepatocellular carcinoma (HCC) is a common malignant disease with poor prognosis. To improve the clinical outcome, early diagnosis of HCC arising from nonviral agents and hepatitis virus is important. Among several etiological factors, mycotoxins defined as carcinogens by the International Agency for Research in Cancer (IARC) might be one of the critical risk factors for nonviral HCC. Aflatoxin B1 is the most well-known carcinogenic mycotoxin for HCC, but the role of the other types of mycotoxin remains unclear. Several studies have reported that a chromatographic separation technique based on high-performance liquid chromatography can successfully detect the concentration of mycotoxins in plasma. Recently, serum level of ochratoxin A (OTA), a widely distributed mycotoxin classified as Group 2B by IARC, was evaluated in HCC patients in Egypt. The results suggested that serum OTA levels

Matsuda Y, Wakai T, Kubota M, Osawa M, Sanpei A, Fujimaki S. Mycotoxins are conventional and novel risk biomarkers for hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(17): 2587-2590 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2587.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2587>

### COMMENTARY ON HOT TOPICS

Hepatocellular carcinoma (HCC) is a common malignancy, with a high prevalence worldwide<sup>[1,2]</sup>. The prognosis of HCC has remained poor, because many of the patients also have chronic liver diseases and are not suitable for radical surgical treatment<sup>[1-3]</sup>. Another obstacle to HCC treatment is that hepatoma cells display strong

resistance against standard chemotherapeutic drugs<sup>[2,3]</sup>. To enable early diagnosis and treatment, understanding the etiological risk factors of HCC is desirable. Currently, approximately half of all HCC patients suffer from chronic hepatitis B and C virus (HBV and HCV) infection, and remaining cases are affected by various types of etiological factors including alcohol abuse, cigarette smoking, mycotoxins, obesity, and oral contraceptive drugs<sup>[4]</sup>. Intriguingly, growing evidence has suggested that the types of etiological risk factors for HCC might differ between geographic areas. For example, obesity-associated HCC has become one of the most important medical issues in developed countries<sup>[5]</sup>, while food contamination with mycotoxins remains a critical risk factor for HCC in developing countries, including South and East Africa, India and China<sup>[6]</sup>.

### **Mycotoxins as a major dietary risk factor for liver cancer**

In recent years, the relationship between cancer risk and mycotoxin has been universally publicized by global surveillance of food contamination. Mycotoxins are secondary metabolites produced by fungi and are present in various types of stored grains, and most of them are resistant against cooking, freezing and digestion after intake of contaminated food. Several food-contaminating mycotoxins have been defined as harmful carcinogens by the International Agency for Research in Cancer (IARC) (Table 1), that is, deoxynivalenol/nivalenol, zearalenone, ochratoxin, fumonisins and aflatoxins. Of these, aflatoxin B1 (AFB1) is the most well-known bioaccumulative toxin involved in the development of HCC<sup>[7]</sup>. AFB1 is produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*, and mainly contaminates improperly stored cereals and peanuts. When individuals are exposed to AFB1 for a long time, mono-oxygenases produce reactive epoxide in the liver, leading to formation of toxic derivatives with nucleic acids and proteins<sup>[8]</sup>. AFB1 has a strong mutagenic effect, and induces G to T transversion within codon 249 of the tumor suppressor *p53* gene. Point mutation of *p53* has been observed in approximately half of all patients with AFB1-associated HCC<sup>[9-11]</sup>. It has also been reported that geographical distribution of aflatoxin exposure and HBV infection overlap, leading to a synergistic effect on the genetic mutation of *p53*<sup>[10,11]</sup>. AFB1 is now regarded as the representative of orally ingested carcinogens, and has been classified as a Group 1 carcinogen by IARC (IARC 7th Annual Report on Carcinogens, 1987).

### **Ochratoxin A: A possible diagnostic marker of HCC?**

Based on the clinical evidence of AFB1 in HCC, several researchers have turned their attention to the other type of mycotoxins. Ochratoxin A (OTA), which has been classified as a possible human carcinogen (Group 2B) by the IARC, is a secondary metabolite of *Aspergillus* and *Penicillium* fungi. OTA is widely spread in cereals such as barley, wheat, coffee and bread<sup>[12]</sup>, and is well known for its possible contribution to nephritic diseases. Although the evidence is still open to debate, several studies have

**Table 1 Classification of food mycotoxins as human carcinogens or potential human carcinogens**

| Group | Classification of food mycotoxins                               |
|-------|---|
| 1     | Aflatoxin B1, B2, G1, G2  |
| 2A    | -   |
| 2B    | Aflatoxin M1, ochratoxin A, sterigmatocystin                    |
| 3     | Citrinin, patulin, luteoskyrin, cyclochlorotine, deoxynivalenol |
| 4     | -   |

Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans; Group 4: Probably not carcinogenic to humans (classified by the International Agency for Research in Cancer).

reported that OTA might be a causative agent of Balkan endemic nephropathy<sup>[13]</sup>. Moreover, OTA is increased to high levels in the plasma of patients with nephropathy in specific regions such as Tunisia<sup>[14]</sup>, suggesting that OTA plays a critical role in the development of nephritic diseases.

Unfortunately, the role of OTA in hepatocarcinogenesis remains unclear. Although the results of relevant studies are controversial, several have suggested that the carcinogenic effect of OTA is due to increased hepatotoxicity and DNA damage. Ehrlich *et al.*<sup>[15]</sup> have tested the genotoxic effect of OTA in human hepatoma HepG2 cells using both micronucleus and single-cell gel electrophoresis assays, and have found that it causes pronounced dose-dependent effects on DNA damage. Renzulli *et al.*<sup>[16]</sup> have reported that OTA induces DNA damage through oxidative stress, and this could be prevented by rosmarinic acid, a natural phenolic compound contained in many Lamiaceae herbs. Bouaziz *et al.*<sup>[17]</sup> have reported that OTA triggers a p53- and caspase-dependent mitochondrial apoptotic pathway in HepG2 cells. In contrast, El Golli Bennour *et al.*<sup>[18]</sup> have reported that OTA does not induce significant reactive oxygen species generation in cultured HepG2 cells, but induces mitochondrial and caspase-dependent apoptotic cell death mediated by p53 transcription-independent activities. The aforementioned different and controversial studies suggest that etiological analysis of patients with HCC is indispensable for assessing the relationship between OTA and HCC.

Until recent decades, meta-analysis of etiological risk factors for carcinogenesis has been complicated because traditional methods for assessing toxin exposure were mainly performed by questionnaires or standard enzyme linked immunosorbent assay and these cannot assess minute quantities of environmental toxins. During the last decades, however, a chromatographic separation technique based on high-performance liquid chromatography (HPLC) has enabled us to assess the concentration of mycotoxins in clinical samples such as urine and plasma<sup>[19]</sup>. HPLC enables us to detect OTA at a low level of 0.005 ng/mL in plasma<sup>[20]</sup>, which is often less than the mean level in healthy individuals; therefore, this analytical method is preferable for evaluation of the

etiologic significance of OTA. For example, Grosso *et al.*<sup>[21]</sup> and Aslam *et al.*<sup>[22]</sup> have examined the levels of OTA in the serum of bladder cancer patients by HPLC, and have reported that OTA is unlikely to be a risk factor for bladder cancer in Tunisia and Karachi. di Giuseppe *et al.*<sup>[23]</sup> have examined serum OTA in patients in the Molise region in Italy, and have reported that the levels of OTA are significantly associated with C-reactive protein and cardiovascular risk score in men.

Until recently, there have been no studies investigating OTA in HCC patients using HPLC methods. Very recently, however, Ibrahim *et al.* performed a case-control study of HCC patients from 2010 to 2012 in Egypt, and found that OTA was frequently increased in the serum. They measured serum OTA in 39 HCC patients using HPLC and compared it with the level in healthy individuals in Egypt. The highest incidence of OTA was observed in the HCC group. The mean level of serum OTA in the HCC and normal groups was  $1.11 \pm 0.3$  ng/mL (0.129-10.93 ng/mL) and  $0.201 \pm 0.02$  ng/mL (0.005-0.50 ng/mL), respectively ( $P = 0.0002$ ). Multivariate analysis showed that serum OTA was an independent risk factor for HCC. The authors also found that OTA was well correlated with the levels of  $\alpha$ -fetoprotein, which is a major tumor marker for HCC. To date, to the best of our knowledge, there have been no studies regarding the etiologic relationship between OTA and HCC. Further studies on a large scale should be performed in different countries, and they might support the idea that mycotoxins, including OTA, are useful biomarkers of HCC.

### Summary

Recently, progress in the diagnosis and treatment of hepatitis virus infection has significantly improved the outcome of patients with HBV- and HCV-associated HCC. Early diagnosis of HCC arising in patients with nonviral disease, however, has been remained difficult. HPLC-based analysis of clinical samples might offer a useful tool for detecting minute concentrations of environmental toxins that have accumulated in the host. Although food contamination with mycotoxins has so far been recorded in specific geographic regions, it is conceivable that current advances in the transportation system could cause unexpected food contamination in a wide area. Therefore, to address whether OTA would be a real causal agent for HCC, assessment of the level of mycotoxins, including OTA, in clinical samples would be of value.

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**P- Reviewers** Chan SPL, Gao C, Su CW, Desbois-Mouthon C, Su C  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN



## Tumor necrosis factor- $\alpha$ inhibitor therapy and fetal risk: A systematic literature review

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Received: October 17, 2012 Revised: March 1, 2013

Accepted: March 15, 2013

Published online: May 7, 2013

### Abstract

Tumor necrosis factor- $\alpha$  inhibitors (anti-TNFs) are effective in the treatment of inflammatory bowel disease (IBD) recalcitrant to conventional medical therapy. As the peak incidence of IBD overlaps with the prime reproductive years, it is crucial to establish pharmacologic regimens for women of childbearing age that achieve effective disease control without posing significant fetal harm. A systematic literature review was performed to identify all human studies with birth outcomes data after maternal exposure to infliximab, adalimumab, or certolizumab pegol within 3 mo of conception or during any trimester of pregnancy. Live births, spontaneous abortions or stillbirths, preterm or premature births, low birth weight or small for gestational age infants, and congenital abnormalities were recorded. Fifty selected references identified 472 pregnancy exposures. The subsequent review includes general information regarding anti-TNF therapy in pregnancy followed by a summary of our findings. The benefits of biologic modalities in optimizing disease control during pregnancy

must be weighed against the potential toxicity of drug exposure on the developing fetus. Although promising overall, there is insufficient evidence to prove absolute safety for use of anti-TNFs during pregnancy given the limitations of available data and lack of controlled trials.

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**Key words:** Tumor necrosis factor- $\alpha$  inhibitors; Pregnancy; Congenital abnormalities; Safety; Infliximab; Adalimumab; Certolizumab

**Core tip:** A systematic literature review was performed to identify all human studies with birth outcomes data after maternal exposure to infliximab, adalimumab, or certolizumab pegol within 3 mo of conception or during any trimester of pregnancy. After systematic literature review investigating tumor necrosis factor- $\alpha$  inhibitor therapy and fetal risk, there is insufficient evidence to prove absolute safety for the use of biologics (specifically infliximab, adalimumab, and certolizumab pegol) during pregnancy.

Marchioni RM, Lichtenstein GR. Tumor necrosis factor- $\alpha$  inhibitor therapy and fetal risk: A systematic literature review. *World J Gastroenterol* 2013; 19(17): 2591-2602 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2591.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2591>

### INTRODUCTION

Inflammatory bowel disease (IBD) encompasses the diagnoses of Crohn's disease (CD) and ulcerative colitis (UC). These are chronic relapsing gastrointestinal illnesses that involve proinflammatory molecules. The onset of IBD has a bimodal distribution with a higher peak in the younger population aged 15-30 years; fifty percent of patients afflicted by IBD are diagnosed before the age

of 35<sup>[1]</sup>. Hence, the peak incidence for developing these conditions overlaps with the prime reproductive years<sup>[2,3]</sup>.

Effective control of IBD is essential during pregnancy. Active disease or disease flares have been associated with adverse obstetrical outcomes<sup>[4]</sup>. About 50% of the pregnancies in North America are unplanned, and less than half of females realize their pregnancy status by week four of gestation<sup>[5]</sup>. Inadvertent fetal exposure to medications during the crucial stages of organogenesis is thus possible and common. For these reasons, preconception discussions addressing risks and benefits of pharmacologic therapy during pregnancy are clinically warranted for all patients of childbearing potential.

The decision to pursue or maintain certain drug regimens throughout the prenatal and pregnancy periods may pose a significant challenge; the risks of disease activity must be weighed against the potential side effects of medical therapy. Untreated disease may create greater risks to a pregnancy than the drugs themselves<sup>[2]</sup>. Identifying the safest management strategy is crucial, as medication use during pregnancy impacts maternal disease activity, fetal development, and pregnancy outcomes.

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a pleiotropic cytokine that plays a role both in pregnancy and in the pathophysiology of inflammatory conditions including IBD. Mouse models have demonstrated that TNF- $\alpha$  is one of several cytokines bearing a potent regulatory effect on early development<sup>[6]</sup>. It controls cyclooxygenases that affect blastocyst implantation, vascular permeability of the endometrium, and uterine decidualization<sup>[7]</sup>. TNF- $\alpha$  also contributes to the process of labor by stimulating uterine contractions in conjunction with other inflammatory cytokines<sup>[8]</sup>. The production of TNF- $\alpha$  increases throughout pregnancy and reaches a peak at the onset of labor. High levels of TNF- $\alpha$  have been implicated in such pregnancy complications as infection and fetal growth retardation and have even been linked to early and unexplained spontaneous abortions<sup>[8,9]</sup>.

There is a characteristic abundance of gut inflammation in IBD originating *via* various mechanisms at the cellular and subcellular levels. TNF- $\alpha$  is a key cytokine in the development and perpetuation of this abnormal immune response<sup>[10]</sup>. Several studies support the heightened production of TNF- $\alpha$  in the intestinal mucosa of patients with CD, and the levels are increased in both inflamed and histologically normal mucosa<sup>[11-13]</sup>. Increased TNF- $\alpha$  has also been linked to such rheumatologic and dermatologic conditions as rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, and psoriasis.

TNF- $\alpha$  inhibitors (anti-TNFs) are drugs that block the action of TNF- $\alpha$  and neutralize its biologic effect. This class has demonstrated efficacy in controlling disease activity associated with various inflammatory conditions. Infliximab (IFX), adalimumab (ADA), and certolizumab pegol (CTZ) are three such synthetic antibodies available in the United States for the treatment of IBD. Of these, infliximab has been the most highly studied.

Recognizing the effects of maternal drug use on fetal

development is an important aspect of providing care to pregnant patients and women of childbearing age with IBD. There is limited data, though, pertaining to the safety of biologic agents when used during pregnancy. The United States Food and Drug Administration (FDA) lists anti-TNF agents as category B drugs<sup>[14-16]</sup> (category B specifies that animal studies do not indicate fetal risk and there are no controlled studies in women or that animal studies have demonstrated adverse effects but controlled studies in women have failed to demonstrate risk). A recent consensus statement declared anti-TNF agents to be low risk during certain stages of pregnancy<sup>[17]</sup>. Some case reports and small case series reporting anti-TNF exposure and pregnancy outcomes have been published. However, large population-based studies are sparse, and there is a lack of prospective data in pregnant women. In addition, there is a relatively short number of post-marketing years since the advent of biologics, thus narrowing the safety information pool even further. The increasing use of antibody-based therapeutics fosters the need for further study in this group of patients.

A systematic literature review was performed to investigate fetal risks associated with maternal exposure to TNF- $\alpha$  inhibitors (IFX, ADA, and CTZ) during pregnancy.

## SEARCH STRATEGY

The search strategy was developed with the assistance of a medical librarian. Databases searched included MEDLINE, EMBASE, SCOPUS, and BIOSIS Previews through November 2011 and were restricted to studies published in English and performed in humans. Structured searches were conducted using both medical subject heading terms and keyword/exploded terms as follows: (“congenital abnormalities” OR “congenital disorders” OR “pregnancy” OR “safety”) AND (“infliximab” OR “adalimumab” OR “certolizumab”). Titles and abstracts were screened for relevance; reference lists of the applicable publications were hand-searched to identify additional studies.

## ELIGIBILITY CRITERIA

Case reports, case series, or observational studies published in article or abstract form were eligible for inclusion if there was documented female exposure to IFX, ADA, or CTZ within three months of conception or during any trimester of pregnancy and if > one of the following birth outcomes was assessed: live births, spontaneous abortions (SA), stillbirths (SB), preterm or premature births (PTB/PMB), low birth weight (LBW)/small for gestational age (SGA), or congenital abnormalities (CA). Studies were excluded if there was insufficient detail to link specific anti-TNF exposure with birth outcomes. One investigator independently performed the searches described above and reviewed the citations (titles and abstracts) to determine eligibility. Discrepancies were

resolved by the second investigator.

## DATA EXTRACTION

A standardized form was used to abstract the following data points from each study: anti-TNF drug exposure, indication for anti-TNF agent, pregnancy stage(s) of exposure by trimester, live births, and birth outcomes as aforementioned. Spontaneous abortions were defined as fetal death at < 20 wk, stillbirths as fetal death at > 20 wk or at weight > 350-500 g if gestational age unknown, preterm deliveries as < 37 wk gestation, premature deliveries as < 37 wk gestation and prior to completion of organ development, and low birth weight newborns as < 2500 g. Small for gestational age infants were described by authors as smaller than average size given the number of pregnancy weeks.

## SEARCH RESULTS

The initial search yielded 11452 citations. Fifty studies (Table 1)<sup>[18-68]</sup> met inclusion criteria for full review, including 13 case series, 36 case reports, and 2 prospective studies with control groups. Reports in Table 1 are categorized by biologic agent and study type, and details of maternal anti-TNF exposures and pregnancy outcomes are presented.

The total number of patients exposed to anti-TNFs was 472 (IFX 194/ADA 261/CTZ 17). Table 2<sup>[69-73]</sup> displays anti-TNF exposures and birth outcomes for the following categories: live births, spontaneous abortions, stillbirths, preterm/premature births, low birth weight/small for gestational age, and congenital abnormalities. Outcomes in Table 2 have been listed by anti-TNF exposure (IFX, ADA, and CTZ) and indication (for all medical conditions and for IBD patients alone), and results are compared to the general United States population.

Table 3 summarizes the reported congenital abnormalities associated with live births (4.1%). Among 19 congenital anomalies (IFX 9/ADA 10/CTZ 0), no specific pattern of birth defects was identified<sup>[74-76]</sup>.

## DISCUSSION

We performed a systematic literature review to assess the risk of adverse birth outcomes after maternal exposure to IFX, ADA, or CTZ and identified 50 references with a total of 472 fetal exposures.

The subsequent discussion highlights each biologic agent in the context of pregnancy and provides a summary of our data.

### Infliximab

Infliximab (Remicade) is a human-murine chimeric monoclonal antibody that neutralizes the activity of TNF- $\alpha$ . It is composed of a human immunoglobulin G1 (IgG1) constant region and a murine variable region. Its efficacy in IBD has been documented in randomized

controlled trials in the treatment of moderate to severe CD refractory to conventional therapy as well as enterocutaneous fistulae<sup>[77,78]</sup>. The drug can reduce the need for corticosteroids and, in patients who respond to initial dosing, IFX is effective for the maintenance of response and prolonged remission in CD<sup>[79,80]</sup>.

IFX is classified by the United States FDA as pregnancy category B. Murine models show no evidence of teratogenicity or embryotoxicity. However, anti-TNF- $\alpha$  antibodies vary among species; data cannot simply be paralleled to human pregnancy outcomes. Infliximab does not cross-react with TNF- $\alpha$  in species other than humans and chimpanzees, and it has not been tested in animal reproduction studies<sup>[14]</sup>.

IFX is not thought to cross the placenta in the first trimester due to its human IgG1 constant region<sup>[81]</sup>, but this subclass is known to efficiently cross in the late second and third trimesters<sup>[26]</sup>. Given this timing, the infant is somewhat shielded from drug exposure during the critical period of organogenesis. IFX levels can be detected in newborns of exposed mothers, and the drug remains in the system for up to six months after delivery<sup>[19,50]</sup>. This bears important consequences in terms of newborn infection risks and vaccination responses<sup>[17]</sup>. Discontinuing infliximab in the third trimester is an option to decrease late placental transport to the newborn.

### Adalimumab

Adalimumab (Humira) is a fully human monoclonal IgG1 antibody against TNF- $\alpha$ . It has proven effective for inducing and maintaining remission in CD<sup>[82,83]</sup>, especially in those who have lost response to or have become intolerant of infliximab<sup>[84]</sup>.

ADA is classified as an FDA pregnancy category B drug. In an embryo-fetal perinatal developmental toxicity study, cynomolgus monkeys were administered ADA at extreme dosages of up to 100 mg/kg [266 times human area under the curve (AUC) when dispensed as 40 mg subcutaneously with methotrexate weekly or 373 times human AUC when dispensed as 40 mg subcutaneously without methotrexate]. No evidence of fetal harm due to ADA was recorded. Adequate and well-controlled studies have not been conducted in pregnant women. Again, animal reproduction and developmental studies are not always indicative of human response, and ADA must be used with caution in pregnancy<sup>[15]</sup>. There is no long-term data regarding effects of adalimumab on the developing fetus.

Less information exists on the transplacental diffusion of ADA throughout the trimesters compared to infliximab. Determining the time course of drug administration and when to potentially discontinue ADA during pregnancy is not well-defined due to shorter dosing intervals and limited ability to commercially measure ADA levels. Withholding the drug in the third trimester may be considered to reduce late placental transport to the newborn. Mahadevan *et al*<sup>[17]</sup> suggests discontinuation 8-10 wk prior to estimated date of delivery.

Table 1 Summary of reports of maternal exposure to anti-tumor necrosis factor agents during pregnancy

| Ref.                                      | Study type  | Disease                                    | Anti-TNF- $\alpha$ agent | Exposure to other drugs | Exposures in pregnancies with documented outcome | Maternal exposure: Pregnancy stage               | Live births (n) | SA/SB (n) | PTB/ PMB (n) | LBW/ SGA (n) | CA (n) | Pregnancy outcomes: Details/complications   |
|---|-------------|--|--------------------------|-------------------------|--|--|-----------------|-----------|--------------|--------------|--------|---|
| Chambers <i>et al.</i> <sup>[18]</sup>    | Prospective | RA   | IFX                      | NS                      | 4  | T1   | 3               | 1 SA      | 2            |              |        |   |
| Mahadevan <i>et al.</i> <sup>[19]</sup>   | Prospective | CD: (4)<br>UC: (1)                         | IFX                      | NS                      | 5  | T2/T3 other exposure details                     | 5               |           |              |              |        |   |
| Berthelot <i>et al.</i> <sup>[20]</sup>   | Case series | Rheumatologic disease                      | IFX                      | - <sup>1</sup>          | 3  | NS<br>C/T1: 1                                    | 3               |           |              |              |        |   |
| Chakravarty <i>et al.</i> <sup>[21]</sup> | Case series | RA   | IFX                      | Some pts                | 1  | C/T1/T2: 2<br>Pregnancy, not otherwise specified | 1               |           |              |              |        |   |
| Correia <i>et al.</i> <sup>[22]</sup>     | Case series | CD   | IFX                      | Yes: 1<br>No: 1         | 2  | C/T1/T2/T3                                       | 2               | 1         | 1            | 1            | 1      | 1 preterm/ premature birth due to placental detachment (31 wk, 1.6 kg with acute respiratory failure requiring mechanical ventilation $\times$ 24 h and intensive care $\times$ 40 d; healthy at 8 mo follow-up)                              |
| Hyrich <i>et al.</i> <sup>[23]</sup>      | Case series | Rheumatologic disease                      | IFX                      | Some pts                | 3  | C/T1   | 2               | 1 SA      |              |              |        |   |
| Kane <i>et al.</i> <sup>[24]</sup>        | Case series | CD   | IFX                      | Some pts                | 3  | T1/T2/T3: 2<br>T2/T3: 1                          | 3               |           | 1            |              |        |   |
| Katz <i>et al.</i> <sup>[25]</sup>        | Case series | CD: (82)<br>UC: (1)<br>RA: (8)<br>JRA: (2) | IFX                      | Some pts                | 100  | C: 53<br>T1: 30<br>> 3 mo prior to C: 7          | 68              | 10 SA     | 1            | 1            | 3      | CA (3):<br>1 full-term with tetralogy of Fallot<br>1 intestinal malrotation<br>1 developmental delay and hypothyroidism   |
| Mahadevan <i>et al.</i> <sup>[26]</sup>   | Case series | CD   | IFX                      | Some pts                | 10   | Unknown: 6                                       | 10              | 1 SB      | 3            | 1            |        | 1 complicated neonatal course:<br>Respiratory distress/ jaundice/ seizure. Mother was also exposed to several antibiotics for pulmonary and urinary infections, azathioprine, hydrocortisone, and total parental nutrition early in pregnancy |
| Rosner <i>et al.</i> <sup>[27]</sup>      | Case series | Rheumatologic disease                      | IFX                      | Yes                     | 3  | T1: 1<br>T3: 1                                   | 10              |           | 3            | 1            |        | Miscarriages (14):<br>10 SA<br>1 SB (mother exposed to leflunomide)<br>3 unknown type<br>1 neonatal jaundice (resolved)   |
| Schnitzler <i>et al.</i> <sup>[28]</sup>  | Case series | CD/UC/IC                                   | IFX                      | NS                      | 10   | C/T1/T2/T3: 8                                    | 9               | 1         | 2            | 1            |        | 1 complicated neonatal course: term delivery at 39 wk with respiratory distress/ desaturation/ gastric ulcer day 5; healthy at 6 mo follow-up<br>1 premature rupture of membranes   |

| Author   | Case series | NS                    | IFX | NS       | 25             | T1                              | 22 | 2        | 4  | 2                        | CA (2):  |
|--|-------------|-----------------------|-----|----------|----------------|---------------------------------|----|----------|----|--------------------------|--|
| Weber-Schoenderfer <i>et al.</i> <sup>[29]</sup> | Case series | NS                    | IFX | NS       | 4              | C/T1/T2:3<br>C/T1/T2/T3:1       | 4  | SA       | 1  | 1                        | 1 ventricular septal defect<br>1 growing hemangioma requiring therapy<br>CA (1):<br>L hand polydactyly   |
| Zelinkova <i>et al.</i> <sup>[30]</sup>          | Case series | CD: (3)<br>UC: (1)    | IFX | Some pts | 4              |                                 |    |          |    |                          | (Infant also had respiratory depression after anesthetics that resolved spontaneously. Mother was taking methotrexate 2 mo prior to conception without folic acid supplement.)                                   |
| Akinci <i>et al.</i> <sup>[31]</sup>             | Case report | Rheumatologic disease | IFX | Yes      | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Angelucci <i>et al.</i> <sup>[32]</sup>          | Case report | CD                    | IFX | Yes      | 1              | C/T1                            | 1  |          | 1  | 1                        | LBW  |
| Angelucci <i>et al.</i> <sup>[33]</sup>          | Case report | CD                    | IFX | Yes      | 1              | T1                              | 1  |          |    |                          |  |
| Antoni <i>et al.</i> <sup>[34]</sup>             | Case report | Psoriatic Arthritis   | IFX | NS       | 1              | C/T1                            | 1  |          |    |                          |  |
| Arai <i>et al.</i> <sup>[35]</sup>               | Case report | CD                    | IFX | Yes      | 1              | C/T1/T2                         | 1  |          |    |                          |  |
| Aratani <i>et al.</i> <sup>[36]</sup>            | Case report | CD                    | IFX | Yes      | 1              | T2                              | 1  |          | 1  |                          | SGA  |
| Burt <i>et al.</i> <sup>[37]</sup>               | Case report | CD                    | IFX | Yes      | 1              | C/T1                            | 1  |          | 1  |                          |  |
| Chaparro <i>et al.</i> <sup>[38]</sup>           | Case report | CD                    | IFX | NS       | 1              | C/T1/T2/T3                      | 1  |          | 1  |                          |  |
| Cheent <i>et al.</i> <sup>[39]</sup>             | Case report | CD                    | IFX | NS       | 1              | C/T1/T2/T3                      | 1  |          | 1  |                          | Infant developed disseminated BCG after vaccination at 3 mo and died at 4.5 mo   |
| Epping <i>et al.</i> <sup>[40]</sup>             | Case report | CD                    | IFX | Yes      | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Hou <i>et al.</i> <sup>[41]</sup>                | Case report | CD                    | IFX | NS       | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| James <i>et al.</i> <sup>[42]</sup>              | Case report | CD                    | IFX | Yes      | 1              | T2                              | 1  |          |    |                          |  |
| Kinder <i>et al.</i> <sup>[43]</sup>             | Case report | RA                    | IFX | Yes      | 1              | C/T1                            | 0  | 1        |    |                          |  |
| Østensen <i>et al.</i> <sup>[44]</sup>           | Case report | RA                    | IFX | Yes      | 1              | C/T1                            | 1  | SA       |    |                          | Oligohydramnios detected on 18 wk ultrasound that resolved with discontinuation of Nimesulide  |
| Puig <i>et al.</i> <sup>[45]</sup>               | Case report | Psoriasis             | IFX | Yes      | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Srinivasan <i>et al.</i> <sup>[46]</sup>         | Case report | CD                    | IFX | Yes      | 1 <sup>2</sup> | C/T1                            | 1  |          | 1  |                          | Preterm premature birth (24 wk) complicated by intracerebral and intrapulmonary hemorrhages and neonate died at 3 d<br>Mother was also exposed to metronidazole, azathioprine, and mesalamine for fistulizing CD |
| Steenholdt <i>et al.</i> <sup>[47]</sup>         | Case report | UC                    | IFX | Yes      | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Stengel <i>et al.</i> <sup>[48]</sup>            | Case report | CD                    | IFX | Yes      | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Tursi <i>et al.</i> <sup>[49]</sup>              | Case report | CD                    | IFX | NS       | 1              | C/T1/T2/T3                      | 1  |          | 1  |                          |  |
| Vasiliaskas <i>et al.</i> <sup>[50]</sup>        | Case report | CD                    | IFX | NS       | 1              | C/T1/T2/T3                      | 1  |          |    |                          |  |
| Wilbaux <i>et al.</i> <sup>[51]</sup>            | Case report | AS                    | IFX | NS       | 1              | C/T1                            | 1  |          |    |                          |  |
| Xirouchakis <i>et al.</i> <sup>[52]</sup>        | Case report | CD                    | IFX | Yes      | 1              | C/T1                            | 1  |          | 1  |                          | Preterm (29 wk) birth with neonatal hospitalization $\times$ 30 d post-delivery. Baby in "good condition" at follow-up<br>CA (7) (live births):<br>1 undescended testicle<br>1 microcephaly                      |
| Johnson <i>et al.</i> <sup>[53,54]</sup>         | Prospective | CD and RA             | ADA | NS       | 94             | T1<br>Other exposure details NS | 80 | 13<br>SA | 12 | 7<br>(among live births) | 1 congenital hip dysplasia with inguinal hernia<br>1 congenital hypothyroidism<br>1 ventricular septal defect  |

|   |             |                       |     |                |     |                                 |           |  |
|---|-------------|-----------------------|-----|----------------|-----|---------------------------------|-----------|--|
| Berthelot <i>et al</i> <sup>[20]</sup>          | Case series | Rheumatologic disease | ADA | <sup>3</sup> 2 | 2   | C/T1: 1<br>C/T1/T2/T3: 1        | 2         | 1 bicuspid aortic valve and agenesis of corpus callosum (twin sibling had patent ductus arteriosus)<br>1 congenital hydronephrosis   |
| Hyrich <i>et al</i> <sup>[23]</sup>             | Case series | Rheumatologic disease | ADA | Some pts       | 3   | C/T1                            | 2         | CA (9) (all pregnancies):<br>In addition to above 7 defects were:<br>1 spina bifida and hydrocephalus (resulted in elective termination)<br>1 ectopia cordis and caudal regression<br>(twin pregnancy resulting in a spontaneous abortion)   |
| Johnson <i>et al</i> <sup>[53,54]</sup>         | Case series | CD and RA             | ADA | NS             | 122 | T1<br>other exposure details NS | 5         | 5 CA (5):<br>2 chromosomal abnormalities<br>1 atrial septal defect and peripheral pulmonic stenosis<br>1 ventricular septal defect<br>1 congenital hip dysplasia<br>1 infant with autosomal dominant disease (not otherwise specified); paternal inheritance<br>Twin-to-twin transfusion syndrome (1 small due to discordance) |
| Weber-Schoenderfer <i>et al</i> <sup>[29]</sup> | Case series | NS                    | ADA | NS             | 28  | T1                              | 24        | 1 infant with autosomal dominant disease (not otherwise specified); paternal inheritance   |
| Abdul Wahab <i>et al</i> <sup>[55]</sup>        | Case report | CD                    | ADA | Yes            | 1   | C/T1/T2/T3                      | 2 (twins) | 1<br>SGA   |
| Ben-Horin <i>et al</i> <sup>[56]</sup>          | Case report | CD                    | ADA | NS             | 1   | C/T1/T2/T3                      | 1         |  |
| Bosworth <i>et al</i> <sup>[57]</sup>           | Case report | CD                    | ADA | Yes            | 1   | C/T1/T2/T3                      | 1         | 1  |
| Coburn <i>et al</i> <sup>[58]</sup>             | Case report | CD                    | ADA | Yes            | 1   | T2/T3                           | 1         |  |
| Dessinioti <i>et al</i> <sup>[59]</sup>         | Case report | Psoriasis             | ADA | NS             | 1   | C/T1                            | 1         | 1<br>LBW   |
| Jurgens <i>et al</i> <sup>[60]</sup>            | Case report | CD                    | ADA | NS             | 1   | C/T1                            | 1         |  |
| Kraemer <i>et al</i> <sup>[61]</sup>            | Case report | Takayasu's Arteritis  | ADA | Yes            | 1   | C/T1/T2/T3                      | 1         |  |
| Mishkin <i>et al</i> <sup>[62]</sup>            | Case report | CD                    | ADA | Yes            | 1   | C/T1/T2/T3                      | 1         |  |
| Roux <i>et al</i> <sup>[63]</sup>               | Case report | RA                    | ADA | NS             | 1   | C/T1                            | 1         | 1  |
| Vesga <i>et al</i> <sup>[64]</sup>              | Case report | CD                    | ADA | Yes            | 1   | C/T1/T2/T3                      | 1         |  |
| Witbaux <i>et al</i> <sup>[51]</sup>            | Case report | AS                    | ADA | Yes            | 1   | C/T1/T2<br>C/T1/T2              | 1         | 1<br>CA (1):<br>Primary craniostyostosis requiring surgery   |
| Kane <i>et al</i> <sup>[65]</sup>               | Case series | CD                    | CTZ | NS             | 14  | NS                              | 5         | 1<br>SGA   |
| Mahadevan <i>et al</i> <sup>[66]</sup>          | Case report | CD                    | CTZ | Yes            | 1   | T2/T3                           | 1         |  |
| Ousallah <i>et al</i> <sup>[67]</sup>           | Case report | CD                    | CTZ | NS             | 1   | C/T1/T3                         | 1         |  |
| Steinberg <i>et al</i> <sup>[68]</sup>          | Case report | CD                    | CTZ | Yes            | 1   | T2                              | 1         |  |

<sup>1</sup>Specified no exposure to disease-modifying antirheumatic drugs, methotrexate, or non-steroidal anti-inflammatory drugs; <sup>2</sup>Case reported by Srinivasan *et al*<sup>[61]</sup> was also documented by Katz *et al*<sup>[23]</sup>; <sup>3</sup>Specified no exposure to disease-modifying antirheumatic drugs, methotrexate, or non-steroidal anti-inflammatory drugs. TNF: Tumor necrosis factor; CD: Crohn's disease; UC: Ulcerative colitis; IC: Indeterminate colitis; RA: Rheumatoid arthritis; JRA: Juvenile rheumatoid arthritis; AS: Ankylosing spondylitis; IPX: Infliximab; ADA: Adalimumab; CTZ: Certolizumab pegol; NS: Not specified; Pts: Patients; C: Within < 3 mo prior to conception; T1: First trimester (LMP to 13 wk); T2: Second trimester (14 to 27 wk); T3: Third trimester (28 to 40 wk); SA: Spontaneous abortion; SB: Stillbirth; Preterm birth (< 37 wk gestation); PMB: Premature birth (< 37 wk gestation); LBW: Low birth weight (< 2500 g); SGA: Small for gestational age (smaller than average size given the number of weeks of pregnancy).

**Table 2 Summary of anti-tumor necrosis factor exposures and birth outcomes *n* (%)**

| Anti-TNF exposure  | Birth outcomes, <i>n</i> (with relative percents) |             |           |         |           |          |             |
|--|---|-------------|-----------|---------|-----------|----------|-------------|
|  | Fetal exposures                                   | Live births | SA        | SB      | PTB/ PMB  | LBW/SGA  | CA          |
| IFX/ADA/CTZ total  | 472   | 405 (85.8)  | 32 (8.2)  | 2 (0.6) | 41 (19.9) | 8 (6.1)  | 19 (4.1)    |
| IFX <sup>1</sup>   | 194   | 155 (79.9)  | 15 (10.6) | 2 (1.1) | 21 (26.9) | 5 (4.4)  | 6 (4.0)     |
| IFX in IBD <sup>2</sup>                                      | 151   | 117 (77.5)  | 11 (8.9)  | 2 (1.4) | 16 (36.4) | 5 (4.8)  | 4 (3.5)     |
| ADA <sup>1</sup>   | 261   | 242 (92.7)  | 16 (6.9)  | 0 (0.0) | 20 (15.9) | 2 (28.6) | 13 (5.4)    |
| ADA in IBD <sup>2</sup>                                      | 224   | 210 (93.8)  | 13 (5.8)  | 0 (0.0) | 15 (17.0) | 2 (28.6) | 12 (5.7)    |
| CTZ <sup>1</sup>   | 17  | 8 (47.1)    | 1 (5.9)   | 0 (0.0) | 0 (0.0)   | 1 (12.5) | 0 (0.0)     |
| CTZ in IBD <sup>2</sup>                                      | 17  | 8 (47.1)    | 1 (5.9)   | 0 (0.0) | 0 (0.0)   | 1 (12.5) | 0 (0.0)     |
| Outcome percents in general US population <sup>[69-73]</sup> |   | 64.60%      | 16.50%    | 0.60%   | 12.30%    | 8.20%    | 3.00%-5.00% |

<sup>1</sup>Exposure in all reported medical conditions; <sup>2</sup>Exposure in inflammatory bowel disease (IBD) patients. TNF: Tumor necrosis factor; IFX: Infliximab; ADA: Adalimumab; CTZ: Certolizumab pegol; SA: Spontaneous abortion (fetal death at < 20 wk); SB: Stillbirth (fetal death at > 20 wk or > 350 g if gestational age unknown); PTB: Preterm birth (< 37 wk gestation); PMB: Premature birth (< 37 wk gestation and prior to completion of organ development); LBW: Low birth weight (< 2500 g); SGA: Small for gestational age (smaller than average size given number of pregnancy weeks).

**Table 3 Summary of congenital abnormalities reported**

| Congenital abnormalities ( <i>n</i> = 19)             | Affected ( <i>n</i> ) | Anti-TNF exposure |
|---|-----------------------|-------------------|
| Ventricular septal defect                             | 3                     | IFX (1), ADA (2)  |
| Chromosomal abnormalities                             | 2                     | IFX               |
| Congenital hip dysplasia                              | 2                     | IFX (1), ADA (1)  |
| Intestinal malrotation                                | 1                     | IFX               |
| Congenital hypothyroidism                             | 1                     | IFX               |
| Hemangiomas   | 1                     | IFX               |
| L hand polydactyly                                    | 1                     | IFX               |
| Tetralogy of Fallot                                   | 1                     | IFX               |
| Patent ductus arteriosus                              | 1                     | ADA               |
| Atrial septal defect and peripheral pulmonic stenosis | 1                     | ADA               |
| Bicuspid aortic valve and agenesis of corpus callosum | 1                     | ADA               |
| Primary craniostenosis                                | 1                     | ADA               |
| Microcephaly  | 1                     | ADA               |
| Congenital hydronephrosis                             | 1                     | ADA               |
| Undescended testes                                    | 1                     | ADA               |

IFX: Infliximab; ADA: Adalimumab; TNF: Tumor necrosis factor.

### Certolizumab pegol

Certolizumab pegol (Cimzia) is a recombinant humanized anti-TNF- $\alpha$  fragment antigen binding (Fab') fragment. The antibody fragment is bound to a polyethylene glycol molecule that extends the drug's half-life to approximately two weeks in the plasma, thereby reducing dosing frequency<sup>[85]</sup>. Studies have demonstrated the efficacy of CTZ for induction and maintenance of remission in CD<sup>[86]</sup>.

CTZ is a pregnancy category B drug. It does not cross-react with mouse or rat TNF- $\alpha$ . Reproduction studies in rats have thus been performed using a rodent anti-murine TNF- $\alpha$  pegylated Fab' fragment (cTN3 PF) that is similar in function to CTZ. These studies have been conducted using doses up to 100 mg/kg and have revealed no evidence of impaired fertility or fetal adversities due to cTN3 PF. Adequate and well-controlled studies have not been performed in pregnant women. As animal reproduction studies are not always indicative of human response, this drug must be used with caution in pregnancy<sup>[16]</sup>.

The molecular structure of CTZ lacks an Fc portion, so its cross-placental transfer is different from that of IFX and ADA. The Fab' fragment may passively cross the placenta in low levels during the first trimester, an event that is not expected with the IgG1 antibody. Although CTZ therapy would likely not need to be discontinued in the third trimester, it is important to recognize that the transplacental transfer of this drug occurs during a critical period of organogenesis in the first trimester.

In an animal model, pregnant rats received a murinized IgG1 TNF- $\alpha$  antibody and a PEGylated Fab' fragment of the antibody. Lower levels of the drug were detected in the infant and in breast milk with the Fab' fragment versus the full antibody<sup>[87]</sup>. Mahadevan *et al*<sup>[66]</sup> demonstrated these findings in two human patients receiving certolizumab during pregnancy. The drug was administered to both women two weeks prior to delivery. Although the mothers' drug levels were higher on the date of delivery, newborn cord blood levels were low.

There are few published reports on the use of CTZ during pregnancy. As with the other anti-TNF agents, it is possible that the Fab' fragment passively crosses the placenta at low levels in the first trimester. The drug must be further studied in humans to fully appreciate the course of drug transfer during gestation and subsequent effects on fetal development and pregnancy outcomes.

### SUMMARY OF DATA

Our review indicates that rates of SA and CA in anti-TNF-exposed patients are similar to rates in the general United States population<sup>[69-73]</sup> and in women with IBD unexposed to anti-TNF agents<sup>[74-76]</sup>. The live birth rate in the anti-TNF-exposed group (85.8%) is higher than that of the general United States population (64.6%); this holds true for all patients exposed to IFX or ADA regardless of underlying inflammatory disease and perhaps reflects a state of controlled disease activity. The live birth rate for patients exposed to CTZ (47.1%) is lower than that of the general population, although there is a very small collective sample size. The rates of SA and

SB for all groups are similar to the general United States population<sup>[72]</sup> with the exception of IFX-exposed patients, in whom the rate of SB is just slightly higher. The PTB/PMB rate in the anti-TNF-exposed group (19.9%) is higher than in the United States population (12.3%)<sup>[72]</sup>, perhaps due to an underlying predisposition as in the setting of IBD<sup>[76]</sup>. LBW/SGA infants are more common in ADA- and CTZ-exposed patients than in the general United States population<sup>[73]</sup>, again possibly reflecting the underlying disease itself or the severity of disease activity.

In general, pregnancy does not increase the risk of disease exacerbation in CD or UC<sup>[88,89]</sup>. Approximately one-third of women with inactive IBD at the time of conception are expected to flare during pregnancy and the puerperium<sup>[90]</sup>. Alternatively, if pregnancy overlaps with a period of active IBD, the disease may be difficult to control<sup>[91]</sup>. Active disease at the time of conception has been associated with increased rates of PTB<sup>[89]</sup> and fetal loss<sup>[92]</sup>, and disease flares during pregnancy have been associated with PTB and LBW<sup>[4,93]</sup>. Studies are mixed regarding the risk of congenital malformations among IBD progeny, with some data showing an increased risk for both CD and UC patients<sup>[94]</sup> or for UC patients alone<sup>[95,96]</sup> and other data showing no increased risk in CD or UC<sup>[97,98]</sup>. Regardless of disease activity, women with IBD have an increased risk for such adverse pregnancy outcomes as PTB, SB, LBW, SGA, and delivery complications such as cesarean sections compared to the general population<sup>[97-101]</sup>. In our study, no discernible increased risks for SA or CA were identified. Overall, unless there is a clear risk of fetal harm (*i.e.*, an FDA category X drug) that dictates otherwise, maintenance therapy is conventionally continued throughout pregnancy to optimize maternal disease control and prevent relapse or progression<sup>[102]</sup>.

This systematic review has limitations. Pooling data from different studies yields inherent heterogeneity based on study designs, study populations, and recording of birth outcomes data. As evidenced, there are a limited number of reported pregnancy exposures to anti-TNF agents, many published as case reports or case series with small sample sizes; these do not necessarily reflect outcomes that can be extracted to the general population. Our review is affected by the limitations of the individual studies, including the inability to adjust for maternal disease activity and severity, concomitant medication or substance use, comorbidities, or other maternal characteristics. Additionally, there exist potential publication bias against negative outcomes and recall bias involving drug exposure and timing of administration during conception and pregnancy. The decision to exclude studies based on the English language and on the inability to link specific anti-TNF exposure with birth outcomes may have discounted pertinent publications. Although care was taken to account for evident overlap, it is possible that repeated data exists given the nature of our information (for example, a case report that has also been reported within drug registry data).

A growing body of evidence supports that IFX, ADA, and CTZ are low risk in pregnancy<sup>[17]</sup>, and studies beyond those included in our data set are underway to further elucidate fetal risk and optimal timing of biologic administration during pregnancy<sup>[103,104]</sup>. Thus far, it is believed that IFX and ADA are most compatible for use during conception and at least the first and second trimesters considering mechanisms of placental transport<sup>[17,102]</sup>; further human data are needed to generate safety guidelines for the use of CTZ. In a recent study of pregnant women receiving biologic therapy, IFX and ADA were shown to be transplacentally transferred to infants at birth, with high levels of drug in cord blood and detectable drug levels up to six months after birth. CTZ was found to be least detectable in both cord blood and infant serum after birth. Of note, no CA or significant fetal complications were reported in this study<sup>[104]</sup>.

Future efforts are promising and include the expansion of drug safety data registries and the development of larger prospective trials to help definitively quantify fetal risk and to facilitate clinical decision-making in treating women with IBD during their childbearing years. One such project is the highly anticipated Pregnancy IBD and Neonatal Outcomes study, a prospective data collection from multiple IBD centers in the United States<sup>[105]</sup>. This large cohort registry not only accounts for maternal factors including IBD activity, medication use, delivery methods, and pregnancy complications but also tracks data over time from the neonatal period through children's first year of life. Similarly, post-marketing surveillance data may uncover additional consequences of fetal exposure to biologic agents over time.

While evidence in the field is mounting, caution should indefinitely be exercised. Given the limitations of the available data and lack of controlled trials, there is insufficient evidence to prove absolute safety for use of anti-TNFs during pregnancy. Although the benefits of therapy in optimizing disease activity during gestation may lend to more favorable pregnancy outcomes based on a controlled disease state, definitive safety of drug exposure on the developing fetus has not been confirmed.

Medical management decisions during the preconception and pregnancy periods will inevitably vary by case based on respective risk-to-benefit ratios, details of disease activity, response to alternative therapies, and individual preferences. Women and men of childbearing age should be educated about the effects of IBD on pregnancy and the potential implications of treatment on fetal development. In addition, patients should be encouraged to discuss reproductive plans with their physicians in order to achieve remission prior to conceiving. Ideally, the primary preconception goal should be quiescent disease, as this lends to the most favorable pregnancy outcomes.

## CONCLUSION

After systematic literature review investigating TNF- $\alpha$  inhibitor therapy and fetal risk, there is insufficient evi-

dence to prove absolute safety for the use of biologics (specifically infliximab, adalimumab, and certolizumab pegol) during pregnancy.

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**P- Reviewers** Mayer RB, Ehrenpreis ED **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Zhang DN



## CD133: A cancer stem cells marker, is used in colorectal cancers

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Author contributions: Ren F, Sheng WQ and Du X all contributed to this review; Ren F wrote and revised the manuscript; Sheng WQ initiated the plan; Du X provided guidance throughout the preparation of this manuscript and corrected the final version. Supported by Clinical Key Discipline Fund by Ministry of Health (2010-2012), Chinese National Clinical Key Discipline (2011-2012) and the Shanghai Science and Technology Commission of Shanghai Municipality, No. 10DJ1400500

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Received: December 24, 2012 Revised: February 4, 2013  
Accepted: February 7, 2013  
Published online: May 7, 2013

### Abstract

Colorectal cancer is one of the most common malignant tumors worldwide. A model of cancer development involving cancer stem cells has been put forward because it provides a possible explanation of tumor hierarchy. Cancer stem cells are characterized by their proliferation, tumorigenesis, differentiation, and self-renewal capacities, and chemoradiotherapy resistance. Due to the role of cancer stem cells in tumor initiation and treatment failure, studies of cancer stem cell markers, such as CD133, have been of great interest. CD133, a five-transmembrane glycoprotein, is widely used as a marker to identify and isolate colorectal cancer stem cells. This marker has been investigated to better understand the characteristics and functions of cancer stem cells. Moreover, it can also be used to predict tumor progression, patient survival, chemoradiotherapy resistance and other clinical parameters. In this review, we discuss the use of CD133 in the identification of colorectal cancer stem cell, which is currently controversial. Although the function of CD133 is as yet

unclear, we have discussed several possible functions and associated mechanisms that may partially explain the role of CD133 in colorectal cancers. In addition, we focus on the prognostic value of CD133 in colorectal cancers. Finally, we predict that CD133 may be used as a possible target for colorectal cancer treatment.

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**Key words:** CD133; Colorectal cancer; Cancer stem cells; Prognosis; Chemoradiotherapy resistance

**Core tip:** CD133 is not a reliable marker to identify the entire population of cancer stem cells (CSCs). However, the abundance of CD133 may be a good indicator of CSC identity and consistent with the biological characteristics of CSCs; The expression of CD133 is correlated to the poor survival; CD133(+) cells exhibit more chemoresistant behavior than CD133(-) cells; Whether CD133-targeting therapies can be a specific or efficient treatment for colorectal cancer has not been confirmed.

Ren F, Sheng WQ, Du X. CD133: A cancer stem cells marker, is used in colorectal cancers. *World J Gastroenterol* 2013; 19(17): 2603-2611 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2603.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2603>

### INTRODUCTION

Colorectal cancer is regarded as one of the most common cancers in the world, and a main cause of cancer-related death in western countries. In spite of progressing treatments, a large percentage of advanced tumors have poor prognosis. Recent studies have shown that a small population of tumor cells, known as cancer stem cells (CSCs), may be considered the main initiators of recurrence and metastasis. It is critical to find specific bio-

markers to identify and isolate CSCs as well as to predict patient prognosis. CD133 is one of the best-characterized markers of CSCs. However, its role in colorectal cancer needs further study. Here, we have attempted to elucidate the relationship between CD133 and colorectal cancer based on the results of previous studies.

## INTRODUCTION OF CD133

CD133, a five-transmembrane glycoprotein, was first found to be expressed in hematopoietic stem and progenitor cells by Yin *et al*<sup>[1]</sup>. The protein has a molecular weight of 120 kDa<sup>[1]</sup> and localizes to membrane protrusions. The protein may be expressed as one of two isoforms, CD133-1 and CD133-2. CD133-1 was the first to be discovered, by Yin *et al*<sup>[1]</sup>, and it is mainly expressed in human fetal liver, bone marrow and blood. CD133-2, first cloned and identified by Yu *et al*<sup>[2]</sup>, is another cell surface antigen that is recognized by anti-AC133 monoclonal antibodies. Relative to AC133-1 cDNA, a small exon of 27 nucleotides is deleted in AC133-2 by alternative mRNA splicing<sup>[2]</sup>. CD133-2 mRNA is prominently expressed in human fetal tissue, adult tissues and several carcinomas<sup>[2]</sup>. It has also been suggested that CD133-2 is expressed in multiple stem cell niches<sup>[2]</sup>. Based on these biological characteristics, CD133 is widely used to identify and isolate stem cells and cancer stem cells. However, its function is still unclear. It is hypothesized to be associated with the cell-cell interaction or signal transduction.

## CANCER STEM CELLS AND CD133

Tumor cells show heterogeneity in their morphology, inheritance, functions and other characteristics. However, some tumor cells present not only heterogeneity but also hierarchy. The increasing CSCs model represents a breakthrough in explaining this phenomenon. In this model, CSCs, despite being only a small subset of cancer cells, have the capability to self-renew and sustain the tumor. These CSCs also have the ability to proliferate, resulting in expansion of the CSC pool, and to differentiate into the heterogeneous cancer cell subgroups that may not themselves be tumorigenic but usually constitute the majority of the tumor<sup>[3]</sup>.

More and more studies have indicated that CD133 is a surface marker of CSCs. CD133 has been found in many tumors, including cancers of the brain<sup>[4]</sup>, colon<sup>[5,6]</sup>, liver<sup>[7]</sup>, pancreas<sup>[8]</sup>, kidney<sup>[9]</sup>, lung<sup>[10]</sup>, endometrium<sup>[11]</sup>, ovary<sup>[12]</sup> and bone<sup>[13]</sup>. The exploration of CD133 as a surface marker of colon cancer stem cells (Co-CSCs) is still in progress. In 2007, both O'Brien *et al*<sup>[14]</sup> and Ricci *et al*<sup>[15]</sup> found that CD133(+) cells in colon cancers had the ability to initiate tumor growth. The colon cancer-initiating cells (CC-ICs) represented enrichment in CD133(+) populations. These two studies strongly support CD133 as a marker of Co-CSCs based on the evidence that CD133(+) cells could produce tumors with preserved self-renewal and differentiation capabilities and without phenotypic alterations

after serial transplantation. The long-term tumorigenic potential of CD133(+) colon cancer cells has also been confirmed *in vitro*<sup>[15]</sup>. More importantly, CD133(-) colon cancer cells have no ability to form tumors. However, Shmelkov *et al*<sup>[16]</sup> discovered that CD133 was ubiquitously expressed in differentiated colonic epithelium rather than restricted to stem cells. Furthermore, *in vitro*, both CD133(+) and CD133(-) metastatic tumor subpopulations formed colonospheres. Both subpopulations maintained long-term tumorigenesis in a NOD/SCID serial xenotransplantation model. It should be noted that Shmelkov *et al*<sup>[16]</sup> used subpopulations from metastatic tumors. However, the distinction of CSCs and the function of CD133 in primary and metastatic tumors are still unknown. Despite this fact, the discovery challenged the view of CD133 as a marker of Co-CSCs, and further studies were performed to investigate the discrepancy.

Kawamoto *et al*<sup>[17]</sup> showed that although both CD133(+) and CD133(-) cells could form tumors after injection into NOD/SCID mice, the CD133(+) cells formed larger tumors. There remained a difference in these tumors. CD133(+) cells could not be found in tumors generated by the injection of CD133(-) cells but were observed in tumors from injections of CD133(+) cells, suggesting that CD133(+) cells had self-renewing capability whereas CD133(-) cells did not. The investigation is consistent with Shmelkov's finding that CD133(-) cells also have the ability to form tumors, although it seems that the CD133(+) cells were associated with stronger tumorigenesis than CD133(-) cells. Thus, the presence of CD133 may not be a reliable indicator of CSC *vs* non-CSC identity. A more appropriate distinction is the relative abundance of the CD133 protein<sup>[18]</sup>. Liao *et al*<sup>[19]</sup> attempted to confirm this hypothesis by sorting cancer cells according to the abundance of CD133. CD133 (High), CD133 (Mid), and CD133 (Low) subgroups of SW620 cells (a colorectal cancer cell line) were distinguished, and their biological characteristics were analyzed. The CD133 (High) subgroup exhibited a higher growth rate than the CD133 (Mid) and CD133 (Low) subgroups did. However, despite its much slower growth, the CD133 (Low) subgroup retained its tumorigenicity.

One likely explanation is that CD133 is expressed on not only CSCs but also differentiated tumor cells. However, during CSC differentiation, the specific epitopes recognized by AC133 are masked due to differential glycosylation<sup>[20]</sup>. The expression of CD133 could be modulated by factors in the microenvironment, such as energy supply<sup>[21]</sup>. In addition, the inactivation of CD133 during the progression of colorectal cancer can be considered a result of transcriptional repression, due to promoter hypermethylation of the CD133 CpG islands<sup>[22]</sup>. CD133(-) cells likely lack the AC133 epitopes, the expression of which is influenced by posttranslational modification under certain conditions. It appears that CD133 is not a reliable marker to identify the entire population of CSCs. However, the abundance of CD133 may be a good indicator of CSC identity.



**Table 1 Studies of the relationship between CD133 expression and survival**

| Ref.                                 | n   | Cancer category                       | Tumor stage                                       | Method | Did CD133 expression predict poor survival? |
|--------------------------------------|-----|---------------------------------------|---|--------|---|
| Choi <i>et al</i> <sup>[34]</sup>    | 523 | Colorectal adenocarcinomas            | Stages I-IV                                       | IHC    | No: overall survival                        |
| Kemper <i>et al</i> <sup>[51]</sup>  | 90  | Colorectal cancers                    | Stage II  | RT-PCR | Yes: relapse-free survival                  |
| Saigusa <i>et al</i> <sup>[55]</sup> | 52  | Rectal cancers (post-CRT)             | Unclear   | RT-PCR | Yes: recurrence-free survival               |
| Jao <i>et al</i> <sup>[57]</sup>     | 233 | Colorectal adenocarcinomas (post-CRT) | Stages I-IV                                       | IHC    | Yes: overall survival                       |
| Saigusa <i>et al</i> <sup>[59]</sup> | 33  | Rectal cancers (post-CRT)             | Stage II/III                                      | RT-PCR | Yes: disease-free survival                  |
| Horst <i>et al</i> <sup>[60]</sup>   | 77  | Colorectal adenocarcinomas            | T2/T3, N0, M0                                     | IHC    | Yes: cancer-specific survival               |
| Yasuda <i>et al</i> <sup>[65]</sup>  | 40  | Rectal cancers (post-CRT)             | Advanced  | RT-PCR | Yes: disease-free survival                  |
| Li <i>et al</i> <sup>[66]</sup>      | 104 | Colon carcinomas                      | Stage III B                                       | IHC    | Yes: overall survival                       |
| Artells <i>et al</i> <sup>[67]</sup> | 60  | Colorectal cancers                    | Stages I-III                                      | RT-PCR | Yes: overall survival                       |
| Kojima <i>et al</i> <sup>[69]</sup>  | 160 | Colorectal cancers                    | Stages I-IV (well- and moderately-differentiated) | IHC    | Yes: overall survival                       |
| Kojima <i>et al</i> <sup>[69]</sup>  | 140 | Colorectal cancers                    | Stages I-IV (well- and moderately-differentiated) | IHC    | No: recurrence-free survival                |

IHC: Immunohistochemistry; RT-PCR: Reverse-transcription polymerase chain reaction; CRT: Chemoradiotherapy.

sion at the mRNA level, concluded that CD133 expression is inversely correlated with survival. The majority of these studies included post-chemoradiotherapy patients. However, all of these studies used small sample sizes. Although some studies had deficiency in their design, majority of them came to the similar conclusion that the expression of CD133 was correlated to the poor survival, and more uniform studies should be performed to demonstrate this confusion.

CD133 can be found in not only primary tumors but also metastatic tumors such as liver metastases. It was reported that the CD133(+)/CD44(+) subpopulation was responsible for this metastasis<sup>[30,71]</sup>. Neumann *et al*<sup>[72]</sup> reported that cases with MLH1(+), CD133 (high scores) and  $\beta$ -catenin (high scores) tumors were associated with a very high rate of distant metastases (94.4%). Thus, it seems that CD133, in combination with other markers, may be a good predictor of metastasis risk.

Several studies have investigated whether the CD133 mRNA level in peripheral blood is useful for prognosis in colorectal cancers. Lin *et al*<sup>[73]</sup> first described that an increased level of CD133 mRNA in the peripheral blood could predict colon cancer recurrence, independent of tumor-node-metastasis stage. Pilati *et al*<sup>[68]</sup> investigated patients with liver-confined hepatic metastasis from colorectal cancers. The level of CD133, used as a marker of circulating tumor cells, increased inversely with the survival. Iinuma *et al*<sup>[74]</sup> showed that in patients with Dukes' stage B and C cancer, CEA/CK/CD133 demonstrated significant prognostic value. In contrast, no significant differences were seen in patients with Dukes' stage A disease. These studies support the use of CD133 mRNA in peripheral blood as prognostic marker. However, Gazzaniga *et al*<sup>[75]</sup> reported that there was no correlation between the expression of CD133 in circulating tumor cells isolated from peripheral blood and outcomes in patients with metastatic colorectal cancers. All these studies used an reverse-transcription polymerase chain reaction approach, but this method may have low sensitivity and lead to a high rate of false positive results in detecting circulating tumor cells<sup>[76]</sup>. Due to the limitation of this method and the inclusion of early stage (stage I)

patients with better outcomes as well as advanced stage (stage IV) patients with worse outcomes, current studies may not accurately distinguish between the outcomes in patients with early and advanced stages of colorectal cancer. However, the prognostic value of CD133 mRNA in the peripheral blood of patients with middle-stage disease (stages II and III) has been confirmed by some studies.

Chemoradiotherapy (CRT) resistance is a major problem that affects the survival of patients with colorectal cancer. Furthermore, the acquired resistance has a long-term memory<sup>[77]</sup>. Conventional chemotherapy targets rapidly dividing cells, but CSCs divide slowly. Therefore, CSCs are likely to contribute to the resistance of cytotoxic systemic therapies<sup>[78]</sup>. Efforts have been made to demonstrate that the role of CD133(+) colorectal tumors are more resistant to 5-fluorouracil-based chemotherapy than CD133(-) tumors. Ong *et al*<sup>[79]</sup> conducted a clinical study containing 501 primary colorectal cancer cases that provided evidence supporting this hypothesis. Moreover, recent studies showed that the level of CD133 increased in post-CRT specimens<sup>[65]</sup> and that CD133 expression was detected in 27.5% of non-CRT and 70% of CRT specimens<sup>[80]</sup>. *In vitro*, CD133 was overexpressed in human colon cancer cell lines that were resistant to 5-fluorouracil and oxaliplatin, such as HT29/5FU-R and HT29/OxR<sup>[81]</sup>. These results suggest that CD133 is a good predictor of CRT resistance. However, Hongo *et al*<sup>[82]</sup> recently reported that CD133(-) cells are more resistant to 5-fluorouracil than CD133(+) cells, which challenged the previous view. However, these authors isolated CD133(+) and CD133(-) cells from a single colorectal cancer cell line, and the original characteristics may have been altered during long-term culture. Therefore, the clinical studies are more reliable than *in vitro* studies. Meanwhile, Ong's research was conducted in a large clinical sample size, and other clinical<sup>[57,80,83]</sup> and *in vitro* studies<sup>[81,84]</sup> supported their results. Considering that Hongo's study was based on a single cell line and that no further studies have drawn similar conclusions to date, we feel it is appropriate to conclude that CD133(+) cells exhibit more chemoresistant behavior than CD133(-) cells. However, more studies, especially

clinical studies, should be performed to clarify the role of CD133 in chemoresistance.

The mechanism of chemoresistance is still under investigation. Intrinsic factors such as antiapoptotic proteins and soluble microenvironmental molecules, including growth factors and cytokines, may cause the refractoriness of CSCs. Several studies have demonstrated that the interleukin 4 (IL-4) produced by cancer cells themselves negatively regulated apoptosis<sup>[85-87]</sup>. Todaro *et al*<sup>[88]</sup> reported that IL-4 protected CD133(+) cells from apoptosis in colon cancer. They later found that treatment with an IL-4 receptor alpha chain antagonist or anti-IL-4 neutralizing antibody strongly enhances the antitumor efficacy of standard chemotherapeutic drugs through selective sensitization of CD133(+) cells<sup>[89]</sup>. These studies highlight the importance of IL-4 in chemoresistance. In addition, the secretion of IL-4 induced an immunosuppressive state in the microenvironment of the tumor, which facilitates the tumor progression. These results indicate that IL-4 may be a good target of colorectal cancer treatment.

## COLORECTAL CANCER TARGET THERAPIES

There are two major targets for advanced colorectal cancer therapy: epidermal growth factor receptor and vascular endothelial growth factor. The Wnt pathway is an additional potential target. Data has shown that Wnt pathway activity could be responsible for the chemoresistance of CD133(+) cells in colorectal cancer cells. Deng *et al*<sup>[43]</sup> demonstrated that 5-fluorouracil upregulated Wnt activity in CD133(+) colon cancer stem-like cells. Dickkopf-1, an inhibitor of Wnt signaling, decreased the expression of CD133 and Lgr5. It also reduced the proliferation, migration, and invasion of colon cancer cells<sup>[90]</sup>. This indicates that blocking the Wnt pathway may be one possible solution to the problem of chemoresistance. Furthermore, other pathways, such as the Notch and Hedgehog signaling pathways involved in maintaining CSC identity, and other regulators, such as STAT3<sup>[53,91,92]</sup> and microRNAs, could be conceivable targets.

CSCs can be eliminated by targeting membrane proteins such as CD133 and then delivering medicines that can specifically induce to death. Several efforts have been made to utilize CD133 in the treatment of cancers. Damek-Poprawa *et al*<sup>[93]</sup> conjugated a CD133 monoclonal antibody (MAB) to a genetically modified cytolethal distending toxin (Cdt). The proliferation of CD133(+) cells in cell lines derived from head and neck squamous cell carcinomas was preferentially inhibited in a rate- and dose-dependent manner by the Cdt-MAB complex. Moreover, Rappa *et al*<sup>[94]</sup> decreased the number of CD133 molecules using two different short hairpin RNAs in FEMX-I melanoma cells. The cell growth, cell motility and spheroids-forming capacity were inhibited as a result of downregulation of CD133. In addition, Wang *et al*<sup>[95]</sup> investigated glioblastoma (GBM) using single-walled

carbon nanotubes (SWNTs) conjugated with CD133 monoclonal antibodies. GBM cells were exposed to these SWNTs and then irradiated with near-infrared laser light. They found that the CD133(+) cells were eliminated, and the tumorigenic and self-renewal characters of CD133(+) cells were also blocked. However, some have argued that CD133(-) cells can be reversed to CD133(+) cells through microenvironmental factors and that CD133 is not the only marker able to identify CSCs, indicating that targeting CSCs through CD133 alone is not sufficient to cure tumors. Whether CD133-targeting therapies can be a specific or efficient treatment for colorectal cancer requires further exploration.

## CONCLUSION

Although CD133 has been used as a CSC marker, it cannot be the only marker used to characterize CSCs in colorectal cancers. It seems that a small population of CD133(-) cells also have the biological characteristics of CSCs. Other markers should be used in combination to identify CSCs. Which combination of these markers is the best to identify CSC or shows consistent biological characters with CSC remains a question. Moreover, little is known about the function of CD133. Whether CD133 participates in the biological behavior of CSC or merely acts as a marker of the CSC phenotype is not clear. A variety of studies have confirmed the prognostic value of CD133 in colorectal cancers. However, these studies lack uniform samples, clinical conditions, methods and evaluations. Although CD133 is believed to be a target of advanced colorectal cancer, few efforts have been made to confirm this hypothesis directly. Regardless, it is desirable to explore new strategies of colorectal cancers by the use of CD133.

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**P- Reviewer** Nakajima N **S- Editor** Gou SX **L- Editor** A  
**E- Editor** Zhang DN



## Prevalence of celiac disease in Germany: A prospective follow-up study

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Received: October 31, 2012 Revised: December 18, 2012

Accepted: February 5, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To determine the prevalence of celiac disease in a randomly selected population sample.

**METHODS:** A total of 2157 subjects (1036 males; 1121 females) participating in a population-based cross-sectional study underwent laboratory testing for tissue transglutaminase and antibodies to immunoglobulin A, endomysium and antigliadin. In a second step, all subjects who had been examined serologically were surveyed using a questionnaire that included questions

specific to celiac disease. Subjects with positive antibody titers and those with histories positive for celiac disease then underwent biopsy. At the first follow up, antibody titers were again determined in these subjects and subjects were questioned regarding symptoms specific for celiac disease and disorders associated with celiac disease. The second follow up consisted of a telephone interview with subjects positive for celiac disease.

**RESULTS:** Antibody tests consistent with celiac disease were reported in eight subjects, corresponding to an overall prevalence of 1:270 (8/2157). The prevalence among women was 1:224 and 1:518 in men. Classical symptoms were observed in 62.5% of subjects. Atypical celiac disease was present in 25.0%, and transient celiac disease in 12.5%. False-negative test results were returned in three subjects. This yields a sensitivity and specificity of 62.5% and 50.0%, respectively, for tissue transglutaminase immunoglobulin-A antibody; of 62.5% and 71.4% respectively, for endomysium antibody; and of 62.5% and 71.4%, respectively, for antigliadin antibody.

**CONCLUSION:** The prevalence rate in our collective lies within the middle tertile of comparable studies in Europe. The use of a single antibody test for screening purposes must be called into question.

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**Key words:** Cross-sectional study; Celiac disease; Screening; Prevalence; Serology

**Core tip:** Only limited data on the prevalence of celiac disease in the adult European population are available. Aim of the study was to determine the prevalence of celiac disease in a randomly selected population sample in Germany and to assess the sensitivity and specificity of antibody tests. Eight of 2157 (1:270) subjects tested

positive for celiac disease. Tissue transglutaminase immunoglobulin-A antibody yielded a sensitivity of 62.5% (specificity 50.0%), endomysium antibody of 62.5% (71.4%) and anti gliadin antibody of 62.5% (71.4%). The prevalence rate lies within comparable European study results. The use of a single antibody test for screening purposes must be questioned.

Kratzer W, Kibele M, Akinli A, Porzner M, Boehm BO, Koenig W, Oeztuerk S, Mason RA, Mao R, Haenle MH. Prevalence of celiac disease in Germany: A prospective follow-up study. *World J Gastroenterol* 2013; 19(17): 2612-2620 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2612.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2612>

## INTRODUCTION

The prevalence of celiac disease in population-representative collectives is reported between 1:42 and 1:558<sup>[1-10]</sup> (Table 1) depending on the size of the population studied and the nature of the antisera used for screening. Among blood donors, the reported range is from 1:37 to 1:681<sup>[11-19]</sup> (Table 1) and, for non-population representative samples, between 1:86 and 1:709<sup>[20-24]</sup> (Table 1).

Subjects suffering from celiac disease may present with typical clinical symptoms, such as diarrhea, weight loss and bloating; or, they may be completely asymptomatic or exhibit only unspecific symptoms. For example, anemia or iron deficiency may be the only initial signs of the disease. Age distribution of first onset shows a first peak between nine months and two years, and a second during the fourth decade<sup>[25]</sup>.

It has only been in recent years that the clinical manifestations of this very heterogeneous disorder have been arranged in subclasses<sup>[26]</sup>. The system of subtypes proposed by Holtmeier *et al*<sup>[26]</sup> for the first time integrates clinical symptoms, histology and antibody titers, thus facilitating reliable diagnosis and therapeutic management.

Celiac disease is associated with specific, often serious, complications, including the development of gastric ulcers with the risk of hemorrhage, perforation and stricture, as well as T cell lymphoma<sup>[25,27]</sup>. In fact, the risk of lymphoma in patients with celiac disease is about three times as high as that of the general population, with a peak age of onset at about 60 years<sup>[25]</sup>. The risk of complications and neoplasia is associated with all forms of celiac disease: hence, the recognition of non-classical disease forms is particularly crucial. A strict, gluten-free diet started as early as possible and maintained lifelong significantly reduces the risk of malignancy<sup>[28]</sup>. The protective effects of a gluten-free diet on the development of autoimmune disorders associated with celiac disease, such as diabetes mellitus or autoimmune thyroid diseases, however, remains controversial in the literature<sup>[29-33]</sup>.

Small bowel biopsy has been the conventional gold standard for the diagnosis of celiac disease. Today, however, greater importance is now attached to serological

studies, including antibodies to tissue transglutaminase (tTGA), endomysium (EMA) and gliadin (AGA)<sup>[8]</sup>.

To date, no prospective data on the prevalence of celiac disease in a representative adult population sample in Germany have been published. Objective of the present study was to determine the prevalence of celiac disease in a randomly selected population sample.

## MATERIALS AND METHODS

### Study population

The study "Echinococcus Multilocularis and other Internal Diseases in Leutkirch" (EMIL), a cross-sectional survey assessing the prevalence of *Echinococcus multilocularis* infection and other medical disorders, was conducted in Leutkirch, Germany in 2002. Initially, 4000 of the total 12475 residents were randomly selected by the staff of the municipal registry office from the roster of inhabitants. Out of these 4000 persons, 107 were excluded because their address was unknown or they had not given their informed consent. A total of 2445 individuals finally participated in the study, corresponding to a participation rate of 62.8%. Following exclusion of subjects less than 18 years and subjects with incomplete laboratory results, 2157 subjects were finally included in the present analysis (Figure 1).

The study was conducted in accordance with the principles of the Helsinki Declaration and Good Clinical Practice. It was approved by the ethics committee of the Landesärztekammer Baden-Württemberg. All subjects provided their written informed consent.

### Initial study

All subjects were interviewed by a trained interviewer using a standardized questionnaire. In order to reduce interviewer bias as much as possible, each interviewer underwent in-depth training by an interviewing specialist of the state health office<sup>[34]</sup>.

Because the original EMIL questionnaire did not include specific questions regarding celiac disease, in 2003 all subjects of the EMIL study were mailed a separate questionnaire addressing celiac disease. Subjects were questioned regarding celiac disease that had been diagnosed prior to the date of the EMIL study and were asked whether they were currently (*i.e.*, at the time of the study) prescribed a gluten-free diet. The response rate to this survey stood at 50%.

Each subject underwent phlebotomy of the cubital vein to obtain ca. 25 mL of venous blood. Total immunoglobulin A (IgA) concentration was determined by nephelometry (BN II, Dade Behring, Marburg, Germany). IgA to tTGA was measured using an indirect, non-competitive enzyme immunoassay (Pharmacia Diagnostics Freiburg, Germany). Human recombinant tTGA was used as the antigen. Titers of 5-8 U/mL were considered borderline, while titers > 8 U/L were considered positive. AGA was determined using ELISA (Vita Diagnostics Merzhausen, Germany). Titers of 12-16 U/mL were con-

**Table 1** Prevalence of celiac disease in different countries

| Country                                   | Prevalence | Characteristics of populations studied |                                 |        | Antibody test method |     |     |
|---|------------|--|---------------------------------|--------|----------------------|-----|-----|
|   |            | <i>n</i>                               | Age (yr)<br>mean/median (range) | Males  | tTGA                 | AGA | EMA |
| Population-representative samples         |            |  |                                 |        |                      |     |     |
| Germany (present study)                   | 1:270      | 2157                                   | 42.6 (18-65)                    | 48.03% | -                    | -   | -   |
| New Zealand <sup>[1]</sup>                | 1:82       | 1064                                   | 50.2 (> 18)                     | 39.80% | -                    | -   | -   |
| Iran <sup>[2]</sup>                       | 1:104      | 2799                                   | 33.7 (18-66)                    | 50%    | -                    | -   | -   |
| Ireland <sup>[3]</sup>                    | 1:122      | 1823                                   | NA (15-65)                      | NA     | -                    | -   | -   |
| Sweden <sup>[4]</sup>                     | 1:190      | 1894                                   | 50 (25-74)                      | 50%    | -                    | -   | -   |
| Netherlands <sup>[5]</sup>                | 1:286      | 1440                                   | 40.6 (20-59)                    | 46%    | -                    | -   | -   |
| Spain <sup>[6]</sup>                      | 1:390      | 1170                                   | 44.9 (2-89)                     | 44.70% | -                    | -   | -   |
| Italy <sup>[7]</sup>                      | 1:559      | 2237                                   | 44 (20-87)                      | 46.90% | -                    | -   | -   |
| Greece <sup>[8]</sup>                     | 1:558      | 2230                                   | 46 (18-80)                      | 45%    | -                    | -   | -   |
| Finland <sup>[9]</sup>                    | 1:47       | 2815                                   | NA (52-74)                      | 48%    | -                    | -   | -   |
| Finland <sup>[10]</sup>                   | 1:42       | 4846                                   | NA (30-64)                      | 47%    | -                    | -   | -   |
| Italy <sup>[10]</sup>                     | 1:145      | 2759                                   | NA (30-64)                      | 42%    | -                    | -   | -   |
| Germany <sup>[10]</sup>                   | 1:344      | 3098                                   | NA (30-64)                      | 49%    | -                    | -   | -   |
| Blood donors                              |            |  |                                 |        |                      |     |     |
| Mexico <sup>[11]</sup>                    | 1:37       | 1009                                   | 34 (NA)                         | 68%    | -                    | -   | -   |
| Italy <sup>[12]</sup>                     | 1:100      | 1002                                   | 33 (13-90)                      | 43.40% | -                    | -   | -   |
| United States <sup>[13]</sup>             | 1:105      | 2845                                   | NA                              | 43%    | -                    | -   | -   |
| Iceland <sup>[14]</sup>                   | 1:136      | 813                                    | 36 (17-64)                      | 76.30% | -                    | -   | -   |
| Brazil <sup>[15]</sup>                    | 1:214      | 2045                                   | 32.8 (18-61)                    | 87.60% | -                    | -   | -   |
| Netherlands <sup>[16]</sup>               | 1:333      | 1000                                   | NA                              | NA     | -                    | -   | -   |
| Norway <sup>[17]</sup>                    | 1:340      | 2069                                   | 3 (18-67)                       | 66.30% | -                    | -   | -   |
| Iran <sup>[18]</sup>                      | 1:400      | 2000                                   | 35.5 (18-65)                    | 79%    | -                    | -   | -   |
| Brazil <sup>[19]</sup>                    | 1:681      | 2045                                   | 32.8 (18-61)                    | 87.60% | -                    | -   | -   |
| Non-population representative collectives |            |  |                                 |        |                      |     |     |
| Turkey <sup>[20]</sup>                    | 1:100      | 906                                    | 38.6 (20-59)                    | 50%    | -                    | -   | -   |
| Switzerland <sup>[21]</sup>               | 1:132      | 1450                                   | NA (12-18)                      | 39.90% | -                    | -   | -   |
| Argentina <sup>[22]</sup>                 | 1:167      | 2000                                   | 29 (16-79)                      | 50%    | -                    | -   | -   |
| Tunisia <sup>[23]</sup>                   | 1:709      | 1418                                   | 27.5 (17-57)                    | 73%    | -                    | -   | -   |
| England <sup>[24]</sup>                   | 1:83       | 7550                                   | 59 (45-76)                      | 41%    | -                    | -   | -   |

tTGA: Tissue transglutaminase antibody; EMA: Endomysial antibody; AGA: Antigliadin antibody; NA: Not available; -: Positive.

sidered borderline, while titers > 16 U/L were considered positive. EMA was measured by means of an indirect immunofluorescence technique using a monkey esophagus immunofluorescence kit (The Binding Site, Ltd., Birmingham, United Kingdom). Positive samples exhibit an apple green fluorescence. IgG antibodies to tTGA were determined using an indirect, non-competitive enzyme immunoassay (Phadia GmbH, Freiburg, Germany) in which human recombinant tTGA antigen served as the solid phase. Titers of 7-10 U/mL were considered borderline, while titers > 10 U/L were considered positive.

All subjects testing positive for tTGA IgA underwent a confirmation test together with serological testing for antibodies to EMA and AGA. Subjects with low concentrations of total IgA were tested for tTGA IgG. Subjects with suspected celiac disease also underwent human leukocyte antigen (HLA) typing. Additional specific antibody testing was performed in order to assess the presence of other immunological disorders often associated with celiac disease (islet-cell antibody, anti-GAD, thyroid peroxidase antibody, auto-antibodies to adrenal cortex, parietal cell antibody).

Every effort was made to refer subjects with suspected celiac disease for esophagogastroduodenoscopy in order to obtain tissue samples from the duodenum.

The endoscopies and biopsies were performed by gastroenterologists in private practice. Between one and seven biopsy samples were obtained from each subject. The samples were examined histologically and classified according to the modified Marsh criteria<sup>[35]</sup>.

HLA loci were detected using the reverse sequence specific oligonucleotide (SSO) dot-blot method. The HLA-A, B and C loci were typed using the reverse SSO line-blot assay. Allele assignment was performed using the Helmberg score software with reference to the current nomenclature report and current literature<sup>[36-38]</sup>. Ambiguities were resolved following sequencing of amplicons obtained after SSP with appropriate primers.

### First follow-up screening

At the first follow-up assessment, conducted in December 2005, all antibody-positive subjects and subjects with histories suspicious for celiac disease underwent comprehensive re-examination. A total of 20 subjects were invited, of whom 14 (70%) participated (Figure 1). Subjects completed a diet and digestive symptoms questionnaire, antibody levels were rechecked and laboratory parameters routinely assessed in the work-up of celiac disease (blood count, coagulation parameters, iron, ferritin, hepatic enzymes, parathyroid hormone, calcium, magnesium, phos-

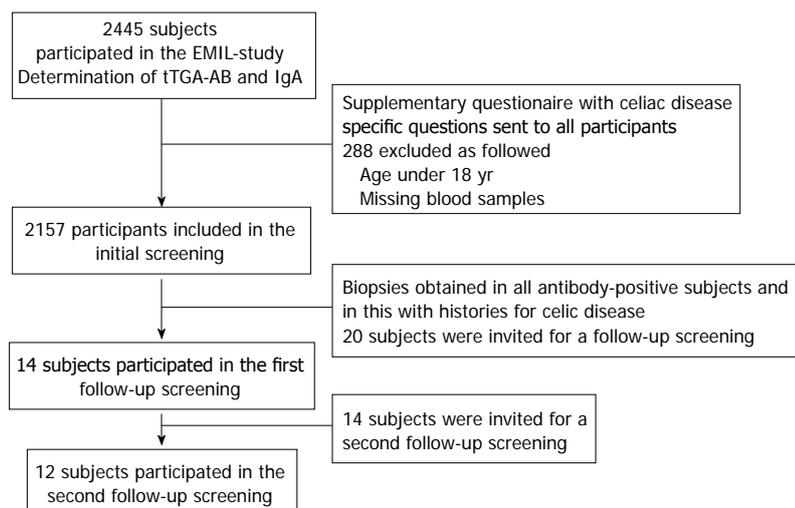


Figure 1 Flow of subjects across the study. tTGA: Tissue transglutaminase antibody; EMIL: Echinococcus Multilocularis and other Internal Diseases in Leutkirch; IgA: Immunoglobulin A.

phate) were obtained.

### Second follow-up screening

As a second follow-up screening, a telephone survey was conducted in March 2008. This survey focused on dietary habits, diet compliance and improvement in symptoms as a result of a gluten-free diet. In this follow-up all celiac positives subjects were included. Also included were the questionable celiac positives. Overall, 12 of 14 (86%) subjects participating in the 2005 follow up were contacted by telephone and re interviewed (Figure 1).

### Statistical analysis

Statistical calculations were performed with the assistance of the Faculty of Medical Documentation and Informatics of the University of Ulm using the SAS statistical software package (version 9.2; SAS Institute Inc., Cary, NC, United States). Data were analyzed descriptively with regard to absolute and relative frequencies, means and standard deviation. Sensitivity and specificity were calculated for all three antibody test methods. Because of the small number of cases, no statistical tests could be applied.

## RESULTS

The study collective available for assessing the prevalence of celiac disease consisted of 2157 subjects (48.0% men, 52.0% women), corresponding to 88% of the total study population. Subjects' age ranged from 18 to 65 years. Their mean age was 42.6 years (standard deviation 12.9 years).

### Initial screening

Fifty-five subjects exhibited a reduced total IgA concentration. However, elevated titers for tTGA IgG were not detected in any of these subjects. A total of 14 subjects (0.65%) were positive for tTGA IgA. Histological findings were available for 11 of these subjects. Celiac disease

was confirmed histologically in five cases (Figure 2).

Six subjects were seronegative but reported histories suspicious for celiac disease (Table 2). Histological findings were available for five of these subjects and confirmed the diagnosis of celiac disease in two cases. In two other subjects, the first diagnosis of celiac disease had already been made at a much earlier date and their histological findings were no longer available. Of these, one subject was HLA positive for DQA1 0101, DQB 0501 and was considered positive for celiac disease in our statistical analysis. The second subject was HLA negative for celiac disease and was considered negative for celiac disease in our statistical analysis (Figure 2). Thus, celiac disease was present in eight subjects, confirmed by biopsy in seven cases and by HLA typing in one case.

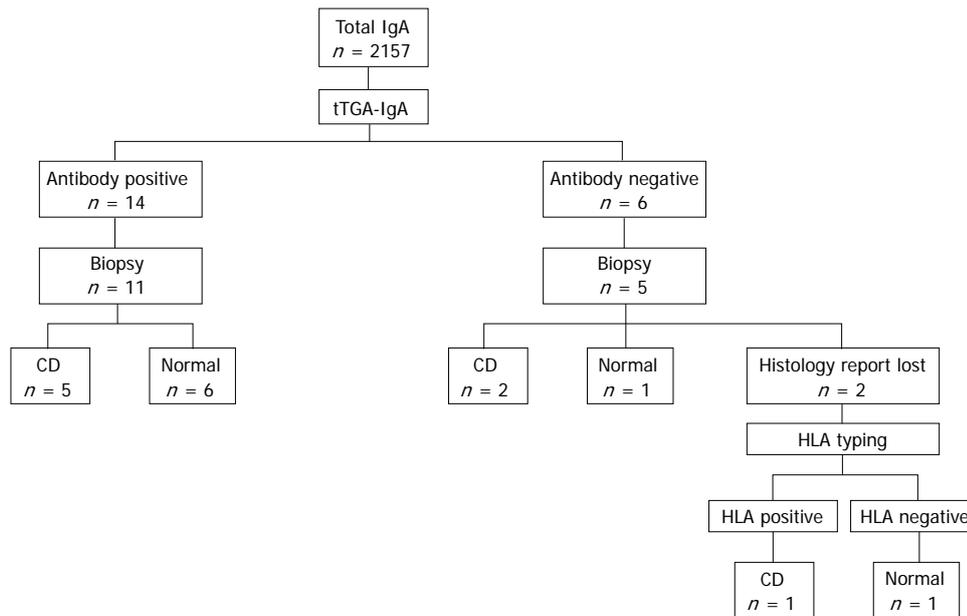
The sensitivity for tTGA IgA antibodies was 62.5%, with a specificity of 50.0%. EMA was associated with a sensitivity and specificity of 62.5% and 71.4%, respectively; and AGA with sensitivity and specificity of 62.5% and 71.4%, respectively. Confirmed false-negative findings were returned for tTGA IgA antibody in three cases, while false positive findings were documented in four cases. Both EMA and AGA were false negative in two cases and false positive in one case (Table 3).

### First follow-up screening

Fourteen of 20 invited subjects participated in the first follow-up screening, corresponding to a participation rate of 70%. tTGA IgA antibodies were detected in three subjects. AGA titers were definitely positive in two subjects. Two subjects were again positive for EMA. Of the six subjects who were initially seronegative but with histories suggestive of celiac disease, three took part in the first follow-up screening: of these, one exhibited positive AGA findings.

### Second follow-up screening

The participation rate at the second follow-up screening stood at 86%, 12 of 14 subjects being reached by tele-



**Figure 2** Subjects positive for celiac disease (based on positive antibody tests or by prior history). Total IgA: Total immunoglobulin A; tTGA IgA: Tissue transglutaminase immunoglobulin A; AB positive: Antibody positive; AB negative: Antibody negative; *n*: Number of subjects; CD: Celiac disease; HLA typing: Human leukocyte antigen typing; HLA: Human leukocyte antigen; HLA positive: Positive for Human leukocyte antigen; HLA negative: Negative for Human leukocyte antigen.

**Table 2** Clinical presentation in subjects with seronegative findings but with histories suggestive of celiac disease

| Age (yr), sex | Clinical presentation   |
|---------------|---|
| 41 yr, female | Diagnosed in 2000, histology unavailable as report destroyed<br>Diarrhea, weight loss of 43 kg prior to diagnosis, adynamia, flatulence, psychiatric symptoms, paresthesias in the fingers<br>In last six months, new onset of epilepsy with petit-mal seizures |
| 18 yr, female | Diagnosed in 2000, histology unavailable as report destroyed; control biopsies in 1990 and 2005 both without evidence of several signs typical for celiac disease   |
| 62 yr, female | Sicca syndrome, lactose intolerance, diarrhea and weight loss<br>Diarrhea, weight loss, fatigue, bone pain, alopecia, osteoporosis, cheilosis. Bronchiectasis known for years   |
| 60 yr, female | Diagnosed in 1989, no other data available  |
| 40 yr, female | Diagnosed in 1989   |
| 56 yr, male   | Sporadic constipation, meteorism, no diarrhea<br>Weight loss, abdominal pain, depressive phases, muscle cramps, eczema on the legs and perianal, meteorism, diarrhea after drinking wheat beer  |

phone and amenable to participation in the standardized interview. Five of eight subjects in our collective had developed classic celiac disease. All subjects maintained a gluten-free diet and reported significant improvement in their intestinal symptoms (Tables 3 and 4). Two further subjects developed atypical celiac disease, which is characterized by mild, atypical symptoms. One subject was monosymptomatic, exhibiting only psoriasis. Subjects were antibody positive and histology revealed changes consistent with Marsh 0-IIIc disease. Despite a strict gluten-free diet, the subject did not report any improvement in his psoriasis (Tables 3 and 4).

The second, female subject was completely asymptomatic. Lactose intolerance was suspected from the subject's history and she avoided all corresponding foods. The subject was antibody positive and histology was consistent with Marsh IIIc disease. Because she continued to be asymptomatic, the subject refused to maintain a gluten-free diet (Tables 3 and 4).

Transient celiac disease was diagnosed in one of the eight subjects. In this disease form, a gluten-free diet leads to complete remission. In this subject, celiac disease had been first diagnosed when she was nine months of age. The subject maintained a strict gluten-free diet until her sixth year; subsequent gastroscopic monitoring failed to return evidence of celiac disease. Since that time, the subject has returned to a diet containing gluten. The subject was negative for DQ2 and DQ8 at HLA typing, but returned positive findings for DQA1\*0101 and DQB1\*0501. This HLA pattern is observed in rare cases in patients with celiac disease. Lactose intolerance was diagnosed in 2004. Gastroscopy performed under the impetus of the present study revealed no evidence of celiac disease. Negative antibody titers during gluten exposure and biopsy findings consistently negative for celiac disease both initially and during the patient's subsequent course correspond to the subtype of transient celiac disease. Based on these HLA findings and improvement in

**Table 3** Change in celiac-specific antibody titers over time

| No.                      | Age (yr), sex | Initial examination |            |          | First follow up |            |     |
|--------------------------|---------------|---------------------|------------|----------|-----------------|------------|-----|
|                          |               | tTG-IgA (U/mL)      | AGA (U/mL) | EMA      | tTG-IgA (U/mL)  | AGA (U/mL) | EMA |
| Classic celiac disease   |               |                     |            |          |                 |            |     |
| 2                        | 58, F         | 5.5                 | 19.4       | Weak Pos | Neg             | Neg        | Neg |
| 4                        | 20, F         | > 100               | 27.6       | Pos      | Neg             | Neg        | Neg |
| 5                        | 52, F         | 44.9                | 24.7       | Pos      | Neg             | Neg        | Neg |
| 17                       | 56, M         | Neg                 | 78.1       | Pos      | Neg             | Neg        | Neg |
| 18                       | 62, F         | Neg                 | Neg        | Neg      | 7               | > 100      | Neg |
| Atypical celiac disease  |               |                     |            |          |                 |            |     |
| 1                        | 40, M         | > 100               | 69.6       | Pos      | 29.9            | 38.1       | Pos |
| 3                        | 42, F         | 41.9                | 56.2       | Pos      | 27              | 20.1       | Pos |
| Transient celiac disease |               |                     |            |          |                 |            |     |
| 16                       | 18, F         | Neg                 | Neg        | Neg      | Neg             | Neg        | Neg |

No.: Subject number; F: Female; M: Male; tTG-IgA: Tissue transglutaminase immunoglobulin A; AGA: Antigliadin antibody immunoglobulin A; EMA: Endomysial antibody; Pos: Positive; Neg: Negative.

symptoms subsequent to starting a gluten-free diet, this patient was considered positive for celiac disease despite lack of initial histological findings.

No other autoimmune disorders were found in our collective of patients with celiac disease. Abnormal laboratory findings suggestive of celiac disease were only moderately pronounced. None of the subjects exhibited iron deficiency anemia. Parathyroid hormone levels were elevated in two subjects with celiac disease.

## DISCUSSION

To date, very few studies have prospectively investigated the prevalence of celiac disease in representative, population-based samples (Table 1). In fact, no corresponding data from a population-based study of adults are available for Germany.

The EMIL study was designed as a cross-sectional investigation in order to obtain a collective that most closely represented the general population. By contrast, a large number of recent studies<sup>[11-19]</sup> have drawn their collectives from blood donors, who are not necessarily representative of a population. A few other studies have investigated non-population representative collectives<sup>[20-24]</sup>.

Our findings correspond to a prevalence of 1:270 (0.37%) for adults in an urban population in Germany. Other population-based studies have reported prevalences between 0.18% and 2.4%<sup>[1-10]</sup>, while, for blood donor collectives, prevalences of 0.15%-2.7%<sup>[11-19]</sup> have been reported. Henker *et al.*<sup>[39]</sup> report a prevalence of 0.19% for asymptomatic celiac disease in children and blood donors. In another study, the same authors report a prevalence of 0.044% in children<sup>[40]</sup>. In a study of the incidence of celiac disease in Berlin, Sandforth *et al.*<sup>[41]</sup> report an incidence rate of 0.05%. Because of the nature of the collectives, however, these data cannot be compared with our findings. In fact, only one study (Monika Project) retrospectively investigated a representative population-based sample in Germany<sup>[10]</sup>. The prevalence of 0.3% reported

in that study is comparable to our findings.

Mustalahti *et al.*<sup>[10]</sup> identified large variability in the prevalence of celiac disease between European nations (Finland, 2.4%; Germany, 0.3%; Italy, 0.7%). Although the precise cause of this difference remains unclear, genetic and environmental factors have been discussed. The study by Mustalahti *et al.*<sup>[10]</sup> must be assessed critically due to its retrospective nature and the quality of the blood samples.

The antibody test methods utilized in the present study to assess for celiac disease have been shown in a comparison of 34 studies to possess both high sensitivity and quite good specificity (tTG-IgA: 93% *vs* 98%, EMA 93% *vs* 99%)<sup>[42]</sup>. The test method for AGA was associated with a lower sensitivity and specificity (80% *vs* 80%-90%)<sup>[43]</sup>. In contrast to these results, Dickey *et al.*<sup>[44]</sup> and Rostami *et al.*<sup>[45]</sup> report a lower sensitivity for AGA and EMA. The results of these tests depend on the severity of mucosal damage. If the damage is slight, the test results may be negative<sup>[45]</sup>. As a consequence, the prevalence of celiac disease is not only underestimated but treatment of affected individuals is delayed, which may be associated with an increased risk of malignancy<sup>[46]</sup>. Compared with data published by Lewis *et al.*<sup>[42]</sup>, the present study found a lower sensitivity (62.50%) and specificity (50%) for tTGA IgA antibody. In the present study, EMA and AGA showed comparably a high sensitivity (62.5%) and specificity (71.4%).

The findings of the present study suggest that the use of tTGA IgA antibody as a suitable method for screening a population for celiac disease should be reconsidered<sup>[42,47]</sup>. It was only by means of our follow-up examinations that we were able to identify subjects with celiac disease with false-negative antibody titers. Otherwise, the prevalence of celiac disease in our collective would have been too low. With the 50% response rate to our celiac disease questionnaire, it cannot be excluded that there may be other undetected false-negative antibody results. A definite conclusion regarding the reliability of this antibody test method is difficult: on the one hand, the number of patients in the different collectives is very small; also, there have been only very few studies to date in which all antibody-positive patients have been biopsied<sup>[2,8,23]</sup>.

Quantitative video capsule endoscopy has been described in the literature as a new method in diagnosing celiac disease<sup>[48,49]</sup>. The findings of these studies show that quantitative image analysis corresponds to the degree of villous atrophy. These studies, however, show some limitations; hence, the value of this new method must be investigated in further studies.

A limiting factor in the present study certainly relates to the study design itself. The EMIL study was not originally conceived to determine the prevalence of celiac disease. As a result, all study participants had to be sent a questionnaire following completion of the initial EMIL study, the response rate to which stood at only 50%. A further disadvantage is the inclusion in our collective of patients who had already been diagnosed with celiac

**Table 4 Clinical presentation, histology and human leukocyte antigen findings in subjects with celiac disease**

| No.                      | Age (yr), sex | Histology        | HLA DQ2 | Clinical presentation   |
|--------------------------|---------------|------------------|---------|---|
| Classical celiac disease |               |                  |         |   |
| 2                        | 58, F         | IIIc             | Pos     | Diarrhea, abdominal cramps, angular cheilitis, osteoporosis, depression, brittle nails, hypothyroidism  |
| 4                        | 20, F         | IIIa-IIIc        | Pos     | Diarrhea, weight variability, circulatory collapse, cheilosis, depression, anxiety, insomnia, brittle nails, restless-leg syndrome  |
| 5                        | 52, F         | IIIb             | Pos     | Fatigue, 1 intestinal cramping, nausea, vomiting, weight loss, paresthesias in the hands, joint, bone and abdominal pains, anxiety, depression, Sicca syndrome, muscle cramps |
| 17                       | 56, M         | IIIc             | Pos     | Weight loss, abdominal cramping, depressive phases, muscle cramps, anal eczema, meteorism, diarrhea following consumption of wheat beer                                       |
| 18                       | 62, F         | IIIc             | Pos     | Diarrhea, weight loss, fatigue, bone pain, alopecia, osteoporosis, cheilosis, bronchiectasia  |
| Atypical celiac disease  |               |                  |         |   |
| 1                        | 40, M         | 0, IIIc          | Pos     | Psoriatic lesion on the head  |
| 3                        | 42, F         | IIIc             | Pos     | No symptoms, suspected lactose intolerance  |
| Transient celiac disease |               |                  |         |   |
| 16                       | 18, F         | NoHisto, ED 1985 | Neg     | Diarrhea, weight loss, Sicca syndrome, lactose intolerance since 2004   |

HLA: Human leukocyte antigen; No.: Subject number; Age: In years; NoHisto: Histological findings unavailable; F: Female; M: Male; Histology: Histological findings classified according to Marsh stage; NA: Not available; Pos: Positive; Neg: Negative.

disease. Also problematic is the impact on the standardization of examination conditions of the retroactive refocusing of the study on determining the prevalence of celiac disease. Patients were referred for endoscopy to several gastroenterologists in private practice in Leutkirch. Biopsies returned between one and seven tissue samples. The histological assessment of the biopsy material was performed by pathologists in different centers.

In conclusion, the findings of the present study show a prevalence of 0.37% for celiac disease, which is comparable to that reported in other European studies. The use of a combination of several antibody test methods for screening examinations appears useful.

## ACKNOWLEDGMENTS

The authors wish to thank the members of the EMIL-Study-Group (in alphabetical order): Adler G, Armsen A, Banzhaf H-M, Bauerdick M, Bertling U, Boehm BO, Brandner BO, Brockmann SO, Deckert M, Dingler C, Eggink S, Fuchs M, Gaus W, Goussis H, Gruenert A, Haenle MM, Hampf W, Haug C, Hay B, Huetter M-L, Imhof A, Kern P, Kimmig P, Kirch A, Klass D, Koenig W, Kratzer W, Kron M, Manfras B, Meitinger K, Mertens T, Oehme R, Pfaff G, Piechotowski I, Reuter S, Romig T, von Schmiesing AFA, Steinbach G, Tourbier M, Voegtle A, Walcher T, Wolff S.

## COMMENTS

### Background

Celiac disease may present with typical clinical signs or patients may be oligo-symptomatic or completely asymptomatic. It is associated with specific, often serious complications. As all forms of celiac disease yield the same risk for complications and especially T cell lymphoma, it is paramount to detect not only the classical form but also patients with atypical disease manifestation.

### Research frontiers

Prevalence rates for celiac disease vary for different populations. This may also be due to the scarcity of data from representative samples. Serological antibody

tests constitute the most important screening tools. In the literature the sensitivity and specificity of transglutaminase immunoglobulin-A, endomysium antibody and antigliadin antibody tests differ.

### Innovations and breakthroughs

This is the first study to prospectively assess the prevalence of celiac disease in a representative population sample of adults in Germany. In comparison to results of the small bowel biopsy the sensitivity and specificity of serological antibody tests for celiac disease were lower than previously described in the literature.

### Applications

Their results add to an accurate estimate of the prevalence of celiac disease in the general population. The use of a single antibody test for screening purposes must be called into question.

### Peer review

Data over recent years have generally noted increased recognition and diagnosis of celiac disease (CD), with rates approaching 2% in some settings. Rates however, appear to vary between geographical regions. This study based in one German city examined several serological tests and gastroenterology symptoms in a large population of adults. The determined prevalence of less than 0.5% is consistent with some previous data, but substantially less than rates in other countries. Interestingly, only 2 of these 2157 adults had previously been thought to have CD, emphasising that CD is often not recognised in routine clinical practice.

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**P- Reviewers** Ciaccio EJ, Rodrigo L **S- Editor** Huang XZ  
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## CYP24A1 inhibition facilitates the anti-tumor effect of vitamin D3 on colorectal cancer cells

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Received: May 23, 2012 Revised: August 21, 2012

Accepted: August 25, 2012

Published online: May 7, 2013

### Abstract

**AIM:** The effects of vitamin D3 have been investigated on various tumors, including colorectal cancer (CRC). 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1), the enzyme that inactivates the active vitamin D3 metabolite 1,25-dihydroxyvitamin D3 (1,25-D3), is considered to be the main enzyme determining the biological half-life of 1,25-D3. During colorectal carcinogenesis, the expression and concentration of CYP24A1 increases significantly, suggesting that this phenomenon could be responsible for the proposed efficacy of 1,25-D3 in the treatment of CRC. The aim of this study was to investigate the anti-tumor effects of vitamin D3 on the human

CRC cell line Caco-2 after inhibition of the cytochrome P450 component of CYP24A1 activity.

**METHODS:** We examined the expression of CYP24A1 mRNA and the effects of 1,25-D3 on the cell line Caco-2 after inhibition of CYP24A1. Cell viability and proliferation were determined by means of sulforhodamine-B staining and bromodeoxyuridine incorporation, respectively, while cytotoxicity was estimated via the lactate dehydrogenase content of the cell culture supernatant. CYP24A1 expression was measured by real-time reverse transcription polymerase chain reaction. A number of tetralone compounds were synthesized to investigate their CYP24A1 inhibitory activity.

**RESULTS:** In response to 1,25-D3, CYP24A1 mRNA expression was enhanced significantly, in a time- and dose-dependent manner. Caco-2 cell viability and proliferation were not influenced by the administration of 1,25-D3 alone, but were markedly reduced by co-administration of 1,25-D3 and KD-35, a CYP24A1-inhibiting tetralone. Our data suggest that the mechanism of action of co-administered KD-35 and 1,25-D3 does not involve a direct cytotoxic effect, but rather the inhibition of cell proliferation.

**CONCLUSION:** These findings demonstrate that the selective inhibition of CYP24A1 by compounds such as KD-35 may be a new approach for enhancement of the anti-tumor effect of 1,25-D3 on CRC.

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**Key words:** Colorectal cancer; CYP24A1 inhibition; Vitamin D3; Tetralone derivatives; Caco-2 cell culture

Kósa JP, Horváth P, Wölfling J, Kovács D, Balla B, Mátyus P, Horváth E, Speer G, Takács I, Nagy Z, Horváth H, Lakatos P. CYP24A1 inhibition facilitates the anti-tumor effect of vitamin D3 on colorectal cancer cells. *World J Gastroenterol* 2013;

## INTRODUCTION

Epidemiologic studies have suggested that maintenance of an adequate level of vitamin D may reduce the incidence and development of several types of tumors, including breast, prostate and colorectal cancers (CRC)<sup>[1-4]</sup>. The role of vitamin D deficiency in the development of CRC, and the potential use of vitamin D in the treatment of CRC have been the focus of a number of studies, as CRC is one of the most common cancers<sup>[5]</sup>.

There is a vast array of evidence suggesting a protective effect of vitamin D against CRC<sup>[6-8]</sup>. There is an inverse association between the serum level of 25-hydroxy vitamin D3 (25-D3) and the risk of CRC<sup>[1,9]</sup>. In ulcerative colitis, low expression of the vitamin D receptor (VDR) is associated with an elevated risk of the development of CRC<sup>[10]</sup>. An inadequate dietary intake of vitamin D and a vitamin D deficiency promote the development and growth of CRC in mice<sup>[3,11]</sup>. In elderly women, higher plasma levels of 25-D3 are accompanied by a lower risk of CRC<sup>[12]</sup>. Further studies have shown that vitamin D may have a preventive role not only in CRC, but also in other cancers of the alimentary tract<sup>[13]</sup>. Nevertheless, the exact cellular pathway for the putative anti-tumor effect of vitamin D remains unclear. The action of 1,25-dihydroxyvitamin-D3 (1,25-D3) through the nuclear VDRs is delayed, but the immediate responses triggered from the cell by cytosolic VDRs acting through Ca<sup>2+</sup> influx might also play an important role in this process<sup>[14]</sup>. However, the application of 1,25-D3 in tumor treatment is restricted due to its tendency to cause hypercalcemia<sup>[15]</sup>.

The anti-tumor efficacy of vitamin D in tumor cell cultures is somewhat contradictory<sup>[16-19]</sup>. Some cancer cell lines are more susceptible to vitamin D treatment than others<sup>[19,20]</sup>, and the vitamin D-sensitive cell cultures have been shown to resemble early-stage tumors<sup>[20]</sup>. During the progression of the cancer, this susceptibility is gradually lost, but the underlying pathophysiological process of this loss is not clear. Though numerous clinical studies have been conducted with vitamin D or its analogs, the anti-tumor results were largely disappointing<sup>[21]</sup>. The current evidence suggests that a relationship does exist between vitamin D and cancer, but the strength of this relationship appears to weaken on progression from the preclinical to the clinical situation<sup>[22]</sup>. Thus, further examinations are needed to identify factors influencing the anti-tumor effect of vitamin D on tumor cells.

The mitochondrial enzyme cytochrome P450 component of 25-hydroxyvitamin-D3-24-hydroxylase (CYP24A1), which is the major 1,25-D3-inactivating enzyme, is considered to be an essential factor determining the biological half-life of 1,25-D3. Previous immunohisto-

chemical studies have shown that the level of CYP24A1 rises significantly as the course of colorectal carcinogenesis progresses<sup>[20,23]</sup>. This fact might explain why 1,25-D3 cannot exert its anti-tumor effect in many pathological situations. It has also been demonstrated that the higher the level of CYP24A1, the more malignant the CRC<sup>[24]</sup>. A concomitantly increased expression of the proliferation marker Ki-67 in human CRC samples suggests that the overexpression of CYP24A1 reduces the local availability of 1,25-D3, and hence its antiproliferative effect<sup>[24]</sup>. Other mechanisms to may be involved in the development of 1,25-D3 insensitivity such as the downregulation of the VDRs<sup>[25]</sup>.

In the present study, we set out to investigate the effects of 1,25-D3 on CRC cells after the inhibition of CYP24A1 activity.

## MATERIALS AND METHODS

### CYP24A1 inhibitors

The ability of tetralones to inhibit CYP24A1 is less than that of theirazole counterparts, but a greater degree of selectivity can be achieved with tetralones through the mechanism of their binding to the active site. Instead of binding to the heme iron, they interact with the active site if the enzyme through hydrogen bonds and van der Waals forces<sup>[26]</sup>. Thirteen new 2-substituted-benzyl-6-methoxy-1-tetralones synthesized in the Department of Organic Chemistry in Szeged were utilized in the present study.

The method employed for the preparation of the tetralones<sup>[27]</sup> involved the condensation of commercially available 6-methoxy-1-tetralone with benzaldehyde or a substituted benzaldehyde (Figure 1). 6-methoxy-1-tetralone (1) was dissolved in 4% ethanolic KOH solution, the appropriate benzaldehyde (2a-m) was added, and the reaction mixture was stirred at room temperature for 1-8 h until the starting material had disappeared (thin layer chromatography monitoring), and then allowed to stand overnight. The precipitate that formed was filtered off, washed with water, purified by flash chromatography on silica gel, and recrystallized from ethanol. The synthesis of the hydroxy derivative necessitated initial protection of the hydroxy group in the 4-hydroxybenzaldehyde with a tetrahydropyranyl group, which was stable under the basic ethanolic KOH condensation conditions. The protecting group was removed by heating with aqueous hydrochloric acid in a mixture of ethyl acetate and ethyl methyl ketone. In the next step, the 2-substituted-benzylidene-6-methoxy-1-tetralones (3a-m) were dissolved in ethyl acetate, and hydrogenated at 1 atm in the presence of Pd/C as catalyst for 1 h at room temperature. The catalyst was subsequently removed by filtration through a bed of silica gel, the solvent was evaporated *in vacuo*, and purification by flash chromatography on silica gel furnished the 2-substituted-benzyl-6-methoxy-1-tetralones (4a-m).

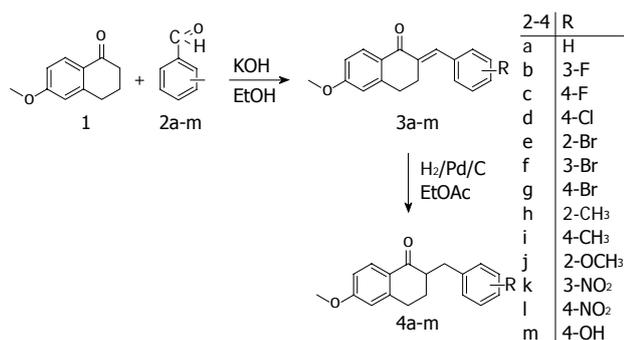


Figure 1 Outline of the procedure for the synthesis of the tetralones.

The resulting tetralones were dissolved individually in dimethyl sulfoxide at a concentration of 10 mmol/L and stored at 4 °C until use. In cell culture experiments, compounds (4a-m) were dissolved in sterile culture medium (GIBCO's OPTI-MEM, Life Technologies-Invitrogen, Carlsbad, CA, United States) to the desired concentration. 1,25-D3 at 1 and 10 nmol/L and an untreated control were also applied in these experiments.

### Cell culturing

The human epithelial colorectal adenocarcinoma cell line Caco-2 obtained from ECACC was maintained in Dulbecco's Modified Eagle Medium (D-MEM, Sigma, St. Louis, MO, United States) supplemented with 10% fetal calf serum (FCS, Sigma) and 1% antibiotic, antimycotic solution (Sigma) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were cultured in 6-, 24- and 96-well plates, and all measurements were carried out in triplicate. The cell line was genotyped and identified as Caco-2 in 2011 on the basis of the results of STR analysis (DSMZ Profile Database, www.dsmz.de). Twenty-four hours before treatment, the medium was changed to GIBCO's OPTI-MEM (Life Technologies-Invitrogen, Carlsbad, CA, United States). All experiments were carried out with cells from passages 5-25.

### Cell viability assay

The protein dye sulforhodamine-B (SRB), was used to test various tetralone derivatives in various concentrations for various incubation times in 96-well plates to determine the effects of the compounds alone and in the presence of 1,25-D3 on the Caco-2 cell number. After removal of the culture medium, 100 µL of trichloroacetic acid was used to fix the cells during an incubation period of 30 min. The plates were then rinsed 5 times with distilled water. The cells were stained with a 0.4% solution of SRB (Sigma) in acetic acid for 30 min. After removal of the excess dye solution the plates were rinsed 4 times with 1% acetic acid solution and allowed to dry at room temperature. The bound SRB was dissolved in unbuffered Tris-Sol and the plates were shaken for 5 min. The plates were measured in an Infinite M200 reader (Tecan AG, Männedorf, Switzerland) at 520 nm.

### Cytotoxicity measurement

Levels of cytotoxicity were quantified after treatment through measurement of the lactate dehydrogenase (LDH) levels in the wells by using the Cytotoxicity Detection Kit<sup>PLUS</sup> (Roche, Indianapolis, IN, United States). The greater the number of cells that die due to the cytotoxic effect, the higher the amount of LDH in the medium. The experiments were carried out in accordance with the kit manufacturer's instructions.

### Cell proliferation assays

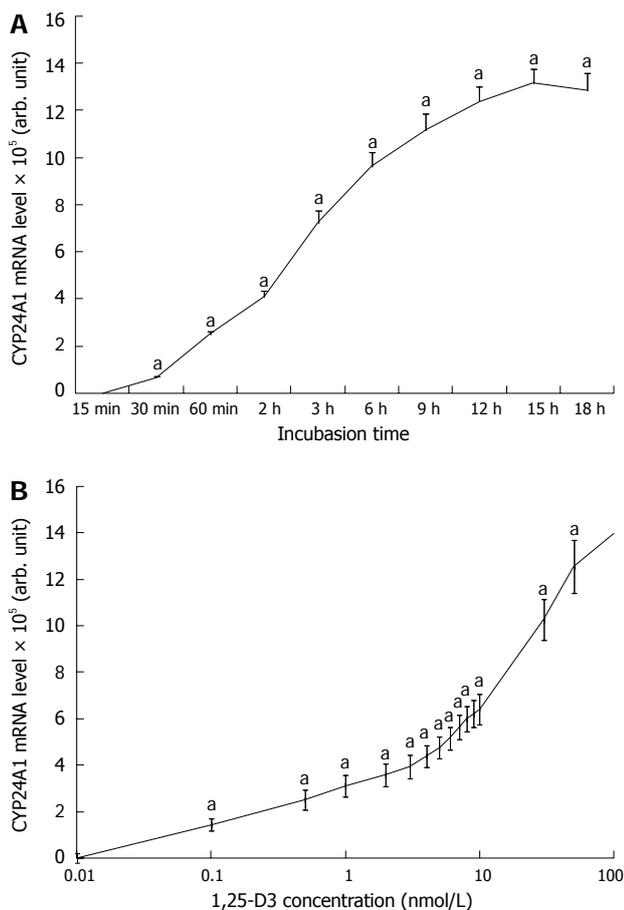
Cell proliferation was quantified by measurement of the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the cellular DNA by means of Cell Proliferation enzyme-linked immunosorbent assay, BrdU (colorimetric) (Roche). The experiments were carried out in accordance with the manufacturer's instructions.

### RNA isolation and Taqman probe-based real-time RT-PCR

RNA was isolated through use of the High Pure RNA Isolation Kit (Roche) as prescribed in the manufacturer's instructions. The isolated RNA was translated by using Moloney murine leukemia virus reverse transcriptase in accordance with the manufacturer's instructions (Promega, Madison, WI, United States). Predesigned and validated gene-specific TaqMan Gene Expression Assays from Life Technologies (Life Technologies, Foster City, CA, United States) were used in triplicate for quantitative real-time polymerase chain reaction (PCR) according to the manufacturer's protocol. Each set contained gene-specific forward and reverse primers and fluorescence-labeled probes. The probes span an exon junction and do not detect genomic DNA [ABI Taqman assay No's are hs00167999\_m1 and hs99999905\_m1, for CYP24A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively]. The PCR assays were carried out with the following protocol: denaturation for 10 min at 95 °C, and 45 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. The PCR reaction volume of 20 µL contained 2 µL cDNA, 10 µL of TaqMan 2x Universal PCR Master Mix NoAmpErase UNG (Life Technologies), 1 µL of gene-specific TaqMan Gene Expression Assay Mix and 7 µL of water. GAPDH was used as a housekeeping gene to normalize for RNA loading. Samples were analyzed using the ABI Prism 7500 real-time PCR system (Life Technologies). Relative quantification (RQ) studies were carried out on collected data (threshold cycle numbers, referred to as Ct) with the 7500 System SDS software 1.3 (Life Technologies).

### Statistical analysis

Data were analyzed by using SPSS for Windows, release 18 (IBM, Armonk, NY, United States). Final data are presented as the means ± SD of at least three independent measurements. Statistical analysis was performed with the unpaired Student *t*-test; results with *P* ≤ 0.05 were con-



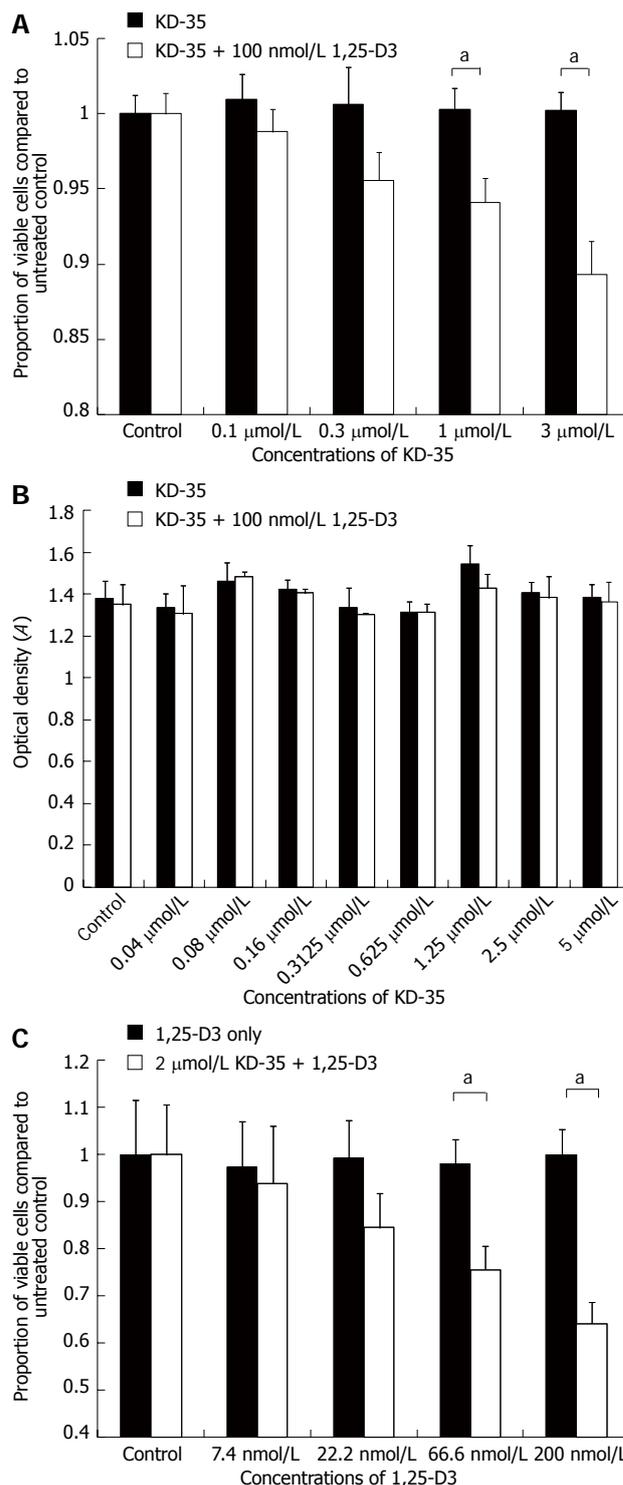
**Figure 2** Time and dose dependent-changes in CYP24A1 mRNA expression in response to 1,25-D3 administration. **A:** Time course of changes in the cytochrome P450 component of the 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1) mRNA expression in Caco-2 cells after the addition of 100 nmol/L active vitamin D3 metabolite 1,25-dihydroxyvitamin D3 (1,25-D3) to the cell culture supernatant. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized CYP24A1 expression levels are shown as a percentage of the CYP24A1 level of the untreated control cells. Points indicate means ± standard deviation (SD) (<sup>a</sup>*P* < 0.05 vs untreated control); **B:** Dose-dependent changes in CYP24A1 mRNA levels in Caco-2 cells after the addition of different amounts of 1,25-D3. GAPDH-normalized CYP24A1 expression levels are shown as a percentage of the CYP24A1 level of the untreated control cells. Points indicate means ± SD (<sup>a</sup>*P* < 0.05 vs untreated control).

sidered statistically significant.

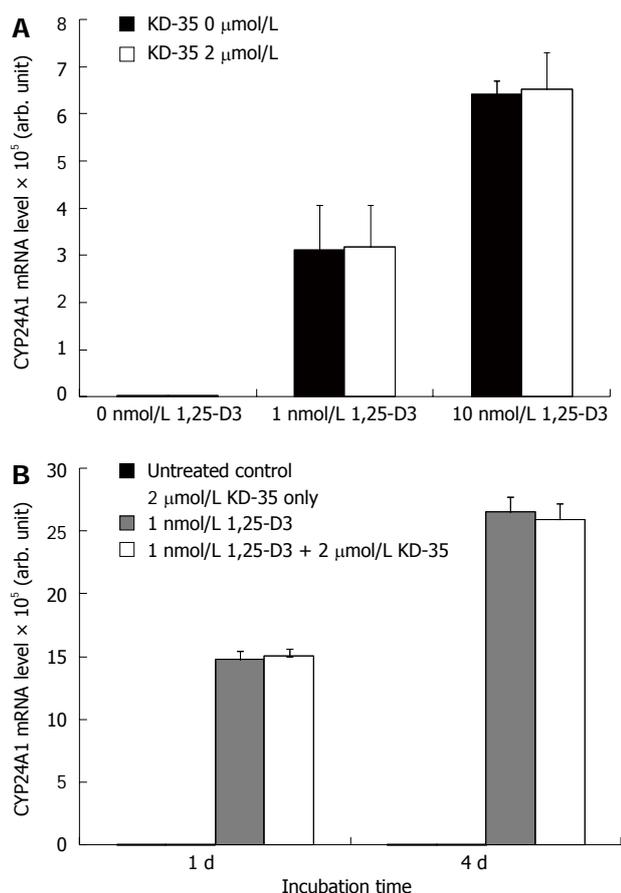
## RESULTS

### Time and concentration-dependent changes in CYP24A1 mRNA expression after vitamin D3 treatment

An increase in CYP24A1 mRNA level of six orders of magnitude was observed after a brief period of 1,25-D3 treatment. The increase in CYP24A1 mRNA expression was very rapid and it could be observed after 30 min of 1,25-D3 administration, and reached a maximum after 12-16 h of incubation (Figure 2A). After 4 h of incubation in the presence of 1 and 10 nmol/L 1,25-D3, the level of CYP24 mRNA was elevated to 311405-fold and 612801-fold, respectively, relative to the untreated controls (Figure 2B).



**Figure 3** Cell proliferation, lactate dehydrogenase activity and proliferation studies in the presence of KD-35 and 1,25-D3. **A:** Changes in the number of viable Caco-2 cells (sulforhodamine-B staining) in the presence of different concentrations of KD-35. Selected wells were treated with 100 nmol/L active 1,25-D3. Data are means ± SD (<sup>a</sup>*P* < 0.05 between KD-35 and KD-35 + 1,25-D3 treated cells); **B:** Changes in the lactate dehydrogenase (LDH) activity of the cell culture supernatant in response to KD-35 with or without 1,25-D3. Data are means ± SD. No significant changes in LDH activity were seen after treatment; **C:** Changes in the proliferation of Caco-2 cells (5-bromo-2'-deoxyuridine incorporation) in response to different concentrations of 1,25-D3. White bars indicate combined treatment with the given 1,25-D3 concentration + 2 μmol/L KD-35. Data are means ± SD. Significance levels were calculated between each sample and the untreated control sample (<sup>a</sup>*P* < 0.05 between 1,25-D3 and 1,25-D3 + KD-35 treated cells).



**Figure 4** KD-35 has no effect on CYP24A1 mRNA expression. A: Changes in CYP24A1 mRNA levels in Caco-2 cells incubated with different concentrations of 1,25-D3 for 4 h with or without KD-35. Data are means  $\pm$  SD. No significant changes in mRNA levels were seen with or without KD-35; B: Effects of KD-35 and KD-35 + 1,25-D3 on CYP24A1 mRNA expression in Caco-2 cells. Data are means  $\pm$  SD. No significant change in mRNA levels was seen with or without KD-35 at any time point.

#### Effects of tetralone derivatives on Caco-2 cell line

Certain of the tetralones were found to decrease the Caco-2 cell viability but only after 2-4 d of incubation with 1,25-D3. These compounds were tested at various concentrations for various periods to optimize the effect of 1,25-D3 in reducing the total Caco-2 cell count. Finally, compound KD-35 was selected for further and detailed investigations.

#### Effects of KD-35 on Caco-2 cell line

When Caco-2 cells were incubated for 4 d in the presence of 100 nmol/L 1,25-D3 with 0.1, 0.3, 1 or 3  $\mu$ mol/L KD-35, the cell number was reduced by 2.17%, 5.07%, 6.18% and 10.93%, respectively, relative to the controls treated with only 100 nmol/L 1,25-D3 or 3  $\mu$ mol/L KD-35 (Figure 3).

#### Results of the cytotoxicity test

To determine the cause of the decrease in viable cell number in the presence of KD-35 and 1,25-D3, we measured LDH concentration in the cell suspension. The concentration of KD-35 ranged between 0.04  $\mu$ mol/L

and 5  $\mu$ mol/L. Half of the wells were treated with KD-35 and 100 nmol/L 1,25-D3, the other half were treated with KD-35 only. All experiments were carried out in triplicate. Incubation lasted for 4 d. In all of the experimental setups, the LDH concentrations did not differ significantly in the presence of KD-35 alone or in combination with 1,25-D3 (Figure 3).

#### Results of the cell proliferation assay

In the presence of 2  $\mu$ mol/L KD-35, the following concentrations of 1,25-D3 were used: 7.4, 22.2, 66.6 and 200 nmol/L. Half of the wells were treated only with 2  $\mu$ mol/L KD-35. Incubation lasted for 4 d. After incubation, the 5-BrdU label was added for an additional 2 h. The reduction in cell number relative to the control was 3.43%, 14.81%, 22.49% and 35.81%, respectively, compared to the wells with 1,25-D3 only (Figure 3).

#### Changes in CYP24A1 mRNA expression

The amount of CYP24A1 mRNA expressed in the presence of various concentrations of KD-35 did not differ from that of the untreated controls. The CYP24A1 mRNA expression did not depend significantly on the duration of incubation with KD-35 (Figure 4).

## DISCUSSION

We have identified a new tetralone compound, KD-35, that effectively and markedly stimulates the anti-proliferative effect of 1,25-D3 in the CRC cell line Caco-2.

CYP24A1, a member of the cytochrome P450 (CYP450) enzyme superfamily is the key enzyme in the metabolism of vitamin D neutralizing the active metabolite 1,25-D3, and thereby controlling its concentration in the tissues. The CYP450 enzymes all display an iron-containing heme domain at the active site. There are two types of enzyme blockers: azoles and non-azoles<sup>[28]</sup>. The *N*-heterocyclic ring of azoles is linked directly to the iron in the heme domain and, although this inhibition is very potent, it is not selective. Since the other enzymes involved in vitamin D metabolism (CYP27A1 and CYP27B1) are also members of the CYP450 superfamily, this type of nonselective inhibition is not specific for CYP24A1.

The enzyme inhibitory effect of non-azoles is mediated through hydrogen bonds and hydrophobic interactions with the active site of the enzyme. This is a more flexible mechanism which may permit significant selectivity though the inhibitory effect may be less than that of azoles<sup>[20]</sup>. We investigated 13 tetralones (non-azoles) in a search for a compound that is effective locally in the colon and is not strongly absorbed, so that the risk of adverse systemic effects is minimized.

Most of the 13 tetralones were either toxic or ineffective, even in the presence of 1,25-D3. Only in the presence of KD-35 did 1,25-D3 markedly inhibit Caco-2 cell proliferation without pronounced cytotoxicity of the tetralone alone. Such inhibition was not observed in the absence of KD-35. Unfortunately, two of the three

most effective tetralones exhibited much higher cytotoxicity at higher concentrations than KD-35. The question arises as to whether KD-35 exerts its effect *via* CYP24A1 inhibition. We did not measure CYP24A1 enzyme activity directly since this is technically extremely difficult. It is also complicated to measure the intermediates of the CYP24A1 reaction. Moreover, a simple enzyme kinetic measurement would not reveal whether the compound enters the cell. We therefore chose an indirect approach: to prove the biological efficacy of the compound. KD-35 was found to exert an effect that allowed 1,25-D3 to reduce Caco-2 cell proliferation effectively, as reflected by an altered BrdU incorporation. Direct cytotoxicity was excluded by the LDH measurements, and no change in CYP24A1 mRNA expression was detected in response to KD-35, which ruled out alterations in protein synthesis. Obviously, no direct evidence was obtained to support direct enzyme inhibition, but an alternative mechanism is highly unlikely with this non-azole.

Two major pathways are mediated through the VDRs: the Wnt-beta-catenin pathway, which is responsible for the loss of adherent cell type, and the E-cadherin pathway, which is responsible for cell-to-cell adhesion and cell differentiation<sup>[29,30]</sup>. The administration of 1,25-D3 suppresses the Wnt-beta-catenin pathway and induces the expression of E-cadherin. The Wnt-beta-catenin pathway is constitutionally overregulated in most CRCs, due to the mutation of several members of the pathway (APC, AXIN2, *etc.*)<sup>[29]</sup>. There are other participants in colorectal carcinogenesis, such as estrogen receptors, which elevate the number of VDRs in the mucosal cells of the alimentary tract, or SNAIL, which inhibits the E-cadherin pathway and expression of VDRs<sup>[30-35]</sup>. Another important factor in the mucosal cell transition toward adenocarcinoma is an elevated level of CYP24A1, the intracellular concentration of which correlates with the dignity of the tumor<sup>[24]</sup>.

Our results corroborate the earlier finding<sup>[36]</sup> that the presence of 1,25-D3 dramatically stimulates the expression of CYP24A1 in CRC cells<sup>[24,37]</sup>. Two vitamin D-responsive elements are present in the promoter region of CYP24A1<sup>[38]</sup>. Through this pathway, 1,25-D3 stimulates its own destruction through metabolism into inactive forms by enhancing the expression of CYP24A1<sup>[39]</sup>.

Besides the genomic effects, there have also been reports of immediate nongenomic mechanisms. A possible mode of action is activation of the RhoA-ROCK-p38MAPK-MSK signaling pathway. This pathway mediates the induction of CST5, which is possibly responsible for tumor suppression and the level of CYP24A1; as a negative feedback mechanism, this eliminates 1,25-D3 from the cell<sup>[40]</sup>. VDRs found in other tumor cell membranes may bind 1,25-D3, and the complex could induce a rapid influx of Ca<sup>2+</sup> into the cell<sup>[40]</sup>, which activates RhoA-ROCK and then the p38MAPK-MSK-1 pathway. Besides the nongenomic activation of this pathway, a vitamin D-responsive element can also be identified in the -1k promoter region of the *RhoA* gene (<http://www.cbil.upenn.edu/cgi-bin/tess/tess>).

RhoA plays an important role in the induction of CDH1/E-cadherin, which is crucial for the acquisition of the polarity and adhesive phenotype of cancer cells<sup>[29]</sup>.

In view of these data, the elevation of CYP24A1 expression might be a self-defense mechanism of tumor cells. By inhibiting the inactivating enzyme, the amount of active vitamin D or its analogs required to elicit their marked anti-tumor effect could be reduced *in vivo*, thereby preventing elevation of the serum Ca<sup>2+</sup> level and avoiding hypercalcemia<sup>[36]</sup>. The inhibition of CYP24A1 may allow 1,25-D3 to exert its anti-tumor effect, in this way leading to a new approach in the treatment of CRC in the future.

## COMMENTS

### Background

The effects of vitamin D3 have been investigated on various tumors, including colorectal cancer (CRC). The cytochrome P450 component of 25-hydroxyvitamin D3-24-hydroxylase (CYP24A1), the enzyme that inactivates the active vitamin D3 metabolite 1,25-dihydroxyvitamin-D3 (1,25-D3) is considered to be the main enzyme determining the biological half-life of 1,25-D3. During colorectal carcinogenesis, the expression and concentration of CYP24A1 increases significantly, suggesting that this phenomenon could be responsible for the controversial efficacy of 1,25-D3 in the treatment of CRC. In the present study, authors set out to investigate the effects of 1,25-D3 on CRC cells after the inhibition of CYP24A1 activity.

### Research frontiers

The anti-tumor effect of vitamin D3 has been a focus of interest during the last 10-15 years. However, vitamin D3 cannot exert this important effect in a number of tumors. The reasons for this have been investigated intensively. One possible explanation for the reduced anti-tumor efficacy of vitamin D3 is the accelerated neutralization of the active vitamin D3 compound in certain cases, *e.g.*, CRC, liver and papillary thyroid cancers.

### Innovations and breakthroughs

The authors synthesized a number of compounds potentially able to inhibit the action of CYP24A1, the enzyme neutralizing the effects of vitamin D3. One of these compounds, KD-35, had inhibitory potential without an apparent toxic effect. In the presence of KD-35, vitamin D3 markedly inhibited the growth of CRC cells.

### Applications

Selective inhibition of the CYP24A1 by compounds such as KD-35 may permit a new approach to enhancement of the anti-tumor effect of 1,25-D3 on CRC.

### Peer review

The authors tackled an interesting topic for investigation. The manuscript is investigating the association between CYP24A1 inhibition and anti-tumor effect of 1a, 25-dihydroxyvitamin-D3 in Caco-2 CRC line. A careful assessment was considered using appropriate cell assays. A major finding of the study was that Caco-2 cell viability and proliferation were markedly reduced in response to 1,25-D3 when the CYP24A1 was inhibited (by KD-35, one of the tetralone compounds).

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**P-Reviewers** Braet F, Lakatos PL **S-Editor** Gou SX  
**L-Editor** Cant MR **E-Editor** Zhang DN



## Establishment of mouse intestinal myofibroblast cell lines

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Supported by Uehara Memorial Foundation and Mishima Kaiun Memorial Foundation; A Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Culture, Sports, Science and Technology

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Received: November 13, 2012 Revised: December 3, 2012

Accepted: January 11, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To establish novel intestinal myofibroblast (IMF) cell lines from mouse colonic mucosa and investigate their biological characters.

**METHODS:** Primary IMFs were isolated from mucosal tissues of mouse colon that was denuded of epithelial cells and smooth muscle layer. For immortalization, primary IMFs were transfected with simian virus 40 large T antigen (designated as LmcMF). We also isolated some primary IMFs that spontaneously became immortalized without transfection (designated as SmcMF). To check immortality and normality of these cells, we examined their proliferative ability and contact inhibition. Moreover, the expression levels of proteins characterizing IMFs [including  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), vimentin, desmin, and type I collagen] and proteins associated with the immune response [such as toll-like

receptor 4 (TLR-4), CD14, MD2,  $I\kappa B\alpha$ , and p-p38] were determined by Western blotting. The localization of several myofibroblast protein markers was also detected by immunofluorescence staining.

**RESULTS:** The cell growth assay results show that both LmcMF and SmcMF cells proliferated logarithmically at least up to passage 20. In addition, the contact inhibition assays show that LmcMF and SmcMF stopped growing after the cells reached confluence. These data suggest that these 2 types of cells were immortalized without losing contact inhibition of growth. Moreover, both LmcMF and SmcMF, like primary IMFs, showed spindle-shaped appearance. The expression levels of key myofibroblast protein markers, including  $\alpha$ -SMA, vimentin, and desmin, were also examined by the Western blotting and immunofluorescence analyses. Our results show that these cells were positive for  $\alpha$ -SMA and vimentin, but not desmin, as well as that both LmcMF and SmcMF expressed type I collagen at a lower level than primary IMFs. Finally, we investigated the expression level of lipopolysaccharide (LPS) receptor-related proteins, as well as the response of the cells to LPS treatment. We found that the TLR4, CD14, and MD-2 proteins were present in LmcMF and SmcMF, as well as in primary IMFs, and that all these cells responded to LPS.

**CONCLUSION:** We established 2 novel IMF cell lines from mouse colonic mucosa, namely, LmcMF and SmcMF, both of which were able to respond to LPS.

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**Key words:** Cell line; Colon; Lipopolysaccharide; Mouse; Myofibroblast

Kawasaki H, Ohama T, Hori M, Sato K. Establishment of mouse intestinal myofibroblast cell lines. *World J Gastroenterol* 2013; 19(17): 2629-2637 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2629.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2629>

## INTRODUCTION

The gastrointestinal mucosa is in contact with the extracorporeal environment and is exposed to various antigens and molecules that are mainly derived from ingested food and commensal bacteria<sup>[1]</sup>. Such antigens do not induce inflammation in healthy individuals, because intestinal epithelial cells form a physical barrier protecting against luminal bacteria and toxic substances. To achieve this protective function, differentiation and proliferation of epithelial cells need to be properly regulated. Accumulating evidence has recently revealed that subepithelial intestinal myofibroblasts (IMFs), which are located subjacent to the epithelium, play crucial roles in regulating epithelial cells<sup>[2-4]</sup>.

IMFs belong to the myofibroblast family, which includes several functionally related cells, such as lung contractile interstitial cells, pancreatic stellate cells, and orbital and synovial fibroblasts<sup>[5]</sup>. IMFs are spindle-shaped or stellate cells that exhibit phenotypic characteristics of both fibroblasts and smooth muscle cells<sup>[6,7]</sup>. In addition, it has been shown that IMFs, like other mesenchymal cells, synthesize collagen<sup>[6,8,9]</sup>. Notably, IMFs orchestrate diverse events in gastrointestinal health and diseases, including epithelial differentiation and development, mucosal repair, carcinogenesis, and inflammatory responses<sup>[3,4,10]</sup>. IMFs express toll-like receptor 4 (TLR-4), which is activated by lipopolysaccharide (LPS), a component of gram-negative bacteria, subsequently leads to secretion of proinflammatory mediators<sup>[10-13]</sup>. Moreover, IMFs, as nonprofessional antigen-presenting cells in the intestinal mucosa, induce proliferation of both resting CD4<sup>+</sup> T cells and regulatory T cells in a major histocompatibility complex (MHC) class II-dependent manner to maintain intestinal mucosal tolerance<sup>[14,15]</sup>. Furthermore, enterotoxins are known to engage MHC class II molecules on IMFs, which in turn leads to secretion of inflammatory mediators<sup>[16]</sup>.

Despite the crucial role of IMFs in gastrointestinal health and diseases, the understanding of their underlying regulatory mechanism is limited. One of the major issues that hinder the advance of IMF research is the lack of IMF cell lines that show myofibroblastic phenotypes without stimulation. Typically, primary IMFs isolated from intestine are used within passages 2-6, because their phenotypes change and the cells stop growing after repeated passages. In addition, CCD-18Co cells, which have been used as a human colon myofibroblast cell line, still need to be treated with transforming growth factor- $\beta$  (TGF- $\beta$ ) for the expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)<sup>[17]</sup>. In this present study, to address these issues, we separated mouse intestinal myofibroblast cell lines (namely, LmcMF and SmcMF) and examined their properties.

## MATERIALS AND METHODS

### Culture of mouse intestinal myofibroblasts and mouse embryonic fibroblasts

C57BL/6J mice purchased from Charles River Japan

(Yokohama, Japan) were maintained in compliance with the guidelines of the Animal Care and Use Committee of Yamaguchi University. Mouse IMFs were isolated as previously described, with slight modifications<sup>[18]</sup>. Briefly, a segment of the proximal colon was detached from the mesenterium, and mucosal layers were completely denuded of epithelial cells by repeated ( $\times 3$ ) 30-min incubation in 1 mmol/L EDTA-Hanks' balanced salt solution at 37 °C. Subsequently, smooth muscle layers were detached from mucosal layers with tweezers. De-epithelialized mucosal samples were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Tokyo, Japan) containing 100 mL/L fetal bovine serum (FBS) at 37 °C in a 50 mL/L CO<sub>2</sub> atmosphere. Consequently, myofibroblast cells that migrated from mucosal tissues formed colonies. In this study, primary IMFs at passages 2-6 were used. Mouse embryonic fibroblasts (MEFs) isolated from embryos of C57BL/6J mice at embryonic day 11.5 were also grown under the same condition described above.

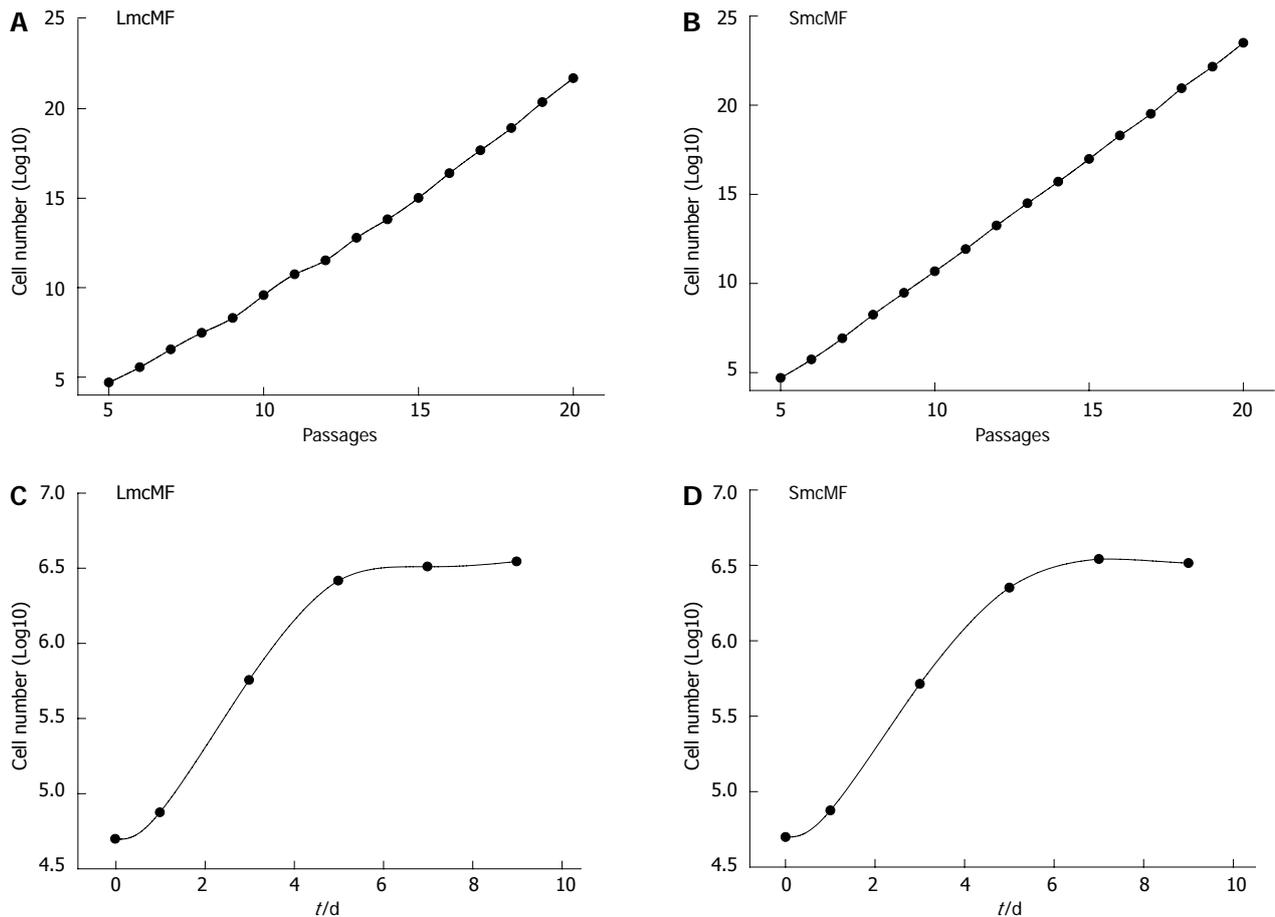
### Antibodies and cell lines

The following antibodies were used in this study: anti- $\alpha$ -SMA and anti-vimentin antibodies from Sigma (Tokyo, Japan); anti-desmin and anti-tubulin antibodies from Thermo Scientific (Yokohama, Japan); anti-TLR4, anti-CD14, and anti-actin antibodies from Santa Cruz (CA, United States); anti-MD2 antibody from AbD Serotec (Kidlington, United Kingdom); anti-I $\kappa$ B $\alpha$  and anti-p-38 antibodies from Cell Signaling (MA, United States); anti-Collagen type I antibody from Merck (Tokyo, Japan); anti-valosin-containing protein (VCP) antibody from Gene Tex (CA, United States); horseradish peroxidase (HRP)-conjugated mouse anti-rabbit Ig Light Chain from ECM biosciences (KY, United States); and HRP-conjugated donkey anti-mouse IgG from R and D Systems (MN, United States). A mouse mammary gland epithelial cell line, Eph4, and a mouse macrophage-like cell line, RAW264.7, were grown in DMEM supplemented with 100 mL/L FBS at 37 °C in a 50 mL/L CO<sub>2</sub> atmosphere.

### Immortalization of IMFs

EGIP-EF1a-Large T-IRES-Puro, a lentiviral plasmid expressing simian virus 40 (SV40) large T antigen (LT) but not small T antigen (ST), was obtained from Addgene (ID No. 18922; MA, United States)<sup>[19]</sup>. Using Lipofectamine LTX (Invitrogen), lentivirus was produced by transfecting Lenti-X 293T cells in 60-mm dishes with 3  $\mu$ g of EGIP-EF1a-Large T-IRES-Puro, 2.3  $\mu$ g of a packaging plasmid (psPAX2), and 1.3  $\mu$ g of a coat protein plasmid expressing vesicular stomatitis virus G protein (pDM2.G). Viral supernatants were collected after 48 h, and filtered (0.22  $\mu$ m). Primary IMFs were infected with virus for 8 h. With puromycin treatment for 6 d, immortalized LT-positive cells were selected and designated as LmcMF.

To obtain spontaneously immortalized IMFs, pri-



**Figure 1** Proliferation and contact inhibition of LmcMF and SmcMF. A, B: The cells were subcultured every 3 d, and the growth rate of LmcMF (A) and SmcMF (B) were assessed as described; C, D: Contact inhibition of LmcMF (C) and SmcMF (D).

many IMFs were repeatedly passaged until cells that kept proliferating emerged. These spontaneously immortalized cells were designated as SmcMF.

### Cell growth and contact inhibition assays

To determine the proliferative ability of IMF cells,  $5 \times 10^4$  cells were plated in 35-mm dishes and passaged every 3 d, and the number of cells was subsequently counted using a hemocytometer. For the contact inhibition assay,  $5 \times 10^4$  cells were plated in 35-mm dishes, and the number of cells was counted on the next day and then every other day.

### Immunofluorescence staining

Primary IMFs, as well as LmcMF and SmcMF, were grown on glass coverslips and subsequently fixed with 4% formaldehyde for 20 min at room temperature. Cells were permeabilized with 0.2% Triton X-100 in PBS-T (PBS containing 0.05% Tween 20) for 60 s and then blocked with 3% skim milk in PBS-T. After incubation with the first antibodies overnight at 4 °C, Alexa594-conjugated secondary antibodies (Alexa Fluor<sup>®</sup> 594 goat anti-mouse IgG or Alexa Fluor<sup>®</sup> 594 donkey anti-goat IgG; Invitrogen) were added and incubated for 1 h at room temperature. Nuclei were counterstained with SYTOX Green (Life Technologies, CA, United States).

Finally, fluorescence images were captured by a confocal laser-scanning microscope (LSM510; Zeiss, Tokyo, Japan).

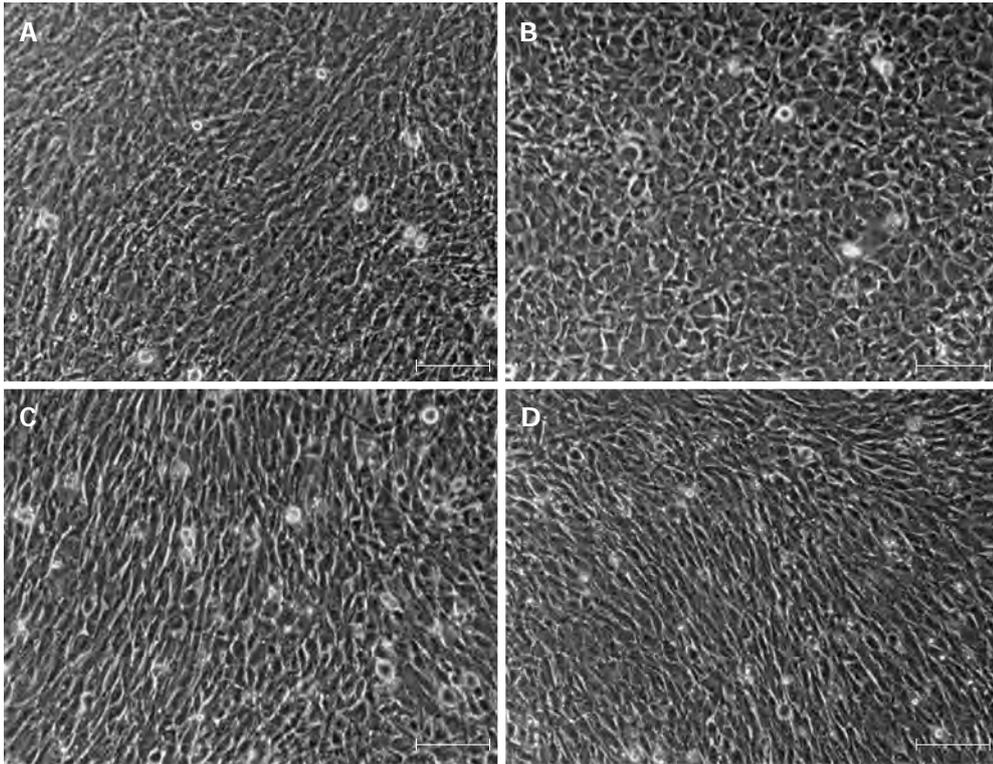
### Western blotting

The western blot analysis was performed as previously described<sup>[18]</sup>. Briefly, cells were lysed in a buffer consisting of 50 mmol/L Tris-HCl (pH 8.0), 5 mmol/L EDTA (pH 8.0), 5 mmol/L EGTA (pH 8.0), 1% Triton X-100, 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 20 mmol/L sodium pyrophosphate, and Roche Complete Protease Inhibitor Cocktail (Roche, Tokyo, Japan). Amersham Hybond ECL Nitrocellulose Membranes (GE Healthcare, Buckinghamshire, United Kingdom) were blocked with 0.5% skim milk and treated with specific antibodies. Protein bands were detected using the ECL Western blotting Detection Reagents (GE Healthcare) or the Western Lightning ECL Pro (PerkinElmer, MA, United States) and visualized using a LAS-3000 mini luminescence imager (Fujifilm, Tokyo, Japan).

## RESULTS

### Immortalization of IMFs without losing contact inhibition of growth

First, we investigated the immortalization of 2 differ-



**Figure 2** Phase-contrast microscopic images of primary intestinal myofibroblasts, mouse embryonic fibroblasts, LmcMF, and SmcMF. A: Primary intestinal myofibroblasts; B: Mouse embryonic fibroblasts; C: LmcMF; D: SmcMF were cultured to confluence, and the phase-contrast microscopic images were taken. Representative images are shown. Scale bars indicate 200  $\mu\text{m}$ .

ent IMF cell lines, LmcMF and SmcMF. The cells ( $5 \times 10^4$  cells) were seeded in 35-mm dishes and subcultured every 3 d during the examination of the cell growth rate. As shown in Figure 1A and B, both LmcMF and SmcMF cells proliferated logarithmically at least up to passage 20, and their doubling times were 19.1 and 17.2 h, respectively. Primary IMFs, on the other hand, immediately stopped growing under this experimental condition, since they require a high cell density for proliferation.

For the contact inhibition assay, the cells ( $5 \times 10^4$  cells) were seeded in 35-mm dishes and cultured up to 9 d (Figure 1C and D). The cells became confluent around day 5 and reached the plateau phase by day 6 or 7. These data indicate that LmcMF and SmcMF were immortalized without losing contact inhibition of growth.

#### **LmcMF and SmcMF show myofibroblastic phenotypes**

IMFs have been defined as spindle-shaped or stellate cells that are positive for  $\alpha$ -SMA and vimentin, and very weakly positive or negative for desmin<sup>[7]</sup>. As shown in Figure 2A and B, primary IMFs exhibited spindle-shaped appearance, whereas MEFs were round-shaped. Both LmcMF and SmcMF, like primary IMFs, showed spindle-shaped appearance (Figure 2C and D).

The expression levels of key myofibroblast protein markers, including  $\alpha$ -SMA, vimentin, and desmin, were subsequently examined by the Western blotting and immunofluorescence analyses. We observed that primary IMFs and LmcMF expressed an equivalent amount of

$\alpha$ -SMA, whereas the  $\alpha$ -SMA protein level in SmcMF was relatively low (Figure 3A and B). As expected,  $\alpha$ -SMA was not detectable in the mouse mammary gland epithelial cell line, Eph4, which was used as a negative control. We also found that vimentin was present in both LmcMF and SmcMF, almost at the same level as that in primary IMFs, but was not detectable in the negative control RAW 264.7 cells (Figure 3C and D). Moreover, desmin, though not present in LmcMF, was detected at very low levels in SmcMF, compared to its expression level in intestinal smooth muscle tissues (positive control) (Figure 3E and F). Furthermore, both LmcMF and SmcMF expressed type I collagen, however, at a lower level than that in primary IMFs (Figure 3G). As the negative control, RAW 264.7 cells did not express type I collagen. Overall, these results suggest that LmcMF and SmcMF share almost the same characteristics with intestinal myofibroblasts.

To confirm the expression pattern of  $\alpha$ -SMA and vimentin in IMFs, we next performed the immunofluorescence analysis. In a cell, actin transits between its globular (G-) and filamentous (F-actin) states, and G-actin can be visualized by immunofluorescence microscopy in a spreading pattern, because they are monomeric actin<sup>[20]</sup>. As shown in Figure 4A, we observed strong filamentous  $\alpha$ -SMA expression in primary IMFs. While LmcMF also showed filamentous  $\alpha$ -SMA fibers, the amount of globular  $\alpha$ -SMA was higher than that in primary IMFs. Most of the  $\alpha$ -SMA protein in SmcMF existed in a

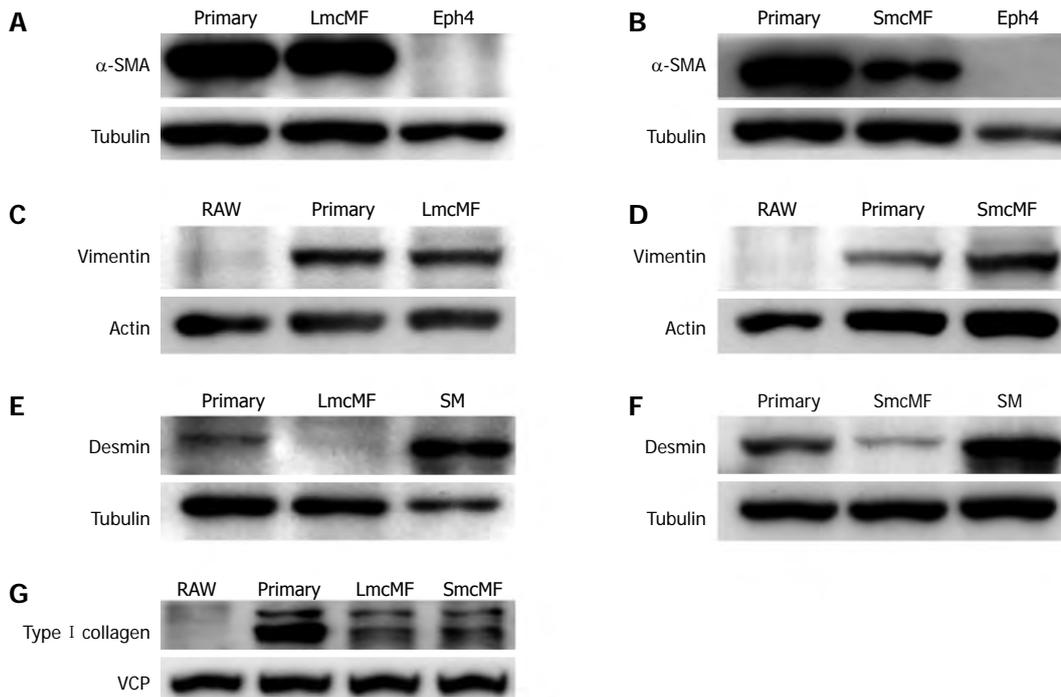


Figure 3 Expression levels of characteristic markers in LmcMF and SmcMF. A, B: The expression levels of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA); C, D: Vimentin; E, F: Desmin; G: Type I collagen in primary intestinal myofibroblast (primary); LmcMF (A, C, and E), and SmcMF (B, D, and F) were investigated by Western blotting. Representative blots from 2 independent experiments are shown. Tubulin, actin, and valosin-containing protein (VCP) were used as loading controls.

globular  $\alpha$ -SMA form. On the other hand, vimentin was predominantly localized around the nucleus of primary IMFs and LmcMF but diffused throughout SmcMF cells (Figure 4B).

### Responses of LmcMF and SmcMF to LPS

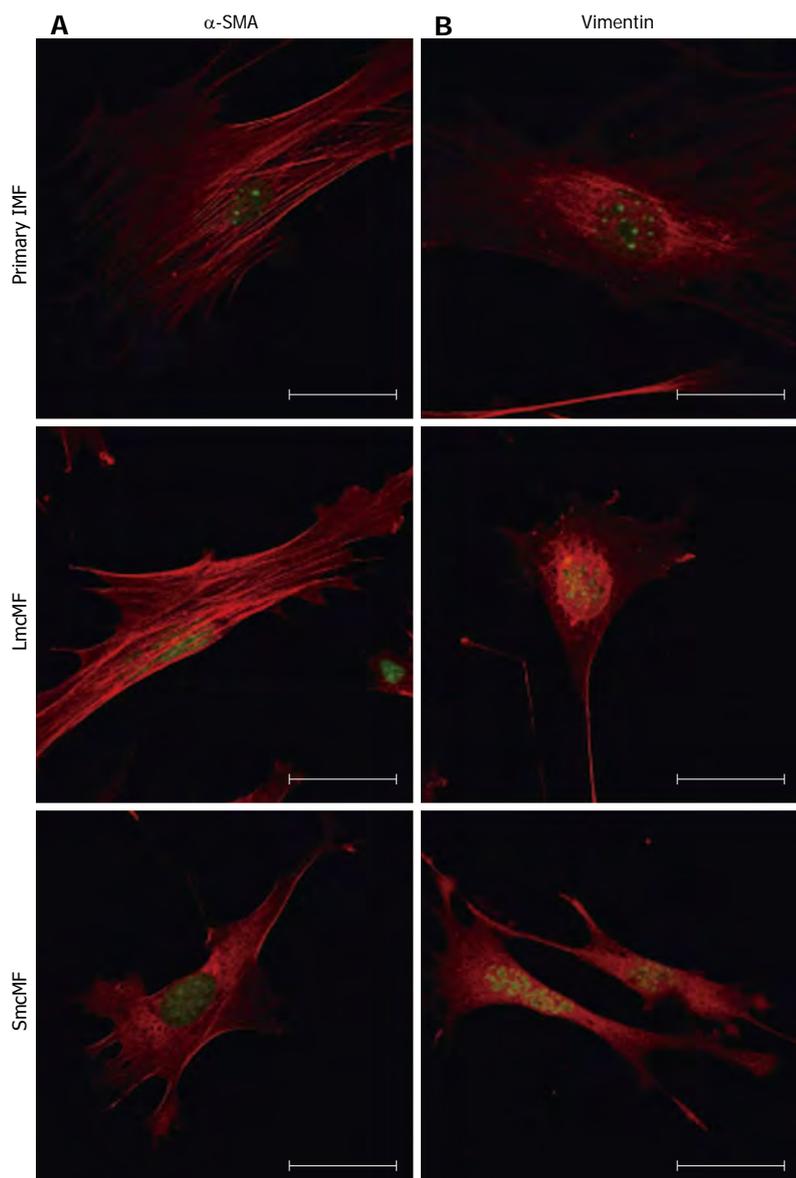
It is well known that the LPS receptor complex is composed of TLR4, CD14, and MD-2 and that both CD14 and MD-2 are required for the activation of TLR4<sup>[21]</sup>. A study has previously shown that primary IMFs expressed all these 3 components and that the exposure of cells to LPS activated the NF- $\kappa$ B and p38 MAPK pathways<sup>[10]</sup>; therefore, we assessed whether the same holds true for LmcMF and SmcMF. Our results show that although the expression level of TLR4 was low in primary IMFs, the levels in LmcMF and SmcMF were much higher (Figure 5A). As for CD14, the expression level was lower in LmcMF, compared to primary IMFs and SmcMF, and the expression level of MD-2 was relatively higher in primary IMFs and lower in SmcMF, compared to LmcMF.

Finally, we investigated the reactivity of primary IMFs, LmcMF, and SmcMF to LPS. We found that LPS treatment (20 ng/mL) induced the degradation of I $\kappa$ B $\alpha$ , as well as the phosphorylation of p38 MAPK (Figure 5B-D). While the maximal levels of I $\kappa$ B $\alpha$  degradation and p38 MAPK phosphorylation were detected at 30 min after LPS treatment, their expression was restored to basal levels within 2 h. Consistent with the previous study (performed in primary IMFs)<sup>[10]</sup>, our results demonstrate that both LmcMF and SmcMF preserve myofibroblastic phenotypes.

## DISCUSSION

IMFs play an important role in intestinal injury, inflammation, fibrosis, and tissue repair; however, their exact function and involvement are still not completely understood<sup>[6,8,10,22]</sup>. One issue that hinders the development of IMF research is the lack of IMF cell lines. In this present study, we established 2 IMF cell lines, namely, LmcMF and SmcMF, both of which were immortalized without losing contact inhibition of growth. LmcMF was established by transfecting primary IMFs with plasmid DNA encoding the LT of replication origin-defective SV40. It is known that 2 oncoproteins, LT and ST, encoded by the SV40 early region play an essential role in inducing transformation of mammalian cells<sup>[23]</sup>. In addition, previous studies have also shown that LT can immortalize cells but cannot induce neoplastic transformation without ST<sup>[23]</sup>. Moreover, it has been shown that immortalization of mouse cells can be achieved by transfecting only with LT<sup>[24]</sup>. Based on these findings, we used a plasmid that expresses LT, but not ST, for our immortalization experiments of IMFs. On the other hand, SmcMF was derived from cells that spontaneously immortalized. It has been reported that murine cells tend to immortalize spontaneously in culture, because mouse somatic cells have the telomerase activity<sup>[25]</sup>.

IMFs can be distinguished from other types of mesenchymal cells, such as smooth muscle cells and fibroblasts, by the expression of 3 protein markers:  $\alpha$ -SMA, vimentin, and desmin. It was reported that IMFs are positive for  $\alpha$ -SMA and vimentin, but negative or very

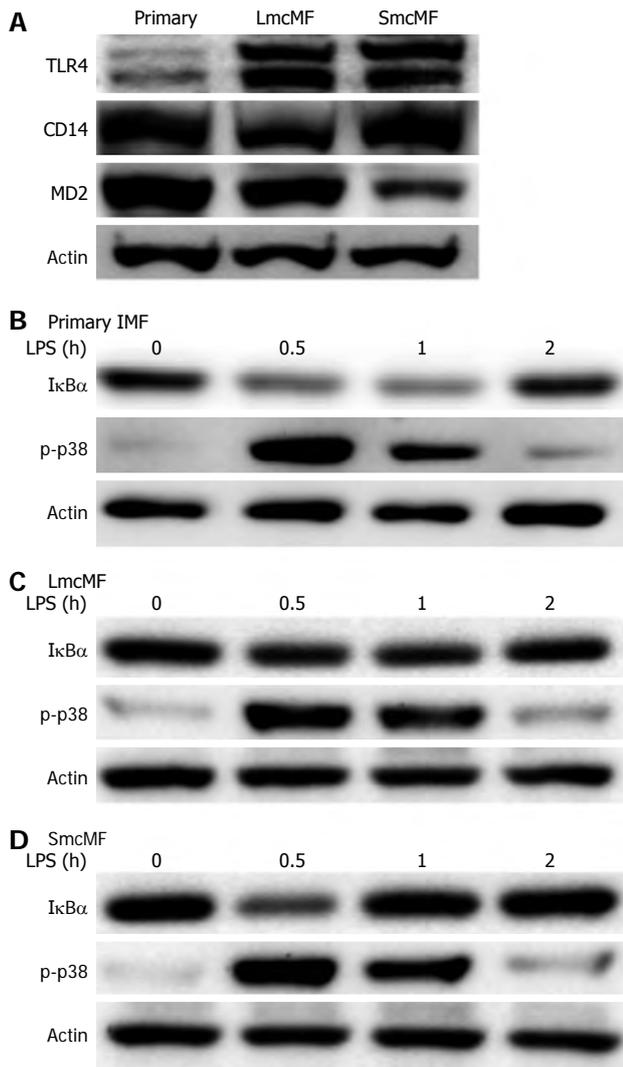


**Figure 4** Immunofluorescence staining for  $\alpha$ -smooth muscle actin and vimentin in primary intestinal myofibroblasts, LmcMF, and SmcMF. Primary intestinal myofibroblasts (IMFs), LmcMF, and SmcMF were immunostained with  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (A) and vimentin (B) (red). SYTOX Green was used for nuclear labeling (green). Representative images are shown. Scale bars indicate 40  $\mu$ m.

weakly positive for desmin<sup>[7]</sup>. In contrast, smooth muscle cells are  $\alpha$ -SMA-positive, vimentin-negative, and strongly positive for desmin, whereas typical fibroblasts, such as dermal fibroblasts, are negative for  $\alpha$ -SMA and desmin, but positive for vimentin<sup>[7,8,26]</sup>. In the present study, we observed that all 3 cell lines (primary IMFs, LmcMF, and SmcMF) expressed both  $\alpha$ -SMA and vimentin, suggesting that these cells share the same characteristics and that LmcMF and SmcMF can be used as intestinal myofibroblast cell lines. It is also noted that the doubling time of these cells is within a day, whereas that of human primary IMFs is about 5 d<sup>[27]</sup>. Therefore, the use of LmcMF and SmcMF will be helpful for improving the efficiency of IMF research.

There are, however, some differences among these cells. For instance, the  $\alpha$ -SMA expression level was

slightly lower in SmcMF, and  $\alpha$ -SMA was organized into stress fibers in primary IMFs and LmcMF, but not in SmcMF. The vimentin expression level was slightly higher in SmcMF, whereas the type I collagen levels in LmcMF and SmcMF were relatively lower than that in primary IMFs. We propose that these differences may depend on the differentiation stage of the cells. In the present study, very low levels of desmin were detected in primary IMFs and SmcMF. It has been reported that primary human colonic myofibroblasts and CCD-18Co cells do not express desmin<sup>[28]</sup>, whereas some lesion myofibroblasts do<sup>[7]</sup>. These differences in the desmin expression may occur by cell differentiation or by the differences between animal species tested (for example, mice *vs* humans); however, to clarify this, additional experiments are necessary.



**Figure 5** Expression of lipopolysaccharide-related proteins and responses of primary intestinal myofibroblasts, LmcMF, and SmcMF to lipopolysaccharide. **A:** The expression levels of indicated proteins in primary intestinal myofibroblasts (IMFs) (primary), LmcMF, and SmcMF were investigated by Western blotting; **B-D:** Primary IMFs (**B**), LmcMF (**C**), and SmcMF (**D**) were stimulated with lipopolysaccharide (LPS) (20 ng/mL) for indicated periods. The IκBα degradation and p38 MAPK phosphorylation were determined by Western blotting. Representative blots from 3-4 independent experiments are shown. Actin was used as a loading control.

The differentiation of fibroblasts into myofibroblasts is an important step in tissue repair and fibrosis<sup>[29]</sup>; however, it is a complex process and has not been completely elucidated. It was reported that CCD-18Co cells exhibit fibroblastic and myofibroblastic phenotypes and that TGF-β stimulates their differentiation into myofibroblasts<sup>[17]</sup>. CCD-18Co cells have also been used to investigate the mechanism of differentiation into myofibroblasts during wound healing processes. However, CCD-18Co cells poorly differentiate into myofibroblasts<sup>[30]</sup>; therefore, LmcMF and SmcMF may be useful to help reveal the role of differentiated myofibroblasts in tissue repair.

Accumulating evidence has shown that IMFs play a crucial role in local immune regulation in the intestine<sup>[10,14,15]</sup>; therefore, it is important that the developed cell lines show immune responses to external factors. Consistent with previous report<sup>[10]</sup>, all three types of IMF expressed TLR4, CD14, and MD-2. We also demonstrate that LmcMF and SmcMF, similar to primary IMFs, were able to respond to LPS stimulation.

In the present study, we observed that the TLR4 expression levels in LmcMF and SmcMF were higher than that in primary IMFs. It is possible that TLR4 was downregulated in primary IMFs and then recovered in LmcMF and SmcMF, since during the cell isolation procedure the intestinal tissues and cells were exposed to a large amount of LPS that existed in the intestinal lumen. Interestingly, although the TLR4 expression level in primary IMFs was low, LPS induced almost the same responses, including IκBα degradation and p38 MAPK phosphorylation, in all 3 types of IMFs. Together, our results indicate that the amount of TLR4 protein in primary IMFs was sufficient to respond to LPS.

In conclusion, we have successfully established 2 novel types of myofibroblast cell lines from mouse colon, namely, LmcMF and SmcMF, both of which expressed the key myofibroblast markers. Moreover, these cell lines were immortalized (rather than transformed to neoplastic cells) and were able to respond to LPS, like primary IMFs. Furthermore, these cell lines proliferate much faster than primary IMFs, and therefore they can be used efficiently. Nevertheless, for the development of IMF research using LmcMF and SmcMF, more experiments are necessary to determine their properties.

## COMMENTS

### Background

With respect to the gastrointestinal mucosa, accumulating evidence has shown that subepithelial intestinal myofibroblasts (IMFs), which are located subjacent to the epithelium, play crucial roles in regulating epithelial cells. IMFs orchestrate diverse events in gastrointestinal health and diseases, including epithelial differentiation and development, mucosal repair, carcinogenesis, and inflammatory responses. Therefore, it is important to establish IMF cell lines, which can be used for clarifying the role of IMFs.

### Research frontiers

Despite the crucial role played by IMFs in gastrointestinal health and diseases, the understanding of their underlying regulatory mechanism is limited. A major issue that hinders the advance of IMF research is the lack of IMF cell lines that exhibit myofibroblastic phenotypes. In this study, authors established 2 IMF cell lines, namely, LmcMF and SmcMF, both of which were immortalized without losing contact inhibition of growth, and were able to respond to lipopolysaccharide, like primary IMFs.

### Innovations and breakthroughs

For the IMF research, the 2 following cell types are available: freshly isolated IMFs (primary IMFs) and IMF-like cell lines (such as CCD-18Co cells). However, primary IMFs typically change phenotypes within passage 6 and stop growing after repeated passages. In addition, CCD-18Co cells need to be treated with TGF-β for the expression of key myofibroblast protein markers. To the best of our knowledge, this report is the first to establish novel types of myofibroblast cell lines from the mouse colon. Authors developed 2 cell lines, namely, LmcMF and SmcMF, both of which expressed the key myofibroblast markers without

stimulation.

### Applications

Because IMFs play crucial roles in regulating epithelial cells, it is important to develop IMF cell lines. The establishment of 2 cell lines is helpful for improving the efficiency of IMF research.

### Peer review

This study characterizes 2 IMF cell lines isolated from the mouse colon. This is a straightforward descriptive report and is methodological in nature; essentially, the authors have generated a potentially useful cell line resource that may be used to further myofibroblast research into tissue repair and fibrosis, immune regulation within the intestine, and other aspects of myofibroblast function. Because only a few good myofibroblast cell lines are currently available, it is important to create new myofibroblast cell lines for *in vitro* studies, and the authors have generated 2 IMF cell lines by using 2 different approaches.

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**P- Reviewers** MacLeod RJ, Spring KJ **S- Editor** Gou SX  
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## Expression of interleukin-22/STAT3 signaling pathway in ulcerative colitis and related carcinogenesis

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Supported by National Natural Science Foundation of China, No. 81072692 and Natural Science Foundation of Jiangsu Higher Education Institutions of China, No. 10KJB320007

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Received: August 14, 2012 Revised: January 31, 2013

Accepted: February 8, 2013

Published online: May 7, 2013

by Western blotting.

**RESULTS:** Patients with active UC had significantly more IL-22, IL-23, IL-22R1 and p-STAT3-positive cells than the patients with inactive UC and normal controls. Furthermore, IL-22 and related proteins were closely related to the severity of the colitis. The expression of IL-22 and IL-22R1 in the tissue of initial UC was stronger than in that of chronic UC, whereas the expression of p-STAT3 was significantly increased in chronic UC tissues. In dysplasia tissues, the expression level of IL-22 and related proteins was higher compared with controls. Mouse colitis model showed that expression of IL-22, IL-22R1 and IL-23 was increased with time, p-STAT3 and the downstream gene were also remarkably upregulated.

**CONCLUSION:** IL-22/STAT3 signaling pathway may be related to UC and UC-induced carcinogenesis and IL-22 can be used as a biomarker in judging the severity of UC.

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**Key words:** Ulcerative colitis; Ulcerative colitis-related carcinogenesis; Interleukin-22; Interleukin-22R1; STAT3

**Core tip:** This study investigates the expression of interleukin (IL)-22, IL-22R1, IL-23, and STAT3 in ulcerative colitis (UC) and UC-related carcinogenesis (UC-CRC) tissues from human and mouse. The results showed that IL-22 and related proteins were closely related to the severity of colitis, and the expression level of IL-22 and related proteins was higher in dysplasia tissues. IL-22/STAT3 signaling pathway was related to UC and UC-CRC. IL-22 can be used as a biomarker for determining the severity of UC and as an interesting therapeutic target in active UC and UC-CRC.

### Abstract

**AIM:** To investigate the expression of interleukin (IL)-22 and its related proteins in biopsy specimens from patients with ulcerative colitis (UC) and UC-related carcinogenesis.

**METHODS:** Biopsy specimens were obtained from patients with inactive ( $n = 10$ ), mild-to-moderately active ( $n = 30$ ), severely active ( $n = 34$ ), initial ( $n = 30$ ), and chronic UC ( $n = 44$ ), as well as UC patients with dysplasia ( $n = 10$ ). Specimens from patients without colonic abnormalities ( $n = 20$ ) served as controls. Chronic colitis in experimental mice was induced by 2.5% dextran sodium sulfate. The expression levels of IL-22, IL-23, IL-22R1 and phosphorylated STAT3 (p-STAT3) were determined by immunohistochemistry. Bcl-2, cyclin D1 and survivin expression was detected

Yu LZ, Wang HY, Yang SP, Yuan ZP, Xu FY, Sun C, Shi RH. Expression of interleukin-22/STAT3 signaling pathway in ulcer-

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## INTRODUCTION

Ulcerative colitis (UC) is a subtype of chronic inflammatory bowel disease (IBD) of the large intestine. The disease is characterized by a dysregulated mucosal immune response. This aberrant immune response leads to the secretion of harmful cytokines that destroy the gastrointestinal tract epithelium, thereby causing further inflammation.

Several inflammatory cytokines have been associated with IBD, including the interleukins IL-1 and IL-6, tumor necrosis factor (TNF)- $\alpha$ , and interferon (IFN)- $\gamma$ . Among the inflammatory cytokines implicated in IBD pathogenesis, much interest has been focused on the recently-identified cytokine IL-22. IL-22 is a member of the IL-10 subfamily; its production is highly dependent on IL-23 in T-helper 17 (Th17)<sup>[1-3]</sup>, Th1<sup>[4,5]</sup>, NK-22<sup>[6,7]</sup>, and CD11c<sup>+</sup><sup>[8]</sup> cells. IL-22 is expressed by the novel Th22 cell lineages<sup>[9]</sup> and innate lymphoid cells (ILCs)<sup>[10]</sup>. IL-22-producing ILCs in humans are responsive to IL-23 signaling, as potentially important mediators of IBDs<sup>[10]</sup>. Th22 and Th17 cells may be implicated in the pathogenesis of several chronic inflammatory and autoimmune diseases such as IBD, psoriasis, ankylosing spondylitis, and rheumatoid arthritis<sup>[11-16]</sup>.

IL-22 signaling is established when the cytokine binds to a heterodimeric receptor complex of IL-22R1 and IL-10R2. Given that IL-10R2 is a ubiquitous protein, cellular IL-22 responsiveness is mainly determined by IL-22R1 expression. IL-22R1 is specifically expressed in nonleukocytic cells such as those of the pancreas, skin, kidney, liver, and colon. IL-22R1 expression is detectable in epithelial cells of these organs, but not in their immune cells<sup>[17-20]</sup>. Therefore, IL-22 is unique among the cytokines because it cannot mediate autocrine or paracrine functions among leukocytes. Instead, IL-22 transmits information between leukocytes and the nonleukocytic cell compartment.

The STAT3 pathway for transcription activation appears to be a major mode of IL-22 signal transduction. Activated STAT3 is translocated from the cytoplasm to the nucleus, where it regulates genes involved in cell apoptosis, proliferation, migration, and survival. STAT3 has important functions in several autoimmune diseases. IL-22 mediates IL-23-induced acanthosis and dermal inflammation in psoriasis and IBD through the activation of STAT3<sup>[21]</sup>. When activated by IL-22, STAT3 can aggravate colitis by promoting the expression of inflammatory factors such as IL-8, IFN- $\gamma$ , and the matrix metalloproteinases (MMPs)<sup>[22,23]</sup>. Previous studies investigated the role of IL-22 in UC and UC-related carcinogenesis (UC-CRC)<sup>[24-26]</sup>. These studies revealed that the IL-22/

STAT3 pathway is involved in UC pathophysiology and carcinogenesis through the activity of inducible nitric oxide synthase (iNOS), DMBT1, and REG $\alpha$ <sup>[24-26]</sup>. However, the IL-22-induced phosphorylated STAT3 (p-STAT3) was considered a defense mechanism because it enhanced mucus production and goblet cell replacement in mouse models for acute colitis and wound healing<sup>[27-29]</sup>. Thus, the role of the IL-22/STAT3 signaling pathway in UC remains unclear. The current study investigates the significance of IL-22 and the IL-22/STAT3 signaling pathway in UC and UC-CRC, as well as its value as a therapeutic target for both diseases.

## MATERIALS AND METHODS

### Patients and tissue samples

Colon biopsy *via* endoscopy was performed on 74 patients with UC, including 31 females and 43 males (median age, 45.9 years; range, 17-87 years). The controls included 6 females and 14 males (mean age, 45.6 years; range, 33-55 years). All samples were obtained at the First Affiliated Hospital of Nanjing Medical University from 2009 to 2011.

This study was approved by the Research Ethics Committee of the First Affiliated Hospital of Nanjing Medical University. Written informed consent was obtained from each patient. The diagnosis of UC was based on clinical, endoscopic, and histological findings. The patient characteristics and histological data are summarized in Table 1.

### Experimental mouse models of UC

The mouse experiments were conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the United States National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University. Male ICR mice were purchased and maintained at the Centre of Animal Facility, Nanjing Medical University. The mice were sacrificed by cervical dislocation. All efforts were made to minimize suffering. Chronic colitis was induced by administering 2.5% dextran sodium sulfate (DSS; ICN Biomedicals Inc., Irvine, CA, United States) to the drinking water in three seven-day cycles, which had a five-day recovery period after each cycle. During the recovery period, mice drank only normal water. The age-matched control mice received only normal drinking water throughout the entire study.

### Immunohistochemistry

All the tissues were fixed overnight in 4% paraformaldehyde at 4 °C, processed, and cut into 5  $\mu$ m-thick sections. The sections were then deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked by 3% H<sub>2</sub>O<sub>2</sub> for 10 min at room temperature. Antigen retrieval was performed by 15 min of boiling in the preheated buffer (10 mmol/L of sodium citrate, pH = 6.0)

**Table 1** Clinical characteristics of ulcerative colitis patients

|  | Normal control<br><i>n</i> = 20 | Histological activity of UC |                                |                         | Types of UC              |                          | UC with dysplasia<br><i>n</i> = 10 |
|--|---------------------------------|-----------------------------|--------------------------------|-------------------------|--------------------------|--------------------------|------------------------------------|
|  |                                 | Inactive<br><i>n</i> = 10   | Mild-moderate<br><i>n</i> = 30 | Severe<br><i>n</i> = 34 | Initial<br><i>n</i> = 30 | Chronic<br><i>n</i> = 44 |                                    |
| Gender ( <i>n</i> )                        |                                 |                             |                                |                         |                          |                          |                                    |
| Male                                       | 14                              | 5                           | 16                             | 22                      | 16                       | 27                       | 6                                  |
| Female                                     | 6                               | 5                           | 14                             | 12                      | 14                       | 17                       | 4                                  |
| Mean age <sup>3</sup> , yr                 | 40.6 ± 12.5                     | 41.2 ± 13.3                 | 44.7 ± 13.2                    | 43.2 ± 17.1             | 42.8 ± 17.3              | 46.5 ± 13.6              | 44.7 ± 16.6                        |
| Mean duration of disease <sup>3</sup> , yr | -                               | 6.14 ± 4.2                  | 4.3 ± 2.3                      | 3.7 ± 3.1               | 0.8 ± 0.6                | 5.1 ± 0.6 <sup>1</sup>   | 10.5 ± 1.4 <sup>2</sup>            |
| Extent of disease ( <i>n</i> )             |                                 |                             |                                |                         |                          |                          |                                    |
| Extensive colitis                          | -                               | 0                           | 8                              | 19                      | 8                        | 19                       | 3                                  |
| Left-side colitis                          | -                               | 4                           | 13                             | 9                       | 13                       | 13                       | 4                                  |
| Proctitis                                  | -                               | 6                           | 9                              | 6                       | 9                        | 12                       | 3                                  |
| Treatment ( <i>n</i> )                     |                                 |                             |                                |                         |                          |                          |                                    |
| Aminosalicylates                           | -                               | 8                           | 25                             | 20                      | 16                       | 35                       | 5                                  |
| Corticosteroids                            | -                               | 4                           | 5                              | 18                      | 4                        | 15                       | 3                                  |
| Immunosuppressive agent                    | -                               | 0                           | 0                              | 1                       | 0                        | 1                        | 0                                  |
| Biological agent                           | -                               | 0                           | 0                              | 0                       | 0                        | 0                        | 0                                  |
| None                                       | -                               | 3                           | 2                              | 0                       | 10                       | 5                        | 2                                  |

<sup>1</sup>The mean duration of disease in the chronic group (5.1 ± 0.6 years) was significantly longer than in the initial group (0.8 ± 0.6 years;  $P < 0.001$ ); <sup>2</sup>The mean duration of disease in the ulcerative colitis (UC) with dysplasia group (10.5 ± 1.4 years) was significantly longer than in other groups ( $P < 0.05$ ); <sup>3</sup>Data are expressed as mean ± SD.

at 200 W in a microwave. The slides were transferred to a humidifier and blocked by incubating in 5% normal goat serum at room temperature for 1 h. The polyclonal rabbit antibodies used in this study included anti-p-STAT3 tyrosine 727, anti-IL-22, anti-IL-22R1, and anti-IL-23 (ab30647, ab18499, ab5984 and ab115759, respectively); these antibodies were diluted in 5% normal goat serum at ratios of 1:200, 1:200, 1:200 and 1:400, respectively. The sections were incubated in the respective antibodies overnight at 4 °C. The slides were subsequently incubated in the secondary antibody goat anti-rabbit IgG-biotin (B8895; Sigma-Aldrich, St. Louis, MO, United States) at room temperature for 40 min. The sections were incubated in the ABC-peroxidase solution (Ultrasensitive™ S-P kit, kit 9719; Maixin-Bio, China) for 30 min at room temperature, followed by counterstaining with hematoxylin. The immunohistochemistry (IHC) results were analyzed using Image Pro-Plus.

### Western blotting analysis

Proteins were extracted from the mouse tissues and quantified using a commercial protein assay (Bio-Rad Laboratories, CA, United States). The protein samples (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. Immunoblot analysis was conducted using antibodies against total STAT3, p-STAT3 (S727), Bcl-2, cyclin D1, and survivin (Abcam Inc, MA, United States). The results were visualized using the chemiluminescent Pierce ECL Substrate Western blotting detection system (Thermo Scientific, IL, United States) and exposure to autoradiography film (Kodak XAR film).

### Statistical analysis

The results were expressed as the mean ± SD. The two

groups were compared using the Student's *t* test or the Mann-Whitney *U* test, as appropriate. All statistical analyses were performed using the SPSS statistical software (version 13.0).  $P < 0.05$  was considered to be statistically significant.

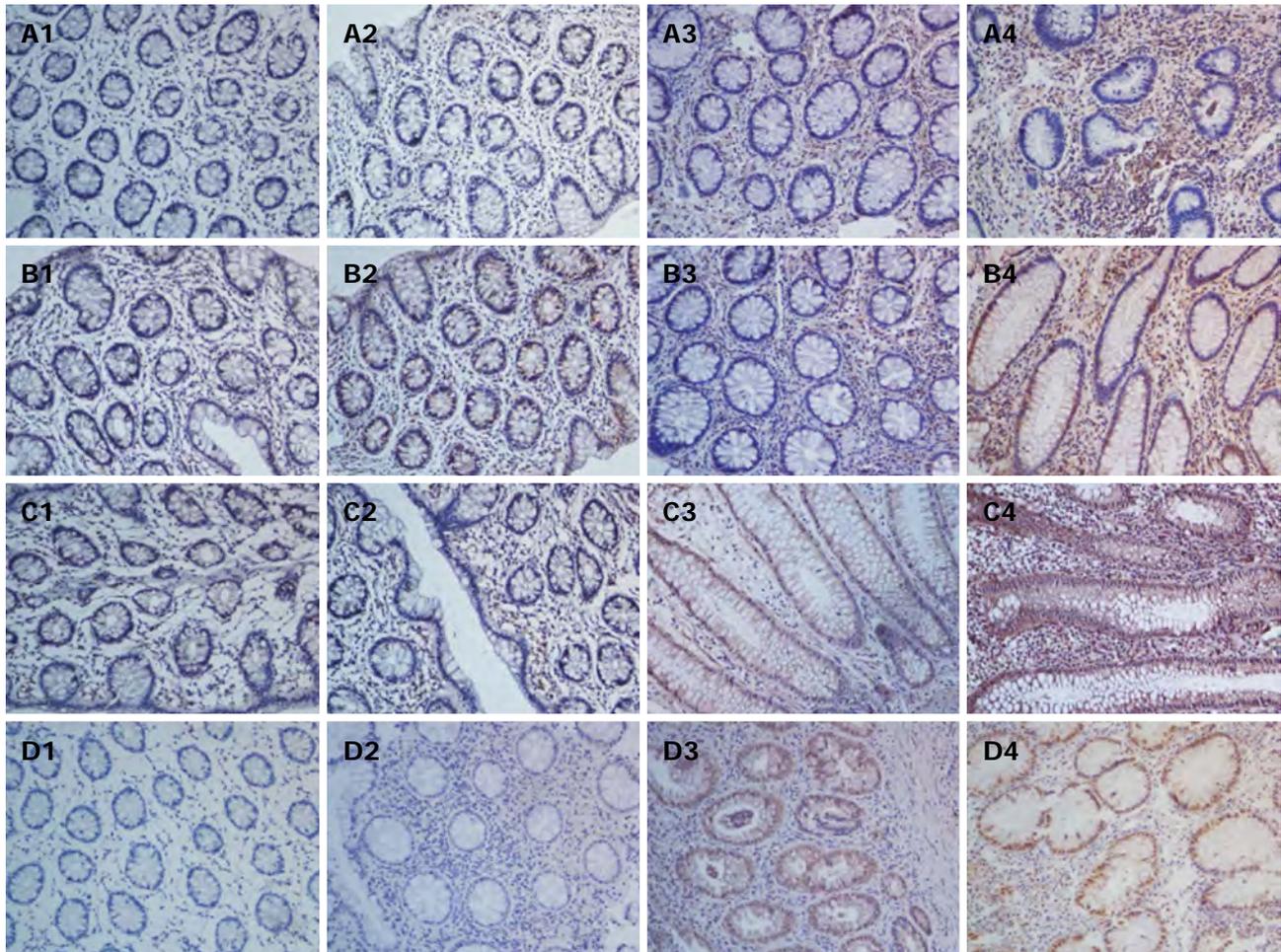
## RESULTS

### Patient characteristics

As shown in Table 1, we collected biopsy specimens from 20 healthy controls, 10 patients with inactive UC, 64 patients with active UC (including 30 patients with mild-to-moderate active UC and 34 patients with severely active UC), 30 patients with initial UC attacks, 44 patients with chronic UC, and 10 UC patients with dysplasia. The duration of disease was significantly longer in the groups with chronic UC and UC with dysplasia than in the other groups ( $P < 0.05$ ). No other significant differences were observed among the other groups, regardless of the treatment used.

### IL-22 expression in patients with different degrees of inflammation

Immunohistochemical analysis showed that the IL-22 protein was mainly expressed in inflammatory cells of the colonic lamina propria, but not in the normal controls (Figure 1A). Tissues with mild-moderate and severe UC had significantly higher expression levels than those with inactive UC and normal colon tissues (mild-moderate *vs* normal,  $P < 0.001$ ; mild-moderate *vs* inactive,  $P = 0.02$ ,  $P < 0.05$ ; severe *vs* normal,  $P < 0.001$ ; severe *vs* inactive,  $P < 0.001$ ; Figure 2A). Moreover, significantly more IL-22-positive cells were present in tissues of severe UC than in those of mild-moderate UC (severe *vs* mild-moderate,  $P < 0.001$ ; Figure 2A). The results indicate that



**Figure 1** Expression and distribution of interleukin-22 and its related proteins in colonic biopsy specimens as analyzed by immunohistochemistry ( $\times 200$ ). A1-A4: Expression and distribution of interleukin (IL)-22 in colonic biopsy specimens from the control, inactive ulcerative colitis (UC), mild-moderate UC, and severe UC tissues, respectively; B1-B4: The expression and distribution of IL-23 in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues; C1-C4: The expression and distribution of IL-22R1 in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues; D1-D4: The expression and distribution of p-STAT3 (S727) in colonic biopsy specimens from the control, inactive UC, mild-moderate UC, and severe UC tissues.

the expression of IL-22 was related to UC severity.

#### **Expression of IL-22-related proteins in patients with different degrees of inflammation**

Previous studies have demonstrated that IL-22 production is highly dependent on IL-23 in Th17, NK-22, and lymphoid tissue-inducer cells<sup>[1-3,6,7]</sup>. Thus, we analyzed IL-23 expression in UC tissues using IHC. The results indicated that IL-23 was significantly highly upregulated in active UC, as compared with inactive UC and the controls (mild-moderate *vs* normal,  $P < 0.001$ ; mild-moderate *vs* inactive,  $P < 0.001$ ; severe *vs* normal,  $P < 0.001$ ; severe *vs* inactive,  $P < 0.001$ ; Figure 2B). Similarly, the IL-23 expression was stronger with severe UC than with mild-moderate UC (severe *vs* mild-moderate,  $P = 0.01$ ,  $P < 0.05$ ; Figure 2B). The positive region of IL-23 in UC was mostly confined to the intestinal epithelial cells (IECs) and inflammatory cells of the colonic lamina propria (Figure 1B).

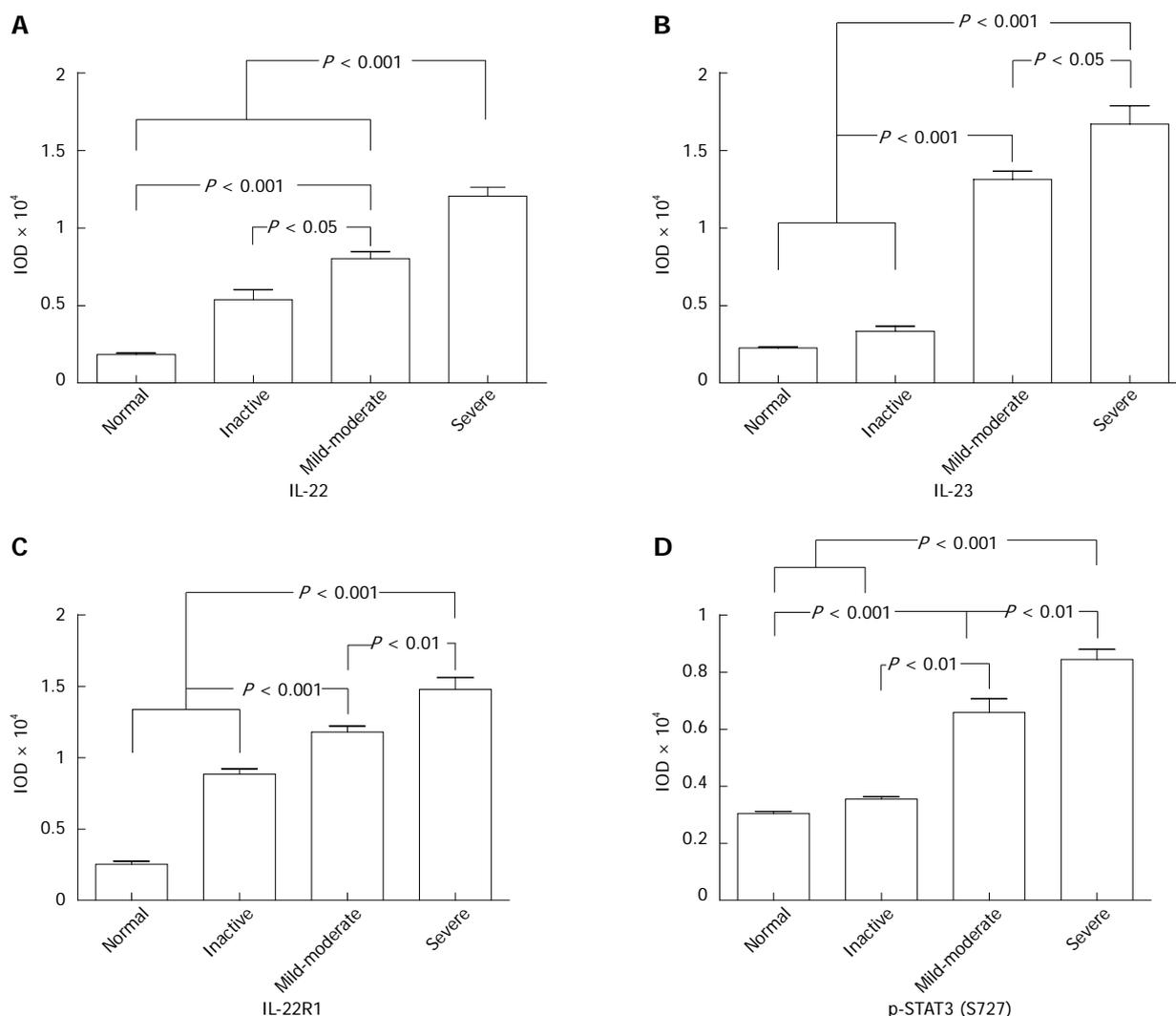
We identified IL-22R1, another key molecule that is necessary for signal transmission. IL-22R1 was mainly

localized in the IECs (Figure 1C). IL-22R1 was overexpressed in active UC (mild-moderate *vs* normal,  $P < 0.001$ ; mild-moderate *vs* inactive,  $P = 0.0002$ ,  $P < 0.001$ ; severe *vs* normal,  $P < 0.001$ ; severe *vs* inactive,  $P = 0.0002$ ,  $P < 0.001$ ; severe *vs* mild-moderate,  $P = 0.007$ ,  $P < 0.01$ ; Figure 2C).

Based on the downstream effects of IL-22, the activation of STAT3 was determined by staining with p-STAT3 at the S727 residue. IHC analysis showed that in the colon tissues resected from patients with UC, the p-STAT3 (S727) protein was mainly expressed in the nucleus of epithelial cells (Figure 1D). Its expression was significantly upregulated in the active UC tissues, particularly in severe UC (severe *vs* normal,  $P < 0.001$ ; severe *vs* inactive,  $P < 0.001$ ; severe *vs* mild-moderate,  $P = 0.0045$ ,  $P < 0.01$ ; mild-moderate *vs* normal,  $P < 0.001$ ; mild-moderate *vs* inactive,  $P = 0.0017$ ,  $P < 0.01$ ; Figure 2D).

#### **IL-22 expression in patients with different types of UC**

According to the clinical diagnostic standards, we classified UC into two types: initial and chronic UC. IHC



**Figure 2** Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from the controls and patients with inactive, mild-moderate, and severe ulcerative colitis. The average integrated optical density (IOD) was obtained by analyzing five random fields for each slide, which were evaluated using Image-Pro Plus software (v. 5.0), for the immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in human colonic biopsy specimens. The expression levels of IL-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (D) were positively correlated with colitis severity.

indicated that IL-22 was significantly upregulated in the initial UC tissues than in the chronic UC tissues (initial *vs* chronic,  $P = 0.0077$ ,  $P < 0.01$ ; Figures 3A and 4A).

**IL-22-related protein expression in patients with different types of UC**

Similar to IL-22, IL-22R1 was more strongly expressed in the tissues of initial UC than in those of chronic UC (initial *vs* chronic,  $P < 0.001$ ; Figures 3C and 4C). By contrast, the number of p-STAT3-positive cells was significantly higher in chronic UC tissues than in initial UC (chronic *vs* initial,  $P = 0.03$ ,  $P < 0.05$ ; Figures 3D and 4D). However, no significant differences were detected in terms of the IL-23 expression in these two groups (Figures 3B and 4B).

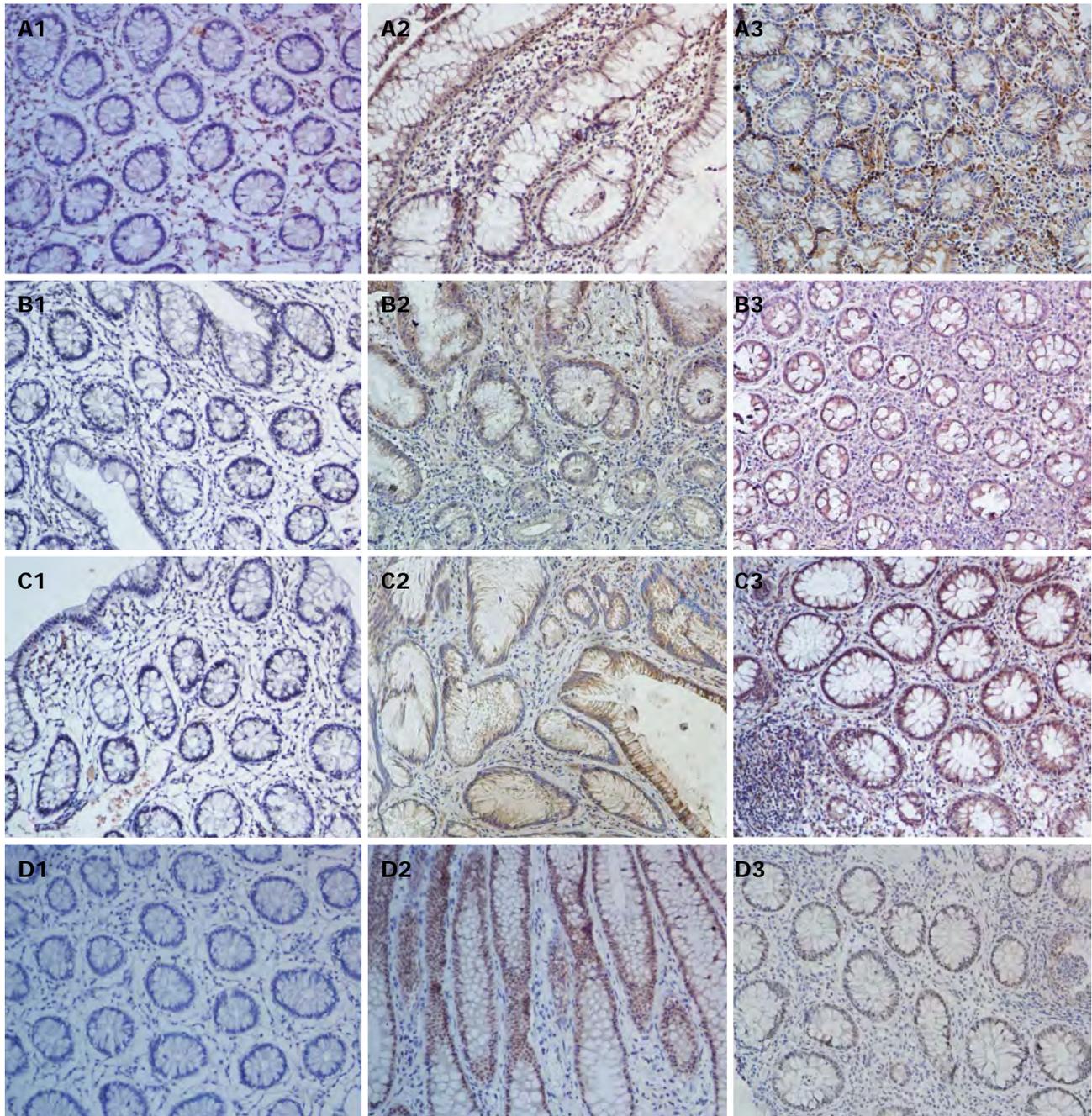
**IL-22 expression in patients with UC-CRC (dysplasia)**

A positive correlation exists between the IL-22-positive cells and the severity of colitis in patients with UC. We investigated IL-22 expression in biopsy specimens from

patients with UC-CRC (dysplasia) and analyzed the IL-22 levels in UC with dysplasia (Figure 5A). Significantly more IL-22-positive cells were observed in the dysplasia group than in the inflammatory group ( $P = 0.02$ ; Figure 6A).

**Expression of IL-22-related proteins in patients with UC-CRC (dysplasia)**

Given that IL-22 was highly upregulated in UC tissue with dysplasia, we studied the expression of the receptor IL-22R1, its upstream IL-23, and its downstream p-STAT3 (S727) in UC tissues with dysplasia, as compared with active and inactive UC tissues. The expression levels of IL-22R1, IL-23, and p-STAT3 were significantly higher in UC tissues with dysplasia than in the control group (IL-22R1: dysplasia *vs* active,  $P = 0.02$ ; IL-23: dysplasia *vs* active,  $P = 0.01$ ; p-STAT3: dysplasia *vs* active,  $P = 0.02$ ; Figure 6B-D). The increased expression was strictly found at the dysplastic tissues of the patients (Figure 5B-D).



**Figure 3** Expression and distribution of interleukin-22 and related proteins in human colonic tissues from controls and patients with chronic and initial ulcerative colitis as detected by immunohistochemistry ( $\times 200$ ). A1-A3: Expression and distribution of interleukin (IL)-22 in ulcerative colitis (UC) tissues from the control, chronic UC, and initial UC groups; B1-B3: Expression and distribution of IL-23 in UC tissues from the control, chronic, and initial UC groups; C1-C3: Expression and distribution of IL-22R1 in UC tissues from the control, chronic, and initial UC groups; D1-D3: Expression and distribution of p-STAT3 in UC tissues from the chronic and initial UC groups.

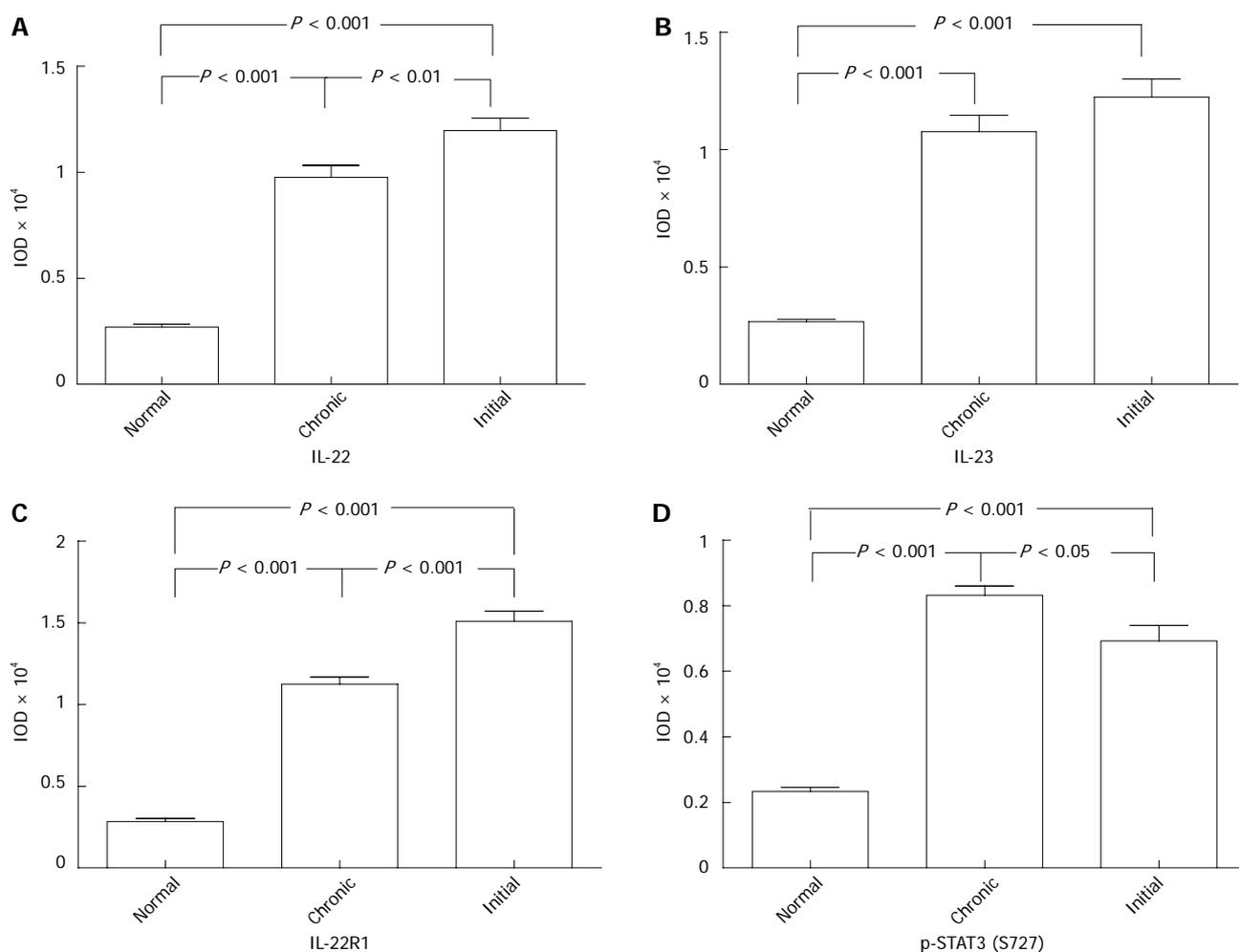
#### **Expression of IL-22 and its related proteins in DSS-induced mouse models of chronic colitis**

We induced experimental colitis by treating mice with DSS to study the role of IL-22 and its related proteins in the disease. Dynamic IL-22, IL-23, and IL-22R1 expression levels were investigated by IHC. p-STAT3 activity and its downstream gene expression were confirmed by Western blotting analysis at different time points (at days 40, 80, and 120). The expression levels of IL-22, IL-22R1, and IL-23 were increased with time (Figure 7A).

STAT3 activation and the activity of its downstream cell proliferation-related genes, such as Bcl-2, cyclin D1, and survivin, were also investigated. All these genes had sustained expression over time (Figure 7B).

## **DISCUSSION**

IBDs such as Crohn's disease and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract. Although their etiology is not completely under-

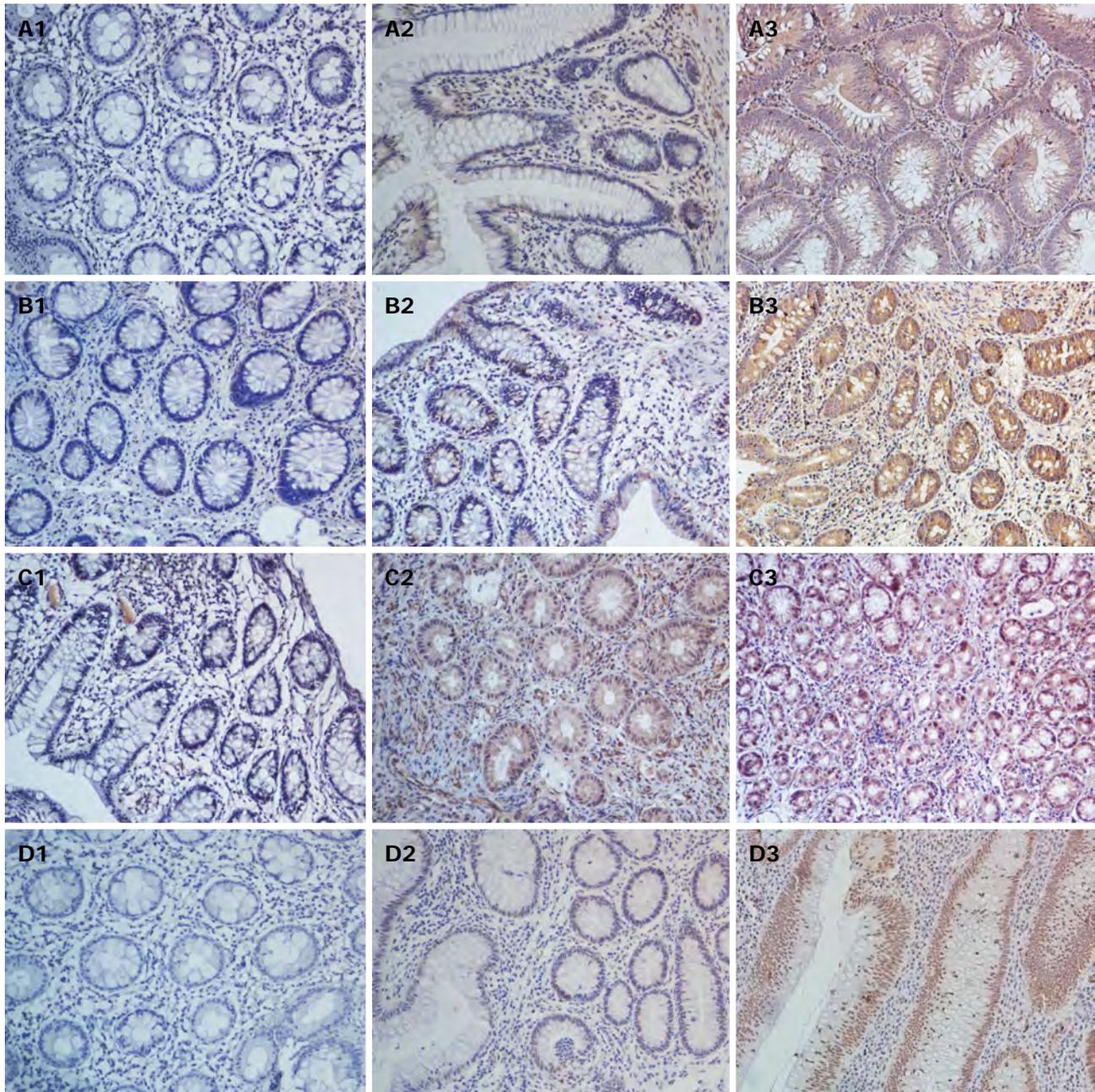


**Figure 4** Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from controls and patients with chronic and initial ulcerative colitis. The average integrated optical density (IOD) for the immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in colonic tissues with initial and chronic ulcerative colitis (UC). Significantly higher expression levels of IL-22 and IL-22R1 were observed in the initial UC group as compared with the chronic UC group. p-STAT3 was highly expressed in the chronic UC group.

stood, initiation and aggravation of the inflammatory process seem to be related to a massive local mucosal immune response. IL-22 belongs to the IL-10 family of cytokines; it has recently been shown to be preferentially expressed by Th17 and Th22 cells. These cells have been identified in the pathogenesis of certain chronic inflammatory diseases, including colitis, psoriasis, and rheumatoid arthritis. IL-22 targets innate immune pathways because of the restricted expression of IL-22 receptors on nonleukocytic cells, such as epithelial cells, keratinocytes, and hepatocytes; however, it does not recognize T- or B-cells<sup>[17-20]</sup>. Studies using genetically-engineered mice have demonstrated that epithelial STAT3 activation in DSS-induced colitis is dependent on IL-22, rather than on IL-6. Both IL-22 and STAT3 activation in epithelial cells is important for wound-healing, as demonstrated by *in vivo* experiments<sup>[27]</sup>. Sugimoto *et al.*<sup>[28]</sup> found that the IL-22/STAT3 pathway contributes to the rapid amelioration of local intestinal inflammation by enhancing the production of membrane-bound mucins (Muc1, -3, -10, and -13) in a mouse model of acute colitis. However, IL-22 is also considered an inflammatory driver in IBD

by acting on human colonic subepithelial myofibroblasts to stimulate secretion of proinflammatory cytokines such as MMPs, IL-1, IL-8, and INF- $\gamma$ <sup>[30]</sup>. Highly elevated serum levels of IL-22 were correlated with disease severity in patients with Crohn's disease (CD)<sup>[17]</sup>. Colitis mouse models have indicated that highly elevated IL-22 expression may directly or indirectly induce inflammation<sup>[31]</sup>. Moreover, the IL-22/STAT3 signaling pathway is important in inflammation and carcinogenesis during UC *via* its upregulation of iNOS and MMP production, respectively<sup>[23,24]</sup>. Here, we demonstrate that IL-22 contributes to the inflammatory severity of UC and UC-CRC by activating STAT3 in IECs.

The UC microenvironment is composed of IECs, macrophages, immunocytes, and so on. The interactions among these cells involve their secreted cytokines and consist of a positive feedback loop with persistent activation of the STAT3-enabling progression of UC. Similar to IL-23 and IL-22R1, an IL-22 positive feedback loop in the UC microenvironment was demonstrated in this study. Our results indicated the IL-22 overexpression in the inflammatory cells of the colonic lamina propria in



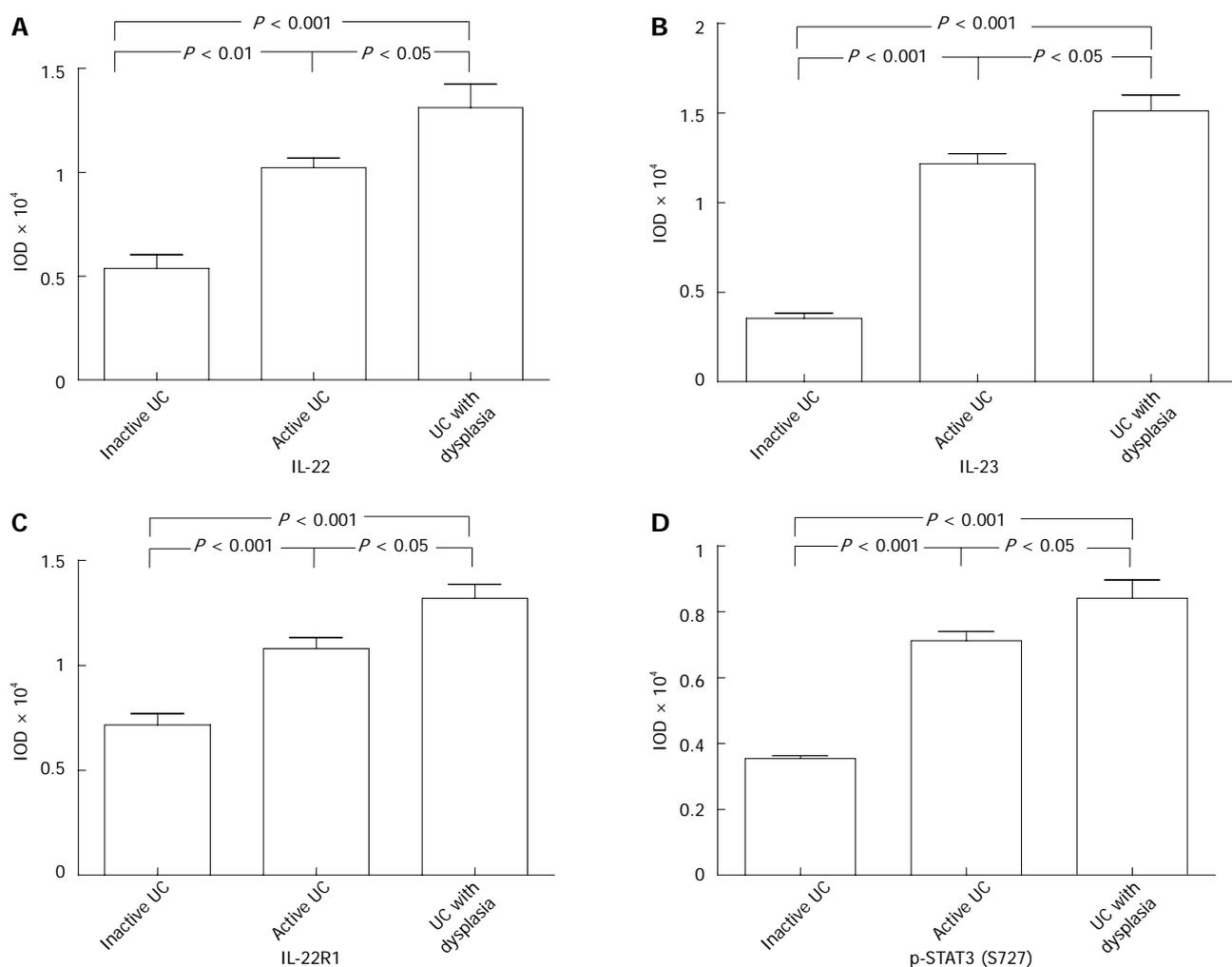
**Figure 5** Expression and distribution of interleukin-22 and its related proteins in human colonic tissues from patients with inactive ulcerative colitis, active ulcerative colitis, and ulcerative colitis with dysplasia, as detected by immunohistochemistry ( $\times 200$ ). A1-A3: Expression and distribution of interleukin (IL)-22 in inactive ulcerative colitis (UC), active UC, and UC with dysplasia tissues; B1-B3: Expression and distribution of IL-23 in inactive UC, active UC, and UC with dysplasia tissues; C1-C3: Expression and distribution of IL-22R1 in inactive ulcerative colitis (UC), active UC, and UC with dysplasia tissues; D1-D3: Expression and distribution of p-STAT3 (S727) in inactive UC, active UC, and UC with dysplasia tissues.

UC tissues. Moreover, the sustained activation of STAT3 signaling in IECs was verified. Simultaneously, IL-22R1 expression was enhanced in IECs, thereby ensuring the transmission of the IL-22 signal.

The STAT3-regulated proinflammatory cytokine IL-23<sup>[32]</sup> was likewise overexpressed in human UC. IL-23 activates innate immune cells to secrete pro-inflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-6, as well as maintains the expansion of Th17 cells that express IL-22<sup>[33]</sup>.

IL-22 is increased in active Crohn's disease and pro-

motes proinflammatory gene expression<sup>[17]</sup>. We demonstrated that IL-22 is more highly expressed in active UC than in inactive UC and the normal control. Thus, the increased IL-22 signaling in active IBD supports the potential of an IL-22 signaling blockade as a therapeutic strategy for IBD. Furthermore, IL-23, IL-22, STAT3, and IL-22R1 are closely related to the colitis severity. Thus, positive feedback loops can further exacerbate inflammation. If left unchecked, these pathways may lead to the chronic immune pathology that is characteristic to IBD. Therefore, IL-22 could be used as a biomarker for deter-

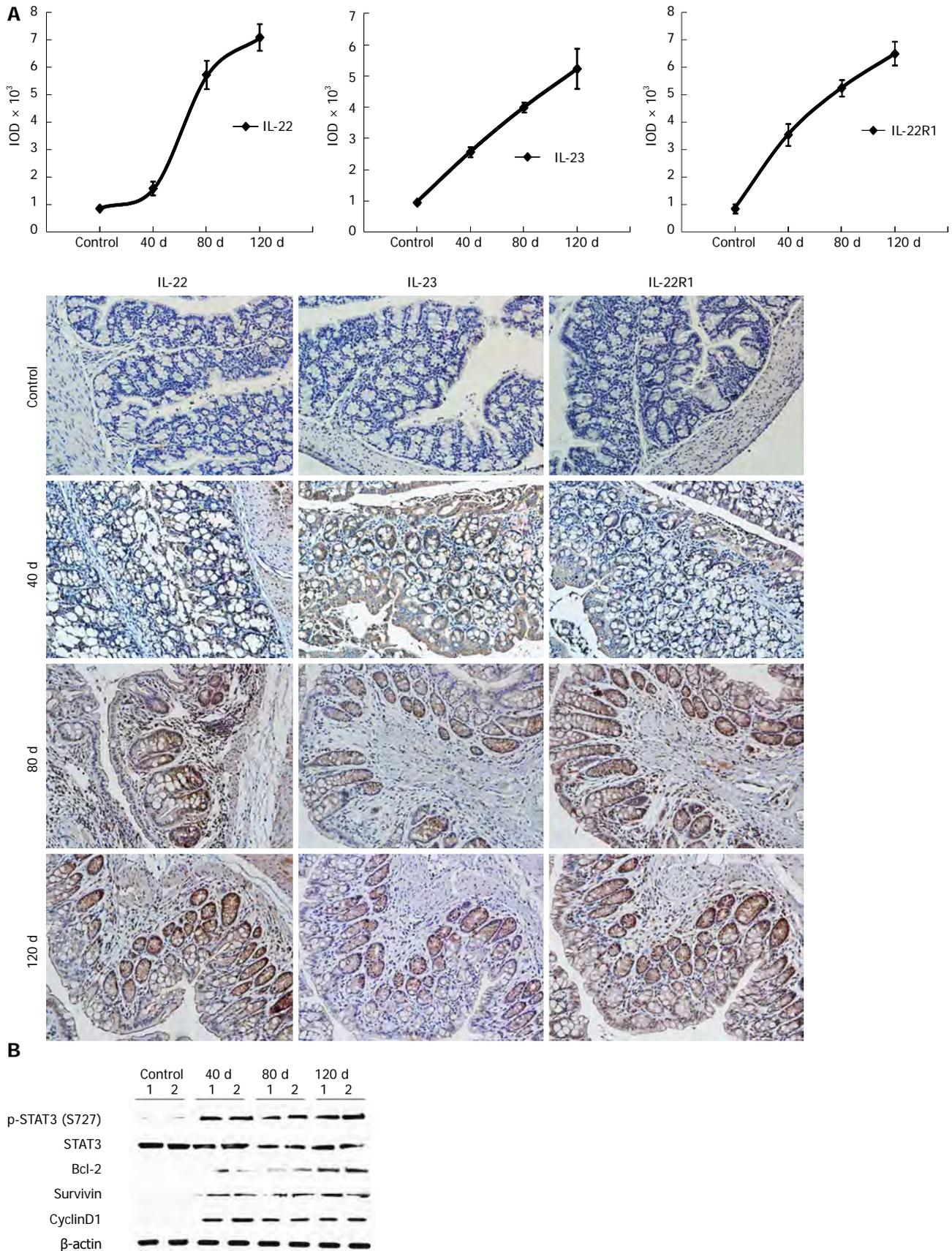


**Figure 6** Statistical evaluation of interleukin-22 and its related proteins in human colonic biopsy specimens from patients with inactive ulcerative colitis, active ulcerative colitis, and ulcerative colitis with dysplasia. The average integrated optical density (IOD) for immunohistochemistry staining of interleukin (IL)-22 (A), IL-23 (B), IL-22R1 (C), and p-STAT3 (S727) (D) in colonic tissues with ulcerative colitis (UC) and UC-related carcinogenesis (UC-CRC). Significantly higher expression levels of IL-22, IL-23, IL-22R1, and p-STAT3 were observed in the dysplasia group, as compared with the inactive and active UC groups.

mining UC severity.

STAT3 is a transcription factor that is activated by the binding of several cytokines, hormones, and growth factors to their respective receptors, including IL-22, IL-6, IL-23, and IL-1 $\beta$ <sup>[34]</sup>. We propose that STAT3 signaling is disrupted in chronic inflammation and carcinogenesis. The expression levels of total STAT3 and p-STAT3 in patients with UC were persistently elevated, with a positive correlation to the degree of inflammation<sup>[22]</sup>. Moreover, STAT3 is constitutively activated in a variety of human cancers, including colorectal cancer. This transcription factor is crucial in cancer cells because it regulates the transcription of genes involved in cell survival, apoptosis, and other cellular processes. Morikawa *et al*<sup>[34]</sup> found that p-STAT3 is significantly associated with poor prognosis in a data set of 724 colorectal cancers. Furthermore, STAT3 signaling has been reported to induce cancer-promoting inflammation and to inhibit antitumor immunity<sup>[35,36]</sup>. IL-6, a main activator of STAT3, has been proven to be important for promoting UC and UC-CRC<sup>[37]</sup>. IL-22, another inflammatory factor that

predominantly activates STAT3, has been verified in the chronic hepatitis and hepatocellular carcinoma (HCC) microenvironment; it induces tumor growth, inhibits apoptosis, and promotes metastasis *via* STAT3 activation<sup>[38]</sup>. Consistent with other studies on chronic hepatitis and HCC, our results demonstrated that the expression levels of IL-23, IL-22, IL-22R1, and STAT3 are consistently highly expressed in human UC tissues. IL-22 and p-STAT3, in particular, are more constitutively upregulated in the chronic colitis than in the controls. During the chronic phase of the DSS-induced mice colitis model, IL-22, IL-22R1, and IL-23 were highly expressed over time. Likewise, p-STAT3 and its downstream Bcl-2, cyclin D1, and survivin genes were remarkably upregulated. Furthermore, the expression of IL-22, p-STAT3, IL-23, and IL-22R1 were significantly elevated in human UC tissues with dysplasia, as compared with inactive and active UC tissues. Our results showed that the expression levels of IL-22 and IL-22R1 were more highly elevated in the acute colitis phase, which is in accordance with earlier studies<sup>[27,28]</sup>. We propose that IL-22 may ameliorate



**Figure 7** Expression of interleukin-22 and its related proteins in the mouse chronic colitis model. A: The average integrated optical density (IOD) for the immunohistochemistry staining of interleukin (IL)-22, IL-23, and IL-22R1 in mouse ulcerative colitis and normal tissues at different time points: days 40, 80, and 120. The expression of IL-22, IL-22R1, and IL-23 gradually increased with time; B: Western blotting detection of p-STAT3 (S727), total STAT3, Bcl-2, survivin, and cyclin D1 after DSS administration. Expression levels were all normalized to β-actin. All genes showed sustained expression over time.

intestinal inflammation by enhancing mucus production and goblet cell replacement in the early phase of inflammatory response. However, the persistent expression of IL-22 during the chronic phase of UC can strongly activate STAT3 phosphorylation in IECs, which is associated with the progression of human UC and UC-CRC<sup>[37]</sup> by upregulating genes for cell proliferation, anti-apoptosis, and survival.

In conclusion, our study provides clinical evidence that the IL-22/STAT3 signaling pathway is related to UC and UC-CRC. Moreover, IL-22 can be used as a biomarker for determining the severity of UC and as an interesting therapeutic target in active UC and UC-CRC.

## COMMENTS

### Background

It has been previously reported that interleukin (IL)-22, one of the cytokines secreted by Th17 cells, promotes a protective and inflammatory effect in inflammatory bowel disease (IBD) through STAT3 signaling activation.

### Research frontiers

The IL-22/STAT3 signaling pathway plays an important role in several autoimmune diseases, such as psoriasis, IBD, and so on. When activated by IL-22, STAT3 can aggravate colitis by promoting the expression of inflammatory factors such as IL-8, interferon- $\gamma$ , and matrix metalloproteinases. However, some studies have found that the IL-22 induced phosphorylation of STAT3 is a defense mechanism that enhances mucus production and goblet cell replacement in mouse models of acute colitis and wound-healing. Thus, the role of the IL-22/STAT3 signaling pathway in ulcerative colitis (UC) remains unclear.

### Innovations and breakthroughs

IL-22 may ameliorate intestinal inflammation by enhancing mucus production and goblet cell replacement during the early phase of the inflammatory response. However, the persistent expression of IL-22 in chronic phase of UC can strongly activate the phosphorylation of STAT3 in intestinal epithelial cells. p-STAT3 is associated with the progression of human UC and UC-related carcinogenesis (UC-CRC) because it upregulates the genes for cell proliferation, anti-apoptosis, and survival.

### Peer review

The authors report about the expression of IL-22, IL-22R1, IL-23, and STAT3 in biopsies of human UC and UC-related carcinogenesis. The study is well performed; it gives an overview on the expression of the before-mentioned factors in active and chronic UC and correlates IL-22 expression with disease severity.

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P-Reviewer Guo JM S-Editor Zhai HH L-Editor Ma JY  
E-Editor Zhang DN



## Prognostic and survival analysis of 837 Chinese colorectal cancer patients

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Supported by The Grants from National Natural Science Foundation of China, No. 81102013, No. 81101580; Zhejiang Provincial Natural Science Foundation of China, No. R2090353; National High Technology Research and Development Program of China, No. 2012AA02A506

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Received: October 31, 2012 Revised: February 27, 2013

Accepted: March 6, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To develop a prognostic model to predict survival of patients with colorectal cancer (CRC).

**METHODS:** Survival data of 837 CRC patients undergoing surgery between 1996 and 2006 were collected and analyzed by univariate analysis and Cox proportional hazard regression model to reveal the prognostic factors for CRC. All data were recorded using a standard data form and analyzed using SPSS version 18.0 (SPSS, Chicago, IL, United States). Survival curves were calculated by the Kaplan-Meier method. The log rank test was used to assess differences in survival. Univariate hazard ratios and significant and independent predictors of disease-specific survival and were identified

by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. Statistical significance was defined as  $P < 0.05$ .

**RESULTS:** The survival rate was 74% at 3 years and 68% at 5 years. The results of univariate analysis suggested age, preoperative obstruction, serum carcinoembryonic antigen level at diagnosis, status of resection, tumor size, histological grade, pathological type, lymphovascular invasion, invasion of adjacent organs, and tumor node metastasis (TNM) staging were positive prognostic factors ( $P < 0.05$ ). Lymph node ratio (LNR) was also a strong prognostic factor in stage III CRC ( $P < 0.0001$ ). We divided 341 stage III patients into three groups according to LNR values (LNR1,  $LNR \leq 0.33$ ,  $n = 211$ ; LNR2,  $LNR 0.34-0.66$ ,  $n = 76$ ; and LNR3,  $LNR \geq 0.67$ ,  $n = 54$ ). Univariate analysis showed a significant statistical difference in 3-year survival among these groups: LNR1, 73%; LNR2, 55%; and LNR3, 42% ( $P < 0.0001$ ). The multivariate analysis results showed that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factors for CRC if we did not consider the interaction of the TNM staging system ( $P < 0.05$ ). When the TNM staging was taken into account, histological grade lost its statistical significance, while the specific TNM staging system showed a statistically significant difference ( $P < 0.0001$ ).

**CONCLUSION:** The overall survival of CRC patients has improved between 1996 and 2006. LNR is a powerful factor for estimating the survival of stage III CRC patients.

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**Key words:** Colorectal cancer; Prognostic factors; Cox proportional hazard regression; Lymph node ratio

**Core tip:** Recent reports and reviews have highlighted the importance of metastatic lymph node and Lymph

node ratio (LNR) in predicting prognosis of colorectal cancer (CRC). We found that the histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factor for CRC without consideration of the interaction of the tumor node metastasis staging system. LNR was a powerful factor for estimating the survival of stage III CRC. This paper presents new results on the 5-year overall survival and prognostic factors in Chinese CRC patients.

Yuan Y, Li MD, Hu HG, Dong CX, Chen JQ, Li XF, Li JJ, Shen H. Prognostic and survival analysis of 837 Chinese colorectal cancer patients. *World J Gastroenterol* 2013; 19(17): 2650-2659 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2650.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2650>

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies and one of the most common causes of cancer-related death worldwide<sup>[1]</sup>. An estimated 143460 new cases of CRC will be diagnosed this year, and 51690 patients will succumb to their disease in the United States alone<sup>[2]</sup>. Meanwhile, with the continuous aging of the population and an increased tendency to adopt a western lifestyle, the incidence of CRC and its related mortality is gradually increasing and it has become the fifth most common of all cancers in China<sup>[3,4]</sup>. Thus, the importance of CRC as a public health problem is increasing in China.

Over the past two decades, the 5-year overall survival of CRC patients has improved. Some advanced CRC patients have received clear survival benefits due to the practice of resecting liver metastases and advances in surgical techniques<sup>[5]</sup>. For those patients who have missed the opportunity for surgery, chemotherapy is still the main treatment. Although the overall survival of advanced CRC patients is still poorer than for early stage patients, it is encouraging that the combination of chemotherapy and targeted drugs may have the potential to improve survival.

In clinical practice, clinicians need an accurate outcome prediction of CRC patients to devise an appropriate therapeutic strategy. However, many variables may influence the prognosis, including both patient and tumor characteristics<sup>[6]</sup>. Therefore, we conducted the present study to explore the relevant factors affecting the prognosis of CRC patients using existing data in the Second Affiliated Hospital of Zhejiang University College of Medicine, China.

## MATERIALS AND METHODS

### *Patients and clinical data*

A total of 837 patients with CRC that underwent surgery

at the Department of Surgical Oncology at the Second Affiliated Hospital of Zhejiang University College of Medicine from January 1996 to December 2006 were enrolled from our database. All clinical cases and their follow-up data were recorded. The data included sex, age at diagnosis, clinical symptoms, severe complications, location of the primary tumor, histological type, tumor differentiation, lymphovascular invasion, depth of invasion, numbers of retrieved lymph nodes and metastatic lymph nodes, date of surgery, date of recurrence (if applicable), cause of recurrence (if applicable), date of death (if applicable), cause of death (if applicable), postoperative treatment, and date of follow-up. This study consisted of stages I-IV CRC patients. No local or systemic treatment had been conducted preoperatively. Patients' blood samples were collected before their operation and their carcinoembryonic antigen (CEA) levels were analyzed. Specimens were fixed in formalin and stained with hematoxylin-eosin (HE) and used for histopathological evaluation. The 6<sup>th</sup> and the 7<sup>th</sup> editions of the Union for International Cancer Control (UICC) classification were used to categorize colorectal carcinomas. Rectal cancer was defined as carcinomas with a distal margin of 15 cm from the anal verge measured with a rigid endoscope.

### *Follow-up duration*

All patients were followed up at 3-mo intervals for the first 2 years, and 6-mo intervals for 3-5 years. Follow-up was completed for the entire study population by March 2011, and the median follow-up period was 45 mo. The baseline of the study cases are shown in Table 1 (six cases of double primary CRC were excluded from Table 1).

### *Statistical analysis*

All data were recorded using a standard data form and analyzed using SPSS version 18.0 (SPSS, Chicago, IL, United States). Survival curves were calculated by the Kaplan-Meier method. The log rank test was used to assess differences in survival. Univariate hazard ratios and significant and independent predictors of disease-specific survival and were identified by Cox proportional hazard analysis. The stepwise procedure was set to a threshold of 0.05. Statistical significance was defined as  $P < 0.05$ .

## RESULTS

A total of 837 patients with CRC were enrolled. The 3-year and 5-year survival for all 837 patients was 74% and 68%, respectively. Table 2 summarizes the univariate analysis results of different clinical and pathological features.

Most patients ( $n = 808$ ) were diagnosed in middle age (median age: 60 years, range: 19-91 years) and 29 were diagnosed at  $\leq 35$  years of age. Patients were divided into four groups according to age at diagnosis: age1  $\leq 35$  years, age2 36-59 years, age3 60-74 years, and age4  $\geq 75$  years (Figure 1A). A significant difference in 5-year

**Table 1 Basic data for patients with colorectal cancer *n* (%)**

| Basic data                         | Colon cancer<br>( <i>n</i> = 437) | Rectal cancer<br>( <i>n</i> = 394) |
|------------------------------------|-----------------------------------|------------------------------------|
| Sex                                |                                   |                                    |
| Male                               | 245 (56.1)                        | 245 (62.2)                         |
| Female                             | 192 (43.9)                        | 149 (37.8)                         |
| Age at operation <sup>1</sup> (yr) | 60.9 ± 13.1                       | 58.3 ± 12.7                        |
| Dukes' staging                     |                                   |                                    |
| A                                  | 38 (8.7)                          | 81 (20.6)                          |
| B                                  | 181 (41.4)                        | 117 (29.7)                         |
| C                                  | 166 (38.0)                        | 172 (43.7)                         |
| D                                  | 49 (11.2)                         | 23 (5.8)                           |
| Status of resection                |                                   |                                    |
| Curative                           | 356 (81.5)                        | 349 (88.6)                         |
| Palliative                         | 62 (14.2)                         | 33 (8.4)                           |
| Undefined                          | 19 (4.3)                          | 12 (3.0)                           |
| Tumor size                         |                                   |                                    |
| ≥ 5 cm                             | 154 (35.2)                        | 64 (16.2)                          |
| < 5 cm                             | 247 (56.5)                        | 263 (66.8)                         |
| Undefined                          | 36 (8.2)                          | 67 (17.1)                          |
| Histological differentiation grade |                                   |                                    |
| Well                               | 93 (21.3)                         | 118 (29.9)                         |
| Moderate                           | 184 (42.1)                        | 180 (45.7)                         |
| Poor                               | 108 (24.7)                        | 61 (15.5)                          |
| Undefined                          | 52 (11.9)                         | 35 (8.9)                           |
| Lymphovascular invasion            |                                   |                                    |
| Positive                           | 14 (3.2)                          | 10 (2.5)                           |
| Negative                           | 423 (96.8)                        | 384 (97.5)                         |
| Perineural invasion                |                                   |                                    |
| Positive                           | 11 (2.5)                          | 3 (0.8)                            |
| Negative                           | 423 (96.8)                        | 391 (99.2)                         |
| Invasion of adjacent organs        |                                   |                                    |
| Positive                           | 37 (8.5)                          | 15 (3.8)                           |
| Negative                           | 396 (90.6)                        | 379 (96.2)                         |
| Undefined                          | 4 (0.9)                           | 0 (0.0)                            |

<sup>1</sup>Data are expressed as mean ± SD. Six cases of double primary colon and rectal cancer were not included.

survival was found between these four groups: age1 65%, age2 66%, age3 74%, and age4 53% (*P* = 0.002).

Among the 837 patients, 495 were male and 342 were female. There was no sex difference in survival (*P* = 0.834). Clinical features of 437 colon cancer patients and 394 rectal cancer patients were recorded. We also found six cases of double primary colon cancer and rectal cancer. In spite of a higher incidence of colon cancer, there were no significant differences in survival between patients with colon cancer and rectal cancer.

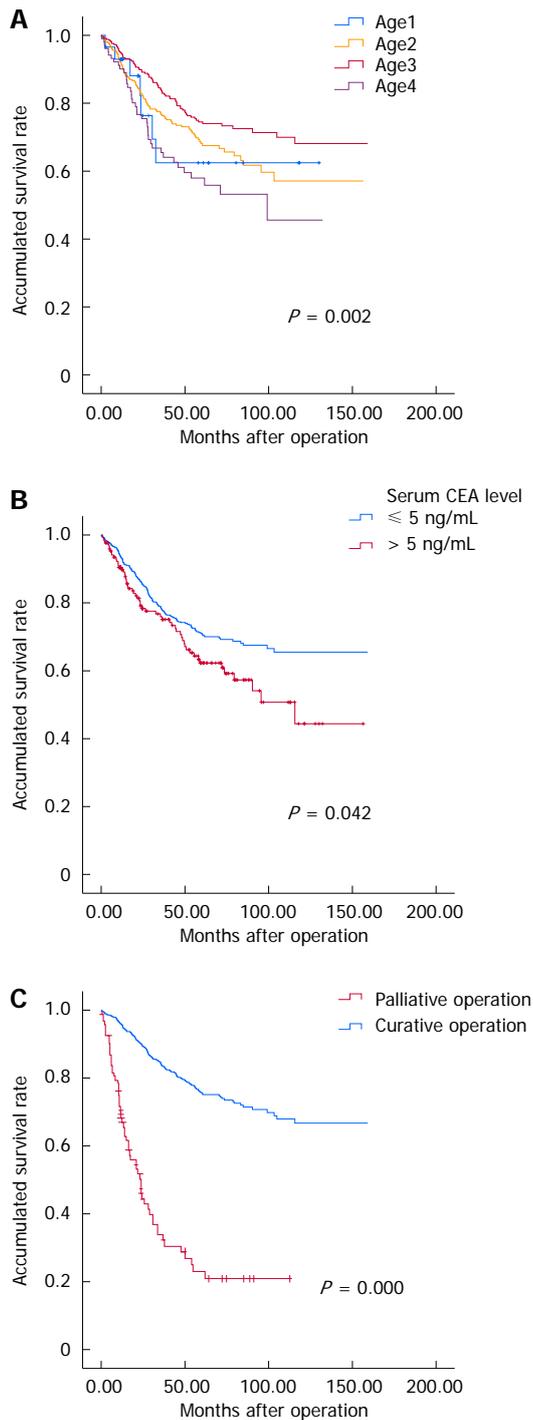
There were 25 patients who had a family history of CRC. It seemed that they had a trend toward better survival than the other 812 patients without a CRC-related family history. The difference was not statistically significant; 3-year survival was 91% *vs* 73% and 5-year survival was 82% *vs* 68% (*P* = 0.391).

According to the results of univariate analysis, patients with obvious clinical symptoms, such as tumor-related obstruction, perforation, diarrhea, constipation, and change of bowel habits had a shorter survival (Table 2). However, only the difference in tumor-related obstruction was statistically significant. The 3-year and 5-year

**Table 2 Univariate analysis of the prognostic factors for patients with colorectal cancer**

|   | <i>n</i> | 3-YSR | 5-YSR | <i>P</i> value <sup>1</sup> |
|---|----------|-------|-------|-----------------------------|
| Age group (yr)                            |          |       |       | 0.002                       |
| Age1 (≤ 35)                               | 29       | 65%   | 65%   |                             |
| Age2 (36–59)                              | 370      | 73%   | 66%   |                             |
| Age3 (60–74)                              | 334      | 78%   | 74%   |                             |
| Age4 (≥ 75)                               | 104      | 61%   | 53%   |                             |
| Sex                                       |          |       |       | 0.834                       |
| Male                                      | 495      | 73%   | 67%   |                             |
| Female                                    | 342      | 74%   | 69%   |                             |
| Family history of CRC                     |          |       |       | 0.391                       |
| Negative                                  | 812      | 73%   | 68%   |                             |
| Positive                                  | 25       | 91%   | 82%   |                             |
| Obstruction                               |          |       |       | 0.000                       |
| Negative                                  | 790      | 76%   | 70%   |                             |
| Positive                                  | 45       | 39%   | 35%   |                             |
| Perforation                               |          |       |       | 0.629                       |
| Negative                                  | 824      | 74%   | 68%   |                             |
| Positive                                  | 11       | 68%   | 68%   |                             |
| Bleeding                                  |          |       |       | 0.116                       |
| Negative                                  | 289      | 69%   | 66%   |                             |
| Positive                                  | 546      | 76%   | 69%   |                             |
| Diarrhea                                  |          |       |       | 0.421                       |
| Negative                                  | 750      | 75%   | 68%   |                             |
| Positive                                  | 85       | 65%   | 63%   |                             |
| Constipation                              |          |       |       | 0.415                       |
| Negative                                  | 776      | 74%   | 68%   |                             |
| Positive                                  | 59       | 72%   | 66%   |                             |
| Habits changes                            |          |       |       | 0.547                       |
| Negative                                  | 531      | 74%   | 69%   |                             |
| Positive                                  | 304      | 73%   | 66%   |                             |
| Serum CEA level                           |          |       |       | 0.042                       |
| ≤ 5 ng/mL                                 | 661      | 74%   | 69%   |                             |
| > 5 ng/mL                                 | 172      | 71%   | 62%   |                             |
| Status of resection                       |          |       |       | 0.000                       |
| Curative                                  | 711      | 80%   | 74%   |                             |
| Palliative                                | 95       | 29%   | 22%   |                             |
| Tumor location                            |          |       |       | 0.705                       |
| Colon cancer                              | 437      | 73%   | 69%   |                             |
| Rectal cancer                             | 394      | 74%   | 66%   |                             |
| Double primary of colon and rectal cancer | 6        | 75%   | 75%   |                             |
| Tumor size                                |          |       |       | 0.004                       |
| < 5 cm                                    | 516      | 77%   | 71%   |                             |
| ≥ 5 cm                                    | 218      | 67%   | 62%   |                             |
| Histological differentiation grade        |          |       |       | 0.001                       |
| Well                                      | 212      | 78%   | 71%   |                             |
| Moderate                                  | 366      | 73%   | 65%   |                             |
| Poor                                      | 170      | 62%   | 60%   |                             |
| Pathological types                        |          |       |       | 0.036                       |
| Non-mucous cell carcinoma                 | 663      | 76%   | 70%   |                             |
| Mucous cell carcinoma                     | 141      | 63%   | 59%   |                             |
| Lymphovascular invasion                   |          |       |       | 0.000                       |
| Negative                                  | 813      | 75%   | 69%   |                             |
| Positive                                  | 24       | 44%   | 36%   |                             |
| Perineural invasion                       |          |       |       | 0.057                       |
| Negative                                  | 820      | 74%   | 68%   |                             |
| Positive                                  | 14       | 42%   | 42%   |                             |
| Invasion of adjacent organs               |          |       |       | 0.000                       |
| Negative                                  | 781      | 75%   | 70%   |                             |
| Positive                                  | 52       | 43%   | 33%   |                             |

<sup>1</sup>*P* values were made by log-rank test. 3-YSR: 3-year accumulative survival rate; 5-YSR: 5-year accumulative survival rate; CEA: Carcino-embryonic antigen; CRC: Colorectal cancer.



**Figure 1** Survival curves of colorectal cancer patients. A: In different age groups; B: With different carcino-embryonic antigen levels; C: With different operation status. CEA: Carcinoembryonic antigen.

survival of 45 patients with preoperative bowel obstruction was 39% and 35% respectively *vs* 76% and 70% in patients without symptoms ( $P < 0.0001$ ). In addition to the clinical symptoms, serum carcino-embryonic antigen (CEA) level is commonly used as a screening and predictive factor for CRC patients (Figure 1B). In our study, the prognosis for patients with high CEA levels of  $> 5$  ng/mL at diagnosis was worse than those who with low CEA levels; 3-year survival was 71% *vs* 74% and 5-year

survival 62% *vs* 69% ( $P = 0.042$ ).

Surgery plays an important role in the treatment of CRC, and radical resection of tumors also has a major influence on prognosis. In our study, 711/837 CRC patients underwent curative surgery, while 95 had palliative surgery due to serious complications or for other reasons (Figure 1C). Compared with patients who had curative surgery, there was a significant decrease in postoperative survival in patients who had palliative surgery; 3-year survival of 80% *vs* 29% and 5-year survival of 74% *vs* 22% ( $P < 0.0001$ ). This confirms that curative surgery is one of the crucial factors affecting prognosis of CRC patients. In addition, the maximum length of the primary lesion, tumor differentiation, histological type, depth of bowel wall invasion, lymphovascular invasion, and invasion of adjacent organs may affect the prognosis of CRC patients ( $P < 0.05$ , Table 2).

Currently, the TNM staging system is widely accepted for tumor staging globally, and also represents the main staging system in our country. The 6<sup>th</sup> revision is regarded as being a significant improvement in CRC staging and the 7<sup>th</sup> revision is considered to be a major turning point in the evolution of cancer staging<sup>[7]</sup>. Regardless of the edition used for staging, survival of CRC patients gradually declined with increase in depth of infiltration of the primary tumor, the number of positive lymph nodes estimated, and status of distant metastases (Table 3, Figure 2). We also found that survival of stage IIIA patients was better than of stage IIB patients regardless of which edition was used to classify postoperative staging: with the 6<sup>th</sup> edition, 5-year survival of stage IIB and IIIA was 75% and 87% ( $P < 0.0001$ ), and for the 7<sup>th</sup> edition, 5-year survival of stages IIB and IIIA was 75% and 91% ( $P < 0.0001$ ).

LNR is defined as the ratio of positive lymph nodes divided by the total number of retrieved lymph nodes, and does not depend on the number of lymph nodes harvested<sup>[8]</sup>. It is considered to be an independent factor that reflects survival of CRC patients, especially those with stage III disease. We calculated the LNR values of 341 stage III cases. The mean LNR was 0.34 (median: 0.25, range: 0-1). Patients were divided into the following three LNR subgroups: LNR1, LNR  $\leq 0.33$ ,  $n = 211$ ; LNR2, LNR 0.34-0.66,  $n = 76$ ; and LNR3, LNR  $\geq 0.67$ ,  $n = 54$  (Figure 3). Survival among these three groups was significantly different ( $P < 0.0001$ ).

After we calculated the positive factors by univariate analysis, we used multivariate analysis (Cox proportional hazard model) to find the most significant prognostic factors (Table 4). First, we analyzed the interaction of the positive clinicopathological factors from univariate analysis, and multivariate analysis showed that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes affected the prognosis of CRC patients ( $P < 0.05$ ). We performed another two separate multivariate analyses with the 6<sup>th</sup> and 7<sup>th</sup> TNM staging systems. We found that histological grade was no longer a positive item when considering the interaction of the

**Table 3 Univariate analysis of tumor node metastasis staging system for patients with colorectal cancer**

| 6 <sup>th</sup> edition of TNM staging system |          |       |       |                | 7 <sup>th</sup> edition of TNM staging system |          |       |       |                |
|---|----------|-------|-------|----------------|---|----------|-------|-------|----------------|
|   | <i>n</i> | 3-YSR | 5-YSR | <i>P</i> value |   | <i>n</i> | 3-YSR | 5-YSR | <i>P</i> value |
| pT  |          |       |       | 0.000          | pT  |          |       |       | 0.000          |
| T1  | 35       | 100%  | 100%  |                | T1  | 35       | 100%  | 100%  |                |
| T2  | 128      | 87%   | 86%   |                | T2  | 128      | 87%   | 86%   |                |
| T3  | 324      | 73%   | 66%   |                | T3  | 324      | 73%   | 66%   |                |
| T4  | 345      | 66%   | 59%   |                | T4a   | 303      | 69%   | 62%   |                |
| Undefined                                     | 5        | 78%   | 78%   |                | T4b   | 42       | 45%   | 33%   |                |
|   |          |       |       | 0.000          | Undefined                                     | 5        | 78%   | 78%   |                |
| pN  |          |       |       | 0.000          | pN  |          |       |       | 0.000          |
| N0  | 445      | 86%   | 80%   |                | N0  | 444      | 86%   | 80%   |                |
| N1  | 224      | 68%   | 61%   |                | N1a   | 103      | 71%   | 63%   |                |
| N2  | 168      | 48%   | 43%   |                | N1b   | 120      | 66%   | 61%   |                |
|   |          |       |       | 0.000          | N1c   | 2        | 50%   | /     |                |
|   |          |       |       |                | N2a   | 82       | 54%   | 43%   |                |
|   |          |       |       |                | N2b   | 86       | 42%   | 42%   |                |
| pM  |          |       |       | 0.000          | pM  |          |       |       | 0.000          |
| M0  | 765      | 78%   | 73%   |                | M0  | 765      | 78%   | 73%   |                |
| M1  | 72       | 28%   | 18%   |                | M1a   | 49       | 29%   | 19%   |                |
|   |          |       |       | 0.000          | M1b   | 23       | 26%   | 17%   |                |
| Stage   |          |       |       | 0.000          | Stage   |          |       |       | 0.000          |
| I   | 121      | 93%   | 93%   |                | I   | 121      | 93%   | 93%   |                |
| II A  | 173      | 88%   | 81%   |                | II A  | 173      | 88%   | 81%   |                |
| II B  | 125      | 85%   | 75%   |                | II B  | 121      | 85%   | 75%   |                |
| III A   | 33       | 87%   | 87%   |                | II C  | 5        | 100%  | 100%  |                |
| III B   | 168      | 68%   | 61%   |                | III A   | 33       | 91%   | 91%   |                |
| III C   | 141      | 53%   | 48%   |                | III B   | 199      | 69%   | 61%   |                |
| IV  | 72       | 28%   | 18%   |                | III C   | 109      | 47%   | 44%   |                |
|   |          |       |       |                | IV A  | 49       | 29%   | 19%   |                |
|   |          |       |       |                | IV B  | 23       | 26%   | 17%   |                |
| Undefined                                     | 4        | 100%  | 100%  |                | Undefined                                     | 4        | 100%  | 100%  |                |

3-YSR: 3-year accumulative survival rate; 5-YSR: 5-year accumulative survival rate; TNM: Tumor node metastasis.

TNM staging system (Table 4). Results for the 6<sup>th</sup> and 7<sup>th</sup> TNM staging systems in multivariate analysis showed significant differences (Table 4, *P* < 0.0001). Another two factors, the depth of bowel wall invasion and the number of metastatic lymph nodes, showed a positive statistical significance, regardless of which TNM staging system was used (Table 4, *P* < 0.05). Besides, with the increase in the number of metastatic lymph nodes with each level, the relative risk of death of CRC patients will increase 1.093 times without consideration of an exact clinical staging. However, this risk decreased to 1.037 times using the 6<sup>th</sup> TNM staging system and 1.047 times using the 7<sup>th</sup> system.

## DISCUSSION

CRC is the fifth most common cancer in China<sup>[3]</sup>. The morbidity and mortality of CRC have shown a clear upward trend in both urban and rural areas over the past 30 years. Although there has been an improvement in surgical techniques and treatment, the 5-year overall survival of CRC is still hovering around 60%. Park *et al*<sup>[9]</sup> have reported a 5-year survival rate of 67.2% in 2230 cases of CRC. In China, Lv *et al*<sup>[10]</sup> has reported 5-year survival rates of 58.4% and 64.5% 383 cases in colon and rectal cancer patients, respectively. In our study, the 3-year and 5-year survival of CRC patients was 74% and 68%, respectively.

The postoperative 5-year survival increased to 74% in our hospital, compared with 66% during 1980-1999<sup>[11,12]</sup>.

From 1980 to the 1990s, rectal cancer accounted for the main part of the incidence of CRC in China<sup>[4,11]</sup>. However, data from Table 2 showed a higher proportion of colon cancer than rectal cancer in our hospital from 1996 to 2006; with 437 cases *vs* 394 cases. Other researchers have reported similar results, which suggests that the proportion of rectal cancer cases is gradually declining<sup>[13-15]</sup>. Although the reason for the change is unclear, some experts have suggested that the higher incidence of colon cancer might be a complex result of changes in dietary habits, the higher rate of diagnosis of colon cancer, etiological changes, and the increased incidence of right colon cancer<sup>[16-20]</sup>.

In addition to the change in location of disease, the age at onset has also changed. Previously, CRC had a higher incidence in elderly people<sup>[21]</sup>. However, recent results at home and abroad have found that detection of CRC in the younger population is increasing<sup>[22]</sup>. CRC in young patients is generally considered a more aggressive disease, which presents at a later stage and has poorer pathological features<sup>[23,24]</sup>. Zhong *et al*<sup>[25]</sup> have reported only a 27.51% 5-year survival rate in young Chinese patients with CRC. In our study, the 5-year survival in the low-age group (age1) was 65%, which was slightly lower than the overall rate (68%), although it had improved

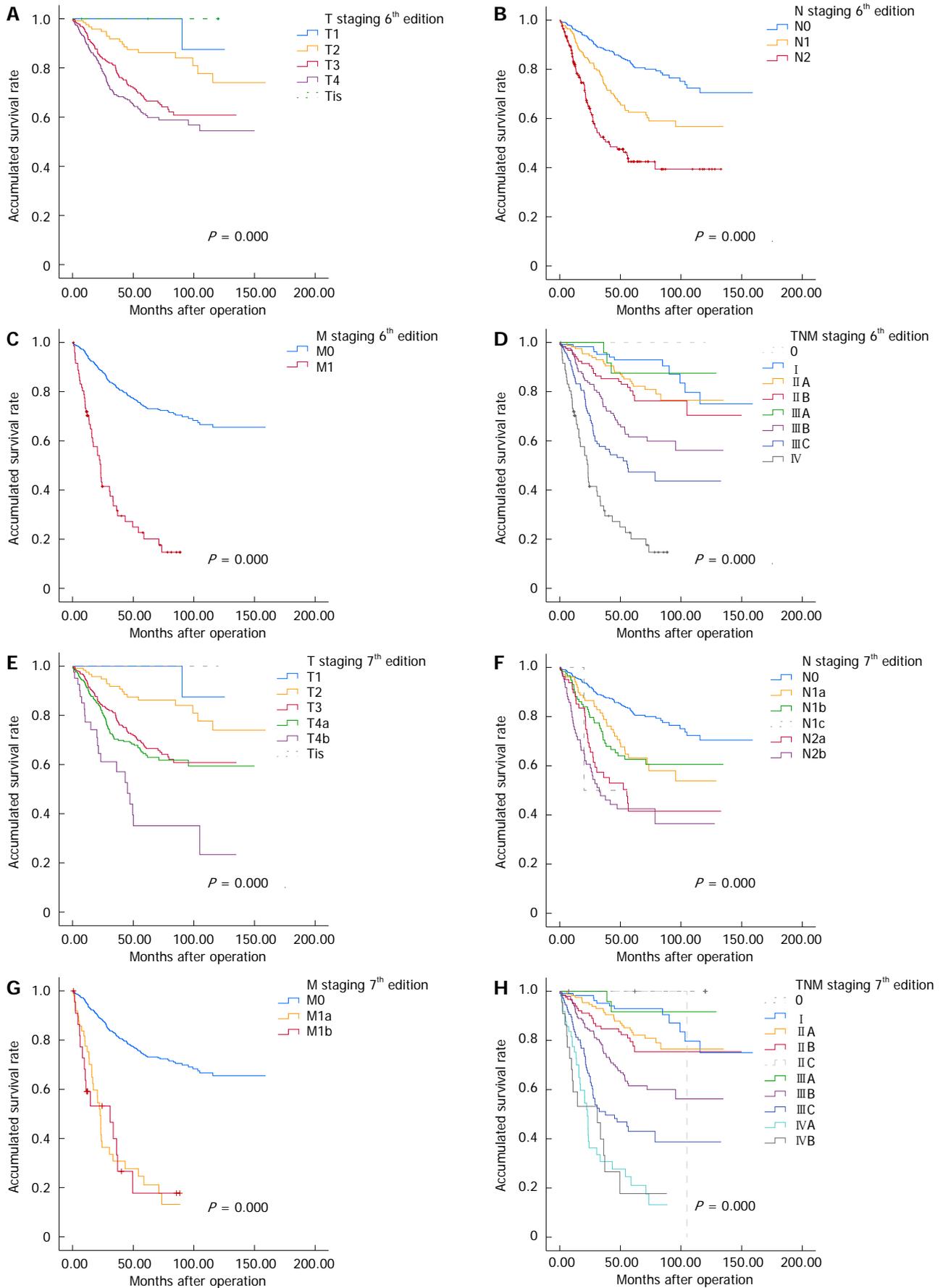


Figure 2 Survival curves of colorectal cancer patients. A-D: According to the 6<sup>th</sup> edition of the tumor node metastasis classification; E-H: According to the 7<sup>th</sup> edition of the tumor node metastasis classification.

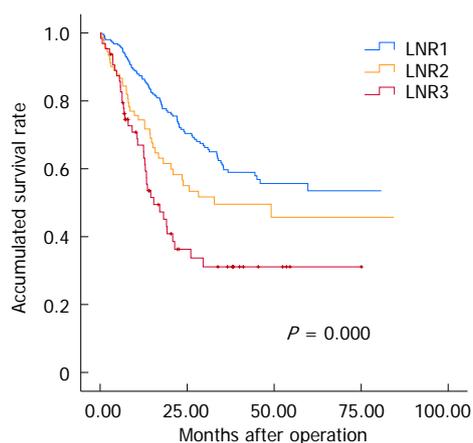


Figure 3 Survival curves of colorectal cancer patients in different lymph node ratio groups. LNR: Lymph node ratio.

from the 5-year survival rate of 53% in patients aged  $\leq 40$  years at our hospital between 1980 and 1999<sup>[26]</sup>. It should be noted that there is no international standard definition of young or old, and the definition of low age in our study is different from that used by Cai *et al.*<sup>[26]</sup>. The overall survival between different age groups showed a significant difference in univariate analysis (Figure 1A), but failed to show a significant difference in the multivariate analysis.

Some reports have suggested that several clinicopathological features contribute to the unfavorable prognosis of CRC in young patients<sup>[27-29]</sup>. A review of the literature has suggested that younger patients with CRC, without relevant predisposing risk factors, have more advanced stages of disease, more aggressive histopathological characteristics, and a poorer prognosis compared with older patients<sup>[24]</sup>. However, there is also some evidence to show that cancer-related survival in young CRC patients seems no less favorable compared with older patients<sup>[30-32]</sup>.

The current international standard for CRC staging is the TNM system. The 7<sup>th</sup> edition of TNM staging, developed by the UICC and American Joint Committee on Cancer, has undergone some significant changes from the 6<sup>th</sup> edition. We tested which of the two versions could predict survival more accurately. Results of univariate analysis showed values in both staging systems were statistically significant prognostic factors ( $P < 0.05$ ). Figure 2D and H demonstrate the differences from stage I to stage IV disease. Similarly, both the 6<sup>th</sup> and 7<sup>th</sup> TNM staging systems were effective for judging the clinical survival and prognosis of CRC based on the results of multivariate analysis. The results also suggest a higher relative risk of death in CRC patients with more metastatic lymph nodes with an unclear clinical staging. It is worth noting that the patients with stage IIIA disease had a better survival than patients with stage IIB disease, as determined from the follow-up data. It might be explained by stage IIIA patients routinely receiving chemotherapy after their operation as part of current clinical practice, while stage IIB patients do not. Some authors also hold the view that lower survival of stage II CRC patients might be

Table 4 Multivariate analysis (Cox proportional hazard model) of prognostic factors

|  | P value | RR    | 95%CI       |
|--|---------|-------|-------------|
| Without interplay tumor node metastasis staging system                 |         |       |             |
| Age group  | 0.060   | 1.193 | 0.993-1.434 |
| Obstruction  | 0.241   | 1.011 | 0.993-1.030 |
| Tumor size   | 0.257   | 1.002 | 0.998-1.006 |
| Serum CEA level  | 0.690   | 0.996 | 0.978-1.015 |
| Status of resection  | 0.082   | 1.005 | 0.999-1.012 |
| Histological grade   | 0.007   | 0.991 | 0.984-0.998 |
| Pathological types   | 0.817   | 0.999 | 0.992-1.006 |
| Depth of bowel wall invasion   | 0.000   | 1.047 | 1.028-1.067 |
| Lymphovascular invasion  | 0.695   | 0.974 | 0.854-1.111 |
| Invasion of adjacent organs  | 0.942   | 0.998 | 0.949-1.050 |
| Number of metastatic lymph nodes                                       | 0.000   | 1.093 | 1.073-1.114 |
| With interplay 6 <sup>th</sup> tumor node metastasis staging system    |         |       |             |
| Age group  | 0.054   | 1.194 | 0.997-1.430 |
| Obstruction  | 0.386   | 1.008 | 0.990-1.028 |
| Tumor size   | 0.259   | 1.002 | 0.998-1.006 |
| Serum CEA level  | 0.789   | 0.997 | 0.979-1.017 |
| Status of resection  | 0.136   | 1.005 | 0.999-1.011 |
| Histological grade   | 0.114   | 0.995 | 0.988-1.001 |
| Pathological types   | 0.290   | 0.996 | 0.989-1.003 |
| Depth of bowel wall invasion   | 0.014   | 1.028 | 1.006-1.050 |
| Lymphovascular invasion  | 0.758   | 0.981 | 0.869-1.108 |
| Invasion of adjacent organs  | 0.840   | 0.994 | 0.935-1.056 |
| Number of metastatic lymph nodes                                       | 0.006   | 1.037 | 1.010-1.065 |
| 6 <sup>th</sup> TNM staging  | 0.000   | 1.471 | 1.344-1.610 |
| With interplay of 7 <sup>th</sup> tumor node metastasis staging system |         |       |             |
| Age group  | 0.094   | 1.168 | 0.974-1.400 |
| Obstruction  | 0.434   | 1.008 | 0.989-1.027 |
| Tumor size   | 0.289   | 1.002 | 0.998-1.006 |
| Serum CEA level  | 0.768   | 0.997 | 0.978-1.016 |
| Status of resection  | 0.184   | 1.004 | 0.998-1.010 |
| Histological grade   | 0.109   | 0.995 | 0.988-1.001 |
| Pathological types   | 0.283   | 0.996 | 0.989-1.003 |
| Depth of bowel wall invasion   | 0.023   | 1.025 | 1.003-1.048 |
| Lymphovascular invasion  | 0.779   | 0.983 | 0.873-1.107 |
| Invasion of adjacent organs  | 0.802   | 0.992 | 0.930-1.058 |
| Number of metastatic lymph nodes                                       | 0.002   | 1.041 | 1.015-1.069 |
| 7 <sup>th</sup> TNM staging  | 0.000   | 1.354 | 1.261-1.454 |

RR: Relative risk; CEA: Carcino-embryonic antigen; TNM: Tumor node metastasis.

related to the particular biological behavior of stage II tumors<sup>[33-35]</sup>.

Lymph node metastasis is a significant component of TNM staging of CRC. Tumor stage and the number of lymph nodes retrieved at resection influence the accuracy of determining nodal status in CRC. They also influence the postoperative treatment strategy of CRC patients. In our study, we took the T, N and M stage as factors in univariate analysis and obtained positive results (Table 3, Figure 2). In addition, multivariate analysis demonstrated a strong relationship between the number of metastatic lymph nodes and survival of CRC patients (Table 4). The relative risk of death is increased with the number of metastatic lymph nodes. The number of lymph nodes found after surgical resection was positively associated with survival of patients with stage II and III colon cancer<sup>[36,37]</sup>. An underestimation of the nodal stage may lead to a high risk of local recurrence and influence decisions regarding adjuvant therapy, as well as influenc-

ing the overall prognosis<sup>[38-41]</sup>. According to the result of the INT-0089 trial, National Comprehensive Cancer Network Colon Cancer Clinical Practice Guidelines recommend that retrieval and examination of  $\geq 12$  lymph nodes can be regarded as adequate lymphadenectomy for accurate staging<sup>[42]</sup>.

There is a difference between the number of metastatic lymph nodes reported during surgery and the actual number of metastatic lymph nodes. The difference may result from many factors, including the extent of surgical dissection and the thoroughness of the pathologists. Cases with insufficient retrieval and undetected lymph nodes are not unusual in clinical practice, although the concept of taking a sufficient number of lymph nodes during surgery to ensure exact postoperative staging is currently agreed. Evaluating lymph node metastasis has become a prognostic factor for CRC, and LNR is an important component of staging. LNR has also been identified as being of significant prognostic value in breast and gastric cancer<sup>[43,44]</sup>. Berger *et al.*<sup>[45]</sup> were the first to suggest LNR as an important prognostic factor after curative resection for CRC. It was then established as a powerful independent index of CRC that reflected the probability of positive lymph nodes based on the number of retrieved lymph nodes<sup>[8,46-48]</sup>. In our study, we found a dramatic decrease in survival with an increase in LNR in stage III CRC patients ( $P < 0.0001$ , Figure 3).

Although the LNR has been emphasized as an important prognostic factor, quantification should be followed for clinical validity. Song *et al.*<sup>[49]</sup> have compared three prognostic factors of CRC and have concluded that LNR classification is a more reliable N classification than the nodal staging in the TNM system and LODDS:

$$\text{defined as } \log \frac{\text{pnod} + 0.5}{\text{tnod} - \text{nnod} + 0.5},$$

pnod is the number of positive lymph nodes, tnod is the total number of lymph nodes retrieved, and 0.5 is added to both numerator and denominator to avoid singularity<sup>[49]</sup>. They believe that LNR is superior to the other two indexes for the following reasons: (1) LNR could contribute to accuracy in prognostic assessment; (2) when the retrieved lymph node numbers is insufficient, TNM nodal staging will be inappropriate for staging migration and will even underestimate prognosis; and (3) as a novel indicator for predicting the status of lymph nodes, evidence of LODDS in CRC is inadequate and is more difficult to calculate and inconvenient for clinical practice<sup>[50]</sup>. When the number of examined lymph nodes is inadequate, LNR is a simple and powerful index to assess the prognosis of CRC patients.

In conclusion, based on the results from our study, we were delighted to find the overall survival in our hospital had improved between 1996 and 2006. Younger patients with CRC have attracted attention because of the increasing number of new cases, their adverse clinicopathological features, and poor prognosis. However, there is still a debate about the prognosis and clinicopathological

features of CRC in young compared to old patients. The pathogenesis and mechanism of disease are still unclear. The overall survival in patients with stage IIIA CRC was better than that in patients with stage IIB disease. This might be a combination of the special biological behavior of stage II CRC and the type of medical intervention for stage III CRC patients. The exact mechanisms of these problems and phenomena need further study.

By using multivariate analysis, we found that tumor histological grade, depth of bowel wall invasion, and metastatic lymph node numbers were independent prognostic factors for patients with CRC if we did not consider the exact clinical staging. We also found other important factors that could affect the prognosis of patients with CRC by univariate analysis, such as patient age, status of resection, and invasion of adjacent organs. The relative risk of death in CRC patients increases with the number of metastatic lymph nodes with an unclear clinical staging, which emphasizes the importance of correct clinical staging.

Surgeons know that a curative operation can greatly improve the overall survival of CRC patients, and resection of a sufficient number of lymph nodes is a necessity for proper postoperative staging. LNR is a powerful factor for assessment of prognosis in stage III CRC patients and is worthy of use in daily practice for evaluating a patient's risk of death. However, we should combine it with other complex factors that together can make a complete assessment so we can devise a proper plan for further treatment.

Besides appropriate treatment, a sensible follow-up plan should be given to CRC patients with full consideration of the factors mentioned above. Moreover, we should devise treatment strategies carefully based on the concept of individualized treatment according to each patient's clinical features, to improve survival and prognosis, especially for those patients with risk factors. In addition, early screening and surveillance by appropriate methods may improve the overall survival of CRC.

## COMMENTS

### Background

In recent years, the morbidity and mortality of colorectal cancer (CRC) has risen in the Chinese population. Although the 5-year overall survival of CRC patients has improved, the overall survival of advanced CRC patients is still poor. There are many impact factors that could influence the prognosis of CRC patients. Thus, a proper model for predicting the prognosis of CRC patients is necessary for both surgeons and physicians.

### Research frontiers

Nowadays, the tumor, node, metastasis (TNM) staging system is approved and widely used for clinical staging of CRC patients. As the latest version of TNM staging system, the 7<sup>th</sup> edition of TNM staging system is considered to represent a major turning point in the evaluation of CRC staging. However, less information of the real assessment validity between the 6<sup>th</sup> and 7<sup>th</sup> versions is available in Chinese populations.

### Innovations and breakthroughs

Recent reports and reviews have highlighted the importance of metastatic lymph node and lymph node ratio (LNR) in predicting prognosis of CRC patients. LNR is an easy but powerful index to evaluate prognosis in stage III CRC patients.

## Applications

Using univariate analysis and Cox proportional hazard regression model, we found that histological grade, depth of bowel wall invasion, and number of metastatic lymph nodes were the most important prognostic factors for CRC without consideration of the interaction of the TNM staging system. LNR is a powerful factor for estimating the survival of stage III CRC patients.

## Terminology

LNR is defined as the ratio of positive lymph nodes divided by the total number of retrieved lymph nodes, and does not depend on the number of lymph nodes harvested. It is considered an independent factor that reflects survival of CRC patients, especially those with stage III disease.

## Peer review

This article is helpful and creative for clinical significance. The results of this article verified the predictive affection of tumor invasion, lymph node metastasis and lymph node ratio. Meanwhile, it concludes that the 6<sup>th</sup> and 7<sup>th</sup> National Comprehensive Cancer Network TNM staging systems are both effective to predict the survival of colorectal cancer patients.

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**P- Reviewers** de Bree E, Zoller M, Ji JF **S- Editor** Huang XZ  
**L- Editor** A **E- Editor** Zhang DN



## Short- and long-term efficacy of endoscopic balloon dilation in Crohn's disease strictures

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Received: October 30, 2012 Revised: December 31, 2012

Accepted: January 18, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To evaluate short- and long-term efficacy of endoscopic balloon dilation in a cohort of consecutive patients with symptomatic Crohn's disease (CD)-related strictures.

**METHODS:** Twenty-six CD patients (11 men; median age 36.8 year, range 11-65 years) with 27 symptomatic strictures underwent endoscopic balloon dilation (EBD).

Both naive and post-operative strictures, of any length and diameter, with or without associated fistula were included. After a clinical and radiological assessment, EBD was performed with a Microvasive Rigiflex through the scope balloon system. The procedure was considered successful if no symptom reoccurred in the following 6 mo. The long-term clinical outcome was to avoid surgery.

**RESULTS:** The mean follow-up time was  $40.7 \pm 5.7$  mo (range 10-94 mo). In this period, forty-six EBD were performed with a technical success of 100%. No procedure-related complication was reported. Surgery was avoided in 92.6% of the patients during the entire follow-up. Two patients, both presenting ileocecal strictures associated with fistula, failed to respond to the treatment and underwent surgical strictures resection. Of the 24 patients who did not undergo surgery, 11 patients received 1 EBD, and 13 required further dilations over time for the treatment of relapsing strictures (7 patients underwent 2 dilations, 5 patients 3 dilations, and 1 patient 4 dilations). Overall, the EBD success rate after the first dilation was 81.5%. No difference was observed between the EBD success rate for naive ( $n = 12$ ) and post-operative ( $n = 15$ ) CD related strictures ( $P > 0.05$ ).

**CONCLUSION:** EBD appears to be a safe and effective procedure in the therapeutic management of CD-related strictures of any origin and dimension in order to prevent surgery.

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**Key words:** Endoscopic balloon dilation; Crohn's disease; Strictures; Endoscopy; Gastrointestinal surgery

de'Angelis N, Carra MC, Borrelli O, Bizzarri B, Vincenzi F, Fornaroli F, De Caro G, de'Angelis GL. Short- and long-term efficacy of endoscopic balloon dilation in Crohn's disease strictures.

World J Gastroenterol 2013; 19(17): 2660-2667 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2660.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2660>

## INTRODUCTION

In the last two decades, the medical therapy for Crohn's disease (CD) has remarkably improved and the introduction of biological therapies has dramatically changed the therapeutic approach in both adults and children<sup>[1,2]</sup>. However, CD still displays an unpredictable clinical course with high incidence of recurrence frequently leading to complications such as strictures, fistulas and abscesses<sup>[3]</sup>. At the time of diagnosis, intestinal strictures may occur throughout the gastrointestinal tract in about 5% of patients, whereas up to one third of the patients develop an intestinal strictures within 10 years of disease activity, with majority of them occurring at the terminal ileum, ileo-colonic, and colonic level<sup>[2,4,5]</sup>.

CD-related strictures is defined as a constant luminal narrowing, which can remain clinically silent or manifest with prestenotic dilatation and obstructive symptoms, such as abdominal bloating, distention, and pain. As the result of the continuous healing response to the chronic inflammation within the intestinal walls, the intestinal stricture induces a progressive narrowing of the lumen and an increased pressure gradient around the stricture, which might ultimately result into the development of internal fistula proximally to the obstruction<sup>[6]</sup>. Stricture-associated fistulas contribute to increase the disease severity and worsen the clinical management.

CD strictures generally show a poor response to medical therapies, and surgical bowel resection or surgical strictureplasty are often required<sup>[5,7,8]</sup>. In patients with CD, intestinal surgery is needed for as many as 80% of CD patients, and a permanent stoma is required in more than 10% of CD patients<sup>[3]</sup>. However, high rate of relapse (defined by recurrence of clinical symptoms) is also observed after surgical resection: 40% at 4 years after bowel resection<sup>[9,10]</sup>, and 50% at 10 to 15 years after ileocecal resection<sup>[11,12]</sup>. Strictureplasty has also been associated with a risk of stricture relapse in 34% of the cases at 7.5 years<sup>[13]</sup>. This implies that up to one third of CD patients will undergo more than one surgery in their life course<sup>[14-16]</sup>. Patients with an early onset disease have an increased risk of surgical relapse and need of repeated resections, which in turn may result in a short bowel syndrome<sup>[13,17-20]</sup>.

Endoscopic balloon dilation (EBD) is a minimally invasive technique that can reduce or delay the need of surgery in patients with CD-related strictures<sup>[21,22]</sup>. With the new generation of double or single endoscopic balloon enteroscopy, this procedure can be performed at almost any level of the gastrointestinal tract. Moreover, the EBD in CD strictures appears to be a safe technique with a low complication rate (0% to 10%)<sup>[22-24]</sup>. It has been shown that the technical success rate of the endoscopic bal-

loon dilation is 95%<sup>[25-27]</sup>, with up to 47% of CD patients showing a long-term global benefit, *i.e.*, a surgery-free period at 3 year follow-up<sup>[22,24]</sup>.

In this prospective study, we aimed to assess the effectiveness and safety of the EBD in a cohort of consecutive CD patients with symptomatic intestinal strictures.

## MATERIALS AND METHODS

### Study cohort

Twenty-six consecutive CD patients (11 males, 15 females), presenting 27 symptomatic strictures (one patient was treated for two strictures), and complaining up to two intestinal symptomatic obstructive episodes as suspected by plain abdominal X-rays or contrast study in the preceding 6 mo, were prospectively enrolled into the trial between March 2004 and March 2011. Diagnosis of CD in both adult and children was based on widely agreed endoscopic and histological criteria<sup>[28,29]</sup>, and disease classification was done according to the Montreal criteria for adults and Paris criteria for children<sup>[30,31]</sup>. A detailed personal and family history was obtained from each patient. The clinical assessment of the patients was performed through the pediatric Crohn's disease activity index (PC-DAI) in children, and through the Crohn's disease activity index (CDAI) in adult<sup>[32,33]</sup>. An PCDAI  $\leq 10$  and a CDAI  $\leq 150$  indicate inactive disease.

Prior to the endoscopic procedure, all patients underwent radiological assessment by abdominal ultrasound with eco-color doppler of mesenteric and the ileocecal region, and magnetic resonance imaging with contrast enhancement to confirm the suspected stricture and to investigate the presence of concomitant fistula or abscess. No exclusion criterion was applied in the selection of strictures to be treated with EBD. Naive and post-surgical strictures, strictures associated with fistula or abscess, and strictures of any length and diameter were included in the study. In all patients surgery was considered for treatment.

### EBD technique

Before the endoscopic procedure, all patients underwent the following tests and medications: standard laboratory blood tests including coagulation tests; mechanical intestinal bowel preparation (approximately 36 h before); and liquid diet (starting at least 12 h before).

The endoscopic dilations were performed under unconscious sedation, obtained by administering IV midazolam +/- meperidine or propofol, under constant monitoring of the vital parameters. All procedures were performed by the same endoscopist (de'Angelis GL), and lasted approximately 1 h. The EBD was carried out using Olympus PCF 140 (Olympus, Germany) and Olympus Ileoscopy single balloon SIF 180 (Olympus, Germany) (according to the stricture site).

The EBD was carried out with a hydrostatic Microvative Rigidflex through the scope balloon system (Microvative Endoscopic, Boston Scientific Corporation<sup>®</sup>, Natick,

**Table 1** Clinical characteristics of the study cohort

| Clinical characteristics of the study cohort ( <i>n</i> = 26)                          | Data               |
|--|--------------------|
| Gender distribution ( <i>n</i> )   |                    |
| Male   | 11                 |
| Female   | 15                 |
| Pediatric/adult patients distribution ( <i>n</i> )                                     |                    |
| Pediatric  | 3                  |
| Adult  | 23                 |
| CD indexes of activity (mean ± SE)   |                    |
| PCDAI ( <i>n</i> = 3)  | 38 ± 7.2           |
| CDAI ( <i>n</i> = 23)  | 365 ± 75           |
| Ongoing medical therapy ( <i>n</i> )   |                    |
| Azathioprine   | 20                 |
| Azathioprine + Infliximab  | 6                  |
| Mean age at the time of the CD diagnosis (yr) [mean ± SE (range)]                      | 22.9 ± 2.8 (2-50)  |
| Mean age at the time of the occurrence of the first stricture (yr) [mean ± SE (range)] | 36.8 ± 3.6 (11-65) |

CD: Crohn's disease; PCDAI: Pediatric Crohn's disease activity index; CDAI: Crohn's disease activity index.

Massachusetts, United States), with a diameter of 15-18 mm. The correct insertion and positioning of the balloon was checked by fluoroscopic control. After reaching the optimal placement through the stricture, the balloon was gradually inflated with water and gastrografin up to 15 mm of diameter, held for 90 s and then deflated. A second inflation up to 18 mm diameter for 90 s was always performed. In case of resilient strictures, the process of inflation was repeated up to 6 times in the same session, reaching progressively larger balloon diameters. Once the balloon dilation was accomplished, a combination of methylprednisolone (40 mg) diluted in 5 mL of normal saline solution were injected intra-lesionally with Olympus single use injector 0, 5 mm (Olympus, Japan). The ultimate step of the procedure consisted in examining the proximal bowel (30 cm above the stricture) in order to detect others possible lesions that were undetected by the pre-procedural assessing imaging.

In combination with underlying treatment, after EBD each patient was treated by administering prednisolone with a dosing scheme determined by body weight: 1.5 mg/kg daily (maximum allowed dose 60 mg daily) for 2 wk, followed by a 4 wk tapering course.

### Clinical outcomes

The technical success of the procedure was defined as the passage of the endoscope through the stricture, reaching a diameter of approximately 15 mm. Procedure-related complications were defined as intestinal perforation, and active bleeding requiring surgery or blood transfusions. The long-term clinical success was defined as surgery was avoided all long the follow-up period by obtaining symptom relief with repeated EBD procedures. The short-term clinical success was defined as 6 mo symptom-free period after the EBD. The need of re-dilation was determined based on clinical and imaging criteria in association with persistence or reoccurrence of

**Table 2** Clinical characteristics of Crohn's disease-related strictures

| Stricture characteristics ( <i>n</i> = 27) | Data             |
|--|------------------|
| Nature of the stricture ( <i>n</i> )       |                  |
| Naive                                      | 12               |
| Post-surgical                              | 15               |
| Location of the stricture ( <i>n</i> )     |                  |
| Upper gastrointestinal                     | 1                |
| Small intestine                            | 2                |
| Ileo-colonic                               | 14               |
| Colonic                                    | 10               |
| Mean length (cm) [mean ± SE (range)]       | 4.6 ± 0.4 (2-12) |
| ≤ 4 cm ( <i>n</i> )                        | 15               |
| > 4 cm ( <i>n</i> )                        | 12               |
| Mean diameter (mm) [mean ± SE (range)]     | 2.5 ± 0.2 (1-6)  |
| ≤ 5 mm ( <i>n</i> )                        | 26               |
| > 5 mm ( <i>n</i> )                        | 1                |
| Stricture associated fistula ( <i>n</i> )  |                  |
| Yes  | 5                |
| No   | 22               |

obstructive symptoms. Surgery was reserved for strictures that did not respond to the medical and the endoscopic therapy.

### Ethical considerations

The work carried out was in accordance with the principles laid down in the Declaration of Helsinki for biomedical research involving humans. All adult patients included in the study gave their consent for the use of their clinical data. For children, written consent was obtained from both parents and those older than 12 year signed a statement of assent.

### Statistical analysis

Data were analyzed by using SPSS (IBM SPSS Statistics, Version 17.0.0 for Macintosh, Chicago, IL, United States). Kaplan-Meier analysis was performed for periods free of surgery and free of endoscopic re-dilation. Regression statistics were used to relate the clinical and demographic variables to the main outcome (*i.e.*, need of surgery). *P* value ≤ 0.05 was considered significant. Data are expressed as median and range, or mean ± SE, unless otherwise stated.

## RESULTS

The patients' demographic and clinical characteristics, and the stricture characteristics are summarized in Tables 1 and 2 respectively. Forty-six EBD were performed for 27 symptomatic strictures occurred in the 26 CD patients. Of these, 15 patients had post-surgical strictures and 11 had naive strictures. The technical success of the endoscopic procedure was achieved in all patients without any endoscopic related complication (Figure 1).

The mean follow-up time of the cohort was 40.7 ± 5.7 mo (range 10-94 mo). All patients survived during the follow-up period. Of the 26 CD patients that were treated with EBD, only two failed to respond to the treat-

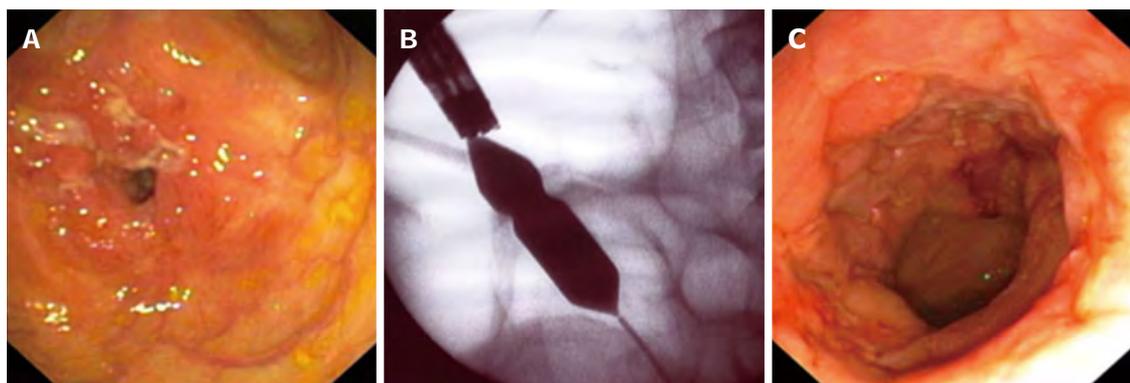


Figure 1 Images of Crohn's disease-related stricture in one patient. A: Direct visualization of the ileal stricture; B: Inflation of the endoscopic balloon under fluoroscopic control; C: Direct visualization of the bowel site after the endoscopic balloon dilation.

ment and underwent elective surgical laparoscopic stricture resection. Both patients requiring surgery presented ileocecal strictures associated with fistula. The overall long-term clinical success rate was 92.6% (24/26 patients remained free of surgery) (Figure 2A).

Of the 24 patients who did not undergo surgery all long the follow-up period, 11 patients received only 1 EBD, and 13 required further dilations over time for the treatment of relapsing strictures (7 patients underwent 2 dilations, 5 patients 3 dilations, and 1 patient 4 dilations). The mean time free of re-dilation between the first and the second EBD was  $21.2 \pm 5$  mo. The cumulative percentages of patients free of re-dilation over the entire follow-up period are shown in Figure 2B.

Throughout the study population, the short-term clinical success rate was 81.5% (2 patients required surgery; 3 patients did not have symptom-free 6 mo) after the first EBD. After the second EBD, the clinical success rate was 92.3% (12/13 patients); after the third EBD, the clinical success rate was 83.3% (5/6 patients). Only one patient underwent a fourth EBD showing a clinical success. The subgroup analysis dividing the study population into two groups based on the nature of the strictures, *i.e.* naive vs post-operative, showed no statistical difference between groups in term of clinical success after repeated EBD. Indeed, after the first dilation, short-term clinical success was obtained in 93.3% of the post-operative strictures and 66.7% of the naive strictures [not significant (NS)]; after the second EBD, success was obtained in 100% of the post-operative strictures and in 80% of the naive ones (NS); after the third EBD, success was observed in 100% of the post-operative strictures and 66.7% of the naive strictures (NS). The cumulative percentages of patients free of re-dilation over the entire follow-up period for both naive and post-operative strictures are shown in Figure 2C.

Of the variables evaluated, the presence of stricture-associated fistula and the stricture location at the ileocecal level resulted significant predictive factors on the long-term negative clinical outcome, *i.e.*, the need of surgery (both  $P = 0.002$ ). In fact, the 2 strictures that required surgery after the first EBD due to the failure of the

endoscopic procedure (*i.e.*, persistency of subocclusion symptoms) were sited at the ileocecal level and were associated with fistula. On the contrary, the sex and age of the patient, the nature of the strictures (naive *vs* post-surgical), the severity of CD activity, the dimension of the strictures (lengths and diameters), and the medical therapy did not influence the long-term clinical outcomes (Table 3).

## DISCUSSION

The present study describes the clinical follow-up of a cohort of 26 CD patients presenting with symptomatic strictures and treated with EBD. The EBD appeared to be a safe technique that prevented the need of surgery in 92.6% of the patients during our follow-up period. The endoscopic treatment associated with the medical therapy influenced the natural history of the disease and thus it can be considered an effective strategy in the management of symptomatic strictures in CD patients.

EBD has become more and more used in the treatment of CD strictures since it demonstrated to be a safe and minimally invasive technique, while conserving the intestinal length. At the same time, the medical therapy is largely applied to manage the clinical course of this inflammatory disease and to control its clinical evolution. A combined medical and endoscopic therapy has shown to be effective in the treatment of CD-related strictures<sup>[22,34]</sup>. However, standardized clinical guidelines and protocols are missing.

Our cohort study presents some points of strength and novelty. In fact, to describe the effectiveness of EBD and its influence of the natural history of the disease, we decided to recruit in the study consecutive CD patients without any strictures related exclusion criterion. Conversely to the other studies<sup>[21,22,34,35]</sup>, we considered strictures of any nature and lengths (up to 12 cm), with and without associated fistula. The objective was to analyze a population that is the most often seen in the everyday clinical practice compared to the highly selected cohorts of patients that are usually described in the literature. Our results demonstrated that the EBD can be

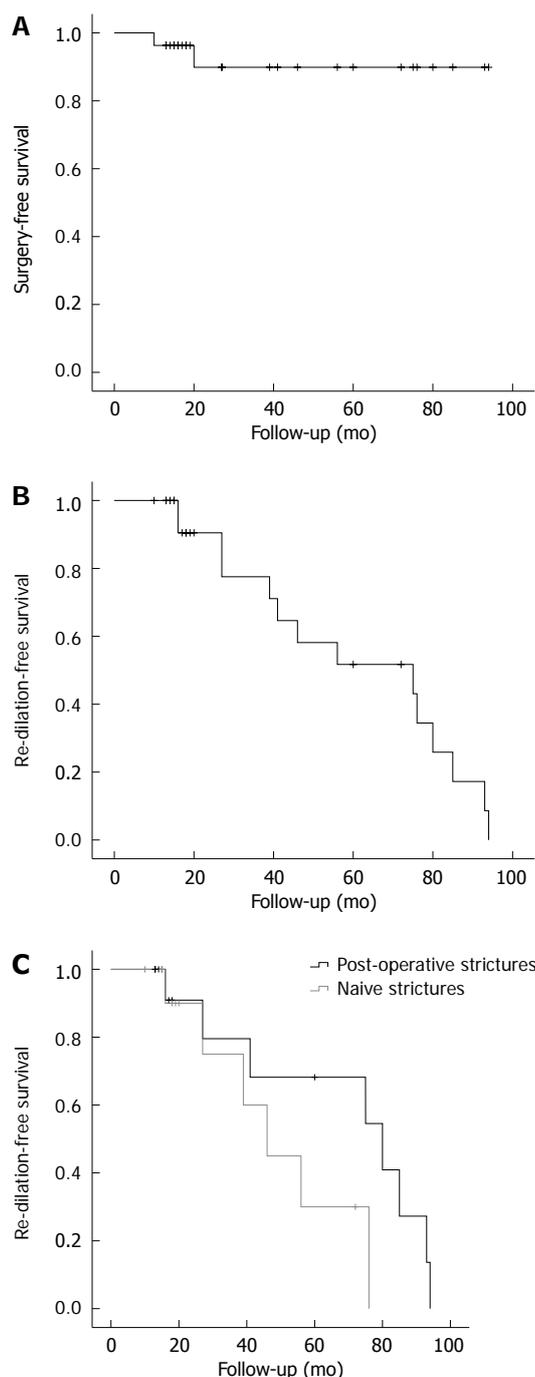


Figure 2 Kaplan-Meier curves for intervals free of surgery or endoscopic dilation. A: Kaplan-Meier curve for interval free of surgery after endoscopic balloon dilation or re-dilation; B: Kaplan-Meier curve for interval free of re-dilation during the follow-up period; C: Kaplan-Meier curve for the interval free of re-dilation over the follow-up period for patients with naive (in gray) and post-operative (in black) strictures.

effectively used also in CD related strictures longer than 4 cm in order to avoid or postpone surgery. The lengths of the strictures did not appear to be a contraindication for performing EBD as a first line treatment in contrast to previous studies<sup>[24,34]</sup>. The EBD overall long-term success rate in our study is higher than the values of Hassan *et al.*<sup>[34]</sup> in a recent systematic review, which reported a cumulative mean success rate of 67% (ranging from 41%

**Table 3 Analyses of the influence of the clinical variables on the occurrence of surgery**

| Variables                              | No surgery (n) | Surgery (n) | P value |
|--|----------------|-------------|---------|
| Male sex                               | 10             | 1           | NS      |
| Adult patients                         | 21             | 2           | NS      |
| Naive strictures                       | 10             | 2           | NS      |
| Moderate disease activity              | 12             | 2           | NS      |
| Strictures length > 4 cm               | 11             | 1           | NS      |
| Strictures diameters ≤ 5 mm            | 24             | 2           | NS      |
| Strictures with fistula                | 3              | 2           | 0.002   |
| Strictures at the ileocecal level      | 3              | 2           | 0.002   |
| Pharmacological therapy (azathioprine) | 18             | 2           | NS      |

NS: Not significant.

to 100%) over an average follow-up time very close to one in the present study. In order to avoid surgery, EBD was repeated-up up to 4 times during the follow with a high short-term clinical success rate after each procedure and a technical success rate of 100% with no procedure-associated complication. This result may be also related to the high-volume endoscopy center in which EBD were performed.

Based on these findings and in accordance with other studies<sup>[22,24,34,35]</sup>, EBD appeared to be a safe procedure with a very low complication rate when an endoscopist experienced in the management of bowel stricture performs it. In our study, we showed that EBD is safe and feasible even in a non-selected sample of CD patients. Moreover, the safety of the procedure may be related to the diameters of the endoscopic balloon used. In our study the 18 mm balloon was the largest applied, for not more than 90 s inflations and no more than 6 dilations per session. If prudence is respected in the selection of balloon dimensions, number of dilations, and progressive inflations, the intra-operative complications may be minimized<sup>[22,23,36]</sup>. The procedural safety is essential in consideration of the need of frequent re-dilations over time in the same patient in order to obtain and maintain symptomatic relief.

EBD can be considered a valuable and safe alternative of surgical resection, but EBD and surgery must not be seen as mutually exclusive solutions for CD strictures. Rather EBD may be a complementary procedure that should be considered in both adult and pediatric patients in order to reach a symptom-free condition with a low risk associated.

As previously reported, we found that the stricture location at the ileocecal level appeared to be a significant negative predictor on the long-term clinical outcome, *i.e.*, the need of surgery<sup>[24]</sup>. In our study, also the presence of fistula was associated with the occurrence of surgery. Notwithstanding, three patients with fistulizing phenotype (2 located at the colo-rectal anastomosis and 1 in the sigma), showed good clinical short and long-term outcomes after EBD, suggesting that the analysis results may represent a type II error and that larger groups could provide different results. Studies on EBD in CD

patients presenting strictures associated with fistula are scarce in the literature, since the majority of the previously published case series considered the presence of fistula as a patient's exclusion criterion<sup>[21,27,35,37]</sup>. Although the paucity of data, in a similar clinical scenario the EBD should not be excluded a priori. Furthermore, it could be used, together with the medical therapy, as an option to bridge the patient to surgery in better performance conditions (*e.g.*, nutrition, inflammatory status), shifting from an emergency to an elective surgery. It is noteworthy that in patients with an adequate nutrition status in which the intestinal obstructive condition has been endoscopically managed (even if suboptimally), the response to surgery and the post-surgical complication rate (*e.g.*, anastomotic leakage) are generally improved<sup>[38,39]</sup>. The other examined variables seem to not affect the clinical outcomes. However, we were not able to detect which variables are associated with clinical success after only one EBD, and which can predict the need of further dilations. These aspects should be studied in a larger sample of patients.

In our cohort, both naive and post-surgical strictures responded to the EBD treatment, without significant difference on the clinical outcomes as seen by other authors<sup>[24,25]</sup>. Thus, EBD can be considered a valuable strategy in the management of both naive and post-surgical CD related strictures. In parallel, EBD resulted equally effective in adult and pediatric CD patients. It must be emphasized that the endoscopic management of CD strictures is cardinal in pediatrics because of the long life expectancy of these patients, who would more probably develop a short bowel syndrome if repeated surgical resections are performed. Moreover, many clinical concerns are related to the malnutrition and subsequent failure to thrive that will follow the obstructive condition in children if this is not immediately managed<sup>[40]</sup>.

Interestingly, all our patients were still medically both before and after the endoscopic treatment, mainly with azathioprine and/or biological therapy. Factors determining the development of strictures are not fully understood, but chronic and trans-mural inflammation probably plays a major role<sup>[41-43]</sup>. Although the lack of data in the literature, azathioprine has been shown to reverse the inflammatory changes at the anastomotic site and to maintaining remission in CD patients<sup>[44,45]</sup>. Conversely, because of reports of complete obstruction after infliximab in patients with or without initial stricture, its use has been contra-indicated in stenotic forms of CD<sup>[46]</sup>. Theoretically, the rapid tissue healing induced by infliximab administration may result in marked architectural changes in the intestinal wall, which may lead to wall stricturing. However, strictures do not occur without inflammation, and chronic inflammation *per se* may lead to strictures. In fact, a long-term inflammatory process sustained by increased cytokine production leads to an excess of fibrotic response. On the other hand, substantial thickening of the mesenchymal layers is observed during mucosal repair. The control of chronic inflammation to prevent fibrosis and stenosis seems more important than the risk of fibrosis induced by treatment, thus justifying inflix-

imab infusions<sup>[41]</sup>. However, the role of biological therapy in case of CD strictures remains controversial<sup>[47,48]</sup>. In our cohort, we were not able to define the role and support of the two medical therapies on the clinical outcomes evaluated. With this objective, multicenter, randomized, controlled, blind clinical trials should be performed. The use of EBD is supported not only by the clinical risk/benefit ratio, but also by the costs associated to this procedure. In Italy, the entire EBD procedure (pre-endoscopy exams; hospitalization; medications; balloon dilation kit) is comprised between 1000 and 1200 Euros (reimbursed by the National Health System).

In conclusion, the EBD is not just an attractive treatment option in the management of CD-related strictures. The available literature provides quite enough evidence to support its use. However, clinical guidelines, especially on the combined medical and endoscopic therapy, are still lacking. Time has come to investigate EBD clinical benefits and success in large clinical studies in order to define and standardize the protocol of use.

## COMMENTS

### Background

Crohn's disease (CD)-related strictures are scarcely responsive to medical therapy and thus they are mainly treated surgically. Recently, endoscopic balloon dilation has been increasingly used in the treatment of CD-related strictures.

### Innovations and breakthroughs

The present study evaluated the efficacy of endoscopic balloon dilation (EBD) in CD-related strictures of any length (up to 12 cm), both naive and post-operative, with or without associated fistula, and in both adult and pediatric patients. Strictures located in both upper and lower gastrointestinal were successfully treated using gastroscope, colonoscope or ileoscope according to the stricture site. Their results confirmed that over a mean follow-up period of 40.7 mo multiple endoscopic balloon dilations were safe and effective to manage CD-related strictures.

### Applications

The present study supports EBD as a valuable option in the treatment of symptomatic CD patients. Moreover, EBD demonstrated as a safe technique, which can be repeated over time in order to avoid surgery, since it can be performed successfully also in relapsing patients who were previously endoscopically dilated. The associated complications are very rare (none in this study).

### Terminology

EBD is an operative endoscopy procedure used to dilate intestinal strictures in unconscious patients. The dilation is carried out with a hydrostatic through the scope balloon system, which, once inserted and positioned through the stricture, is gradually inflated with water, held for 90 s and then deflated to obtain stricture dilation.

### Peer review

de'Angelis *et al* report an interesting series of EBD of strictures in CD. The inclusion of both naive and postoperative strictures with or without associated fistula reflects the usual clinical scenario in CD strictures. Although the number of the cases included in the study is not so large, the conclusions are well presented and discussed. I agree with the authors that until clinical guidelines are available EBD would be a treatment option before surgery in this kind of patients.

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**P- Reviewers** Tsuyoshi K, Sreenivasan S, Di Martino N, Santiago V  
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## Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia

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Received: November 11, 2012 Revised: February 14, 2013

Accepted: March 6, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To investigate the predictive value of narrow-band imaging with magnifying endoscopy (NBI-ME) for identifying gastric intestinal metaplasia (GIM) in unselected patients.

**METHODS:** We prospectively evaluated consecutive patients undergoing upper endoscopy for various indications, such as epigastric discomfort/pain, anaemia, gastro-oesophageal reflux disease, suspicion of peptic ulcer disease, or chronic liver diseases. Patients underwent NBI-ME, which was performed by three blinded, experienced endoscopists. In addition, five biopsies (2 antrum, 1 angulus, and 2 corpus) were taken and examined by two pathologists unaware of the endoscopic

findings to determine the presence or absence of GIM. The correlation between light blue crest (LBC) appearance and histology was measured. Moreover, we quantified the degree of LBC appearance as less than 20% (+), 20%-80% (++) and more than 80% (+++) of an image field, and the semiquantitative evaluation of LBC appearance was correlated with IM percentage from the histological findings.

**RESULTS:** We enrolled 100 (58 F/42 M) patients who were mainly referred for gastro-esophageal reflux disease/dyspepsia (46%), cancer screening/anaemia (34%), chronic liver disease (9%), and suspected celiac disease (6%); the remaining patients were referred for other indications. The prevalence of *Helicobacter pylori* (*H. pylori*) infection detected from the biopsies was 31%, while 67% of the patients used proton pump inhibitors. LBCs were found in the antrum of 33 patients (33%); 20 of the cases were classified as LBC+, 9 as LBC++, and 4 as LBC+++. LBCs were found in the gastric body of 6 patients (6%), with 5 of them also having LBCs in the antrum. The correlation between the appearance of LBCs and histological GIM was good, with a sensitivity of 80% (95%CI: 67-92), a specificity of 96% (95%CI: 93-99), a positive predictive value of 84% (95%CI: 73-96), a negative predictive value of 95% (95%CI: 92-98), and an accuracy of 93% (95%CI: 90-97). The NBI-ME examination overlooked GIM in 8 cases, but the GIM was less than 5% in 7 of the cases. Moreover, in the 6 false positive cases, the histological examination showed the presence of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases). The semiquantitative correlation between the rate of LBC appearance and the percentage of GIM was 79% ( $P < 0.01$ ).

**CONCLUSION:** NBI-ME achieved good sensitivity and specificity in recognising GIM in an unselected population. In routine clinical practice, this technique can reliably target gastric biopsies.

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**Key words:** Narrow-band imaging; Magnification; Gastric intestinal metaplasia; Light blue crest; Gastric cancer; Endoscopy; Precancerous conditions; Gastric biopsy

**Core tip:** Gastric cancer is one of the most common neoplastic diseases in the Western world and has a poor prognosis and inconsistent signs and symptoms in the early phases. Narrow-band imaging with magnifying endoscopy was shown to be a valid method for intestinal metaplasia (IM) detection, this technique can reliably target which patients should be biopsied to evaluate IM and those who do not need biopsies. Moreover, a semi-quantitative evaluation of light blue crest appearance was feasible as there was a good correlation with the histological assessment of IM percentage.

Savarino E, Corbo M, Dulbecco P, Gemignani L, Giambruno E, Mastracci L, Grillo F, Savarino V. Narrow-band imaging with magnifying endoscopy is accurate for detecting gastric intestinal metaplasia. *World J Gastroenterol* 2013; 19(17): 2668-2675 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2668.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2668>

## INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related death worldwide<sup>[1,2]</sup>. However, the mechanism of gastric carcinogenesis is unknown. The intestinal type tumour, according to the Lauren classification, is characterised by the presence of malignant cells forming glandular structures; in contrast to diffuse type carcinomas, these tumours are generally thought to be preceded by a sequence of precursor lesions. In this multistep model of gastric carcinogenesis, described several years ago by Correa *et al*<sup>[3]</sup>, *Helicobacter pylori* (*H. pylori*) causes chronic inflammation of the gastric mucosa, which slowly progresses through the premalignant stages of atrophic gastritis, intestinal metaplasia and dysplasia, intramucosal carcinoma and invasive neoplasia<sup>[4]</sup>.

Intestinal metaplasia (IM) is generally considered as the “field cancerization” in the gastric mucosa. At the cell level, intestinalized glands provide the cellular substrate that allows the gastric non-invasive neoplasia to develop. The reported progression rates of IM and dysplasia to cancer vary greatly from 0% to 10% per year for IM and from 0% to 73% per year for dysplasia<sup>[1]</sup>. The prevalence of this condition in Europe varies by country<sup>[5-9]</sup>; for instance, a Swedish group reported an IM prevalence of 23% in the general population<sup>[10]</sup>, while the prevalence has been estimated to be up to 25% in the Italian population, mainly occurring in the gastric antrum<sup>[7,11-13]</sup>.

In Western countries, premalignant gastric lesions and

early gastric cancer are generally diagnosed upon histologic examination of random biopsies, whereas in Asian countries, especially in Japan, the presence and extension of these lesions are frequently established during an endoscopy<sup>[14-16]</sup>. These remarkable differences are explained by different training and attitudes towards the inspection of the stomach in countries with a high gastric cancer incidence.

The narrow-band imaging system (NBI) is based on the modification of optical filter spectral characteristics in the light source, which improves the visibility of mucosal structures. NBI may be combined with magnification endoscopy to obtain a clear visualisation of surface and vascular patterns<sup>[1,2]</sup>. Earlier studies using the NBI system with magnification endoscopy (NBI-ME) in the gastric mucosa showed that the appearance of a light blue crest (LBC) in the mucosa is a distinctive endoscopic finding that suggests an increased likelihood of detecting IM in the stomach. More precisely, blue-whitish patchy areas are often observed in NBI images of the antrum in patients with gastric IM. Uedo *et al*<sup>[16]</sup> tested NBI-ME on 34 patients with atrophic gastritis and found that the LBC appearance was frequently observed in gastric antrum as blue-white lines visible on the epithelial surface. These authors demonstrated that the appearance of LBCs correlated with histological evidence of IM, with a sensitivity of 89% (95%CI: 83-96), a specificity of 93% (95%CI: 88-97), a positive predictive value of 91% (95%CI: 85-96), a negative predictive value of 92% (95%CI: 87-97) and an accuracy of 91% (95%CI: 88-95).

Despite these promising findings, the added value of NBI-ME in the stomach, and more precisely in detecting premalignant and malignant gastric lesions, requires confirmation from controlled longitudinal trials. Furthermore, a uniform and validated classification system is needed. Thus, the aim of our study was to define the value of NBI-ME for identifying gastric IM in an unselected population through the visualisation of LBC appearance. A secondary aim was to provide a semiquantitative evaluation of LBC appearance and to verify a correlation with the histological measurement of IM percentage.

## MATERIALS AND METHODS

### Subjects

This prospective, blinded study included unselected consecutive patients presenting to the endoscopy unit of the University Hospital of Genoa, Italy. Patients underwent an upper endoscopy for various indications, such as epigastric discomfort/pain, anaemia, gastro-oesophageal reflux disease, suspicion of peptic ulcer disease, or chronic liver diseases. Exclusion criteria included a previous gastrectomy or partial gastric resection; treatment with antiplatelet medication, anticoagulant medication or non-steroidal anti-inflammatory drugs; and the presence of haemorrhagic diseases. The study protocol was approved by the local Ethics Committee and was performed according to the Declaration of Helsinki. All patients pro-

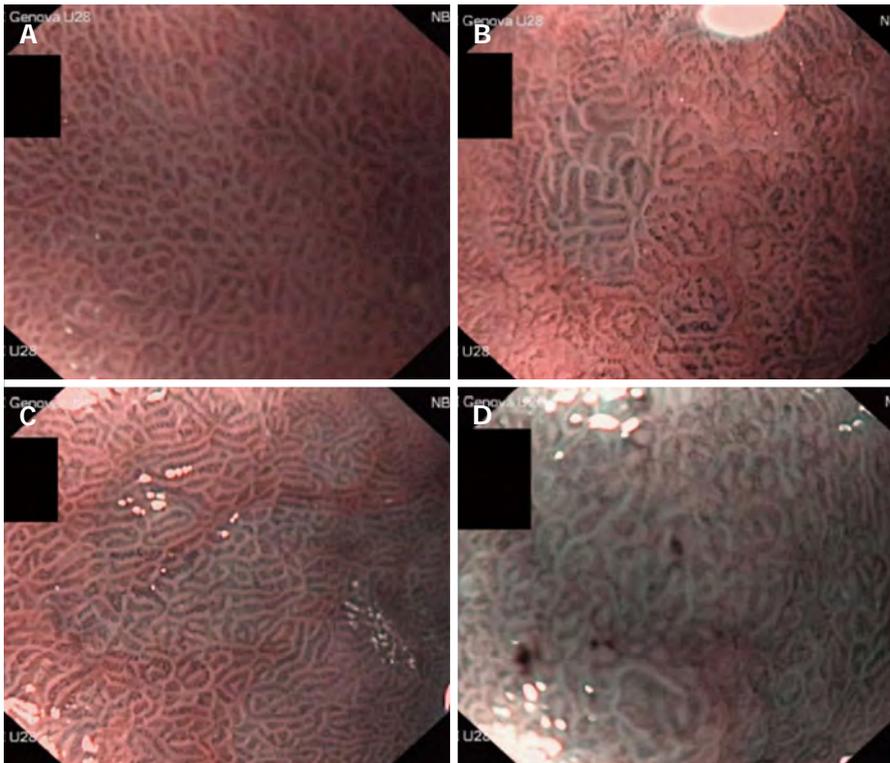


Figure 1 Normal mucosal aspects. A: Narrow-band-imaging with magnification endoscopy examination and the degree of light blue crest appearance; B: Light blue crest (LBC) +, less than 20%; C: LBC ++, 20%-80%; D: LBC +++, 80% or more.

vided written informed consent before the start of the study.

### Study protocol

All subjects who agreed to participate in our investigation underwent a thorough physical and clinical examination, a careful collection of their medical history (including current medications, tobacco use, and alcohol and coffee consumption) and an upper gastric intestinal (GI) endoscopy with the NBI-ME system to determine the presence or absence of gastric IM. Five biopsies (2 antrum, 1 angulus and 2 corpus) were collected from all patients according to the updated Sydney classification<sup>[17]</sup>.

### Endoscopic procedure

The endoscopic examinations were performed by experienced endoscopists (Corbo M, Savarino E, and Dulbecco P) who were blinded to the conditions and medical histories of the patients. Before starting the study, each endoscopist underwent training to obtain good expertise in the detection of IM using NBI-ME. Each endoscopist performed > 300 NBI-ME gastroscopies in patients selected independently of a previous diagnosis of IM and compared their NBI-ME findings with those obtained after a histologic evaluation. At the end of this training, the overall ability to detect histologic IM using NBI-ME was very high (92%). The procedure was conducted with a magnifying endoscope (GIF-Q160Z; Olympus Medical Systems, Tokyo, Japan; 10.9 mm outer diameter insertion tube with a 2.8 mm channel diameter; optical magnifica-

tion  $\times 115$ ). A disposable attachment was fitted to the scope tip to maintain focal distance from the mucosa for magnifying observation (approximately 2 mm). A conscious sedation was performed at the request of the patient. Before the procedure, all patients ingested 66.6 mg of simethicone diluted in 40 mL of water (Istituto Biochimico Italiano Giovanni Lorenzini S.p.A., Aprilia, Italy). If poor visualisation persisted, the gastric mucosal surface was rinsed with an additional 30-60 mL of the simethicone solution. After white light examination, the gastric antrum and body were examined by NBI for blue-whitish patchy areas and then by NBI-ME observation at the maximum magnification, as LBC was frequently observed in these areas. If no blue-whitish areas were observed, NBI-ME was conducted at the proximal and distal portions of the following areas: the anterior wall, the lesser curvature, the posterior wall, and the greater curvature, as previously described<sup>[16]</sup>. During NBI-ME observation, we assessed for the presence or absence of LBCs, defined as a fine, blue-white lines on the crests of the epithelial surface that are similar in appearance to the light reflected from a mirror. The patient was defined as LBC-negative if LBCs were not observed in any of the image fields and as LBC-positive if LBCs were observed in any of the image fields. Moreover, we quantified the degree of LBC appearance as less than 20% (+), 20%-80% (++) and more than 80% (+++) of an image field (Figure 1). In each examination, six pictures were taken and stored (one during NBI and two during NBI-ME observation, both in the antrum and in the corpus).

**Table 1** Demographic and clinical characteristics of unselected patients ( $n = 100$ ) included in the study  $n$  (%)

| Demographic and clinical features | Patients    |
|-----------------------------------|-------------|
| Age, yr (mean $\pm$ SD)           | 67 $\pm$ 12 |
| Sex                               |             |
| Male                              | 42 (42)     |
| Female                            | 58 (58)     |
| <i>H. pylori</i> infection        |             |
| Positive                          | 31 (31)     |
| Negative                          | 69 (69)     |
| Smoking                           |             |
| Non-smoker                        | 68 (68)     |
| Current smoker                    | 16 (16)     |
| Former smoker                     | 16 (16)     |
| Alcohol consuming                 |             |
| Non-drinker                       | 75 (75)     |
| Current drinker                   | 25 (25)     |
| Medication                        |             |
| PPI users                         | 67 (67)     |
| Light blue crests                 |             |
| Present                           | 33 (33)     |
| Absent                            | 67 (67)     |

Data are expressed as absolute numbers (percentages) or mean  $\pm$  SD. *H. pylori*: *Helicobacter pylori*; PPI: Proton-pump inhibitors.

A five biopsy set (2 antrum, 1 angulus, and 2 corpus) of LBC-positive areas was collected in all LBC-positive examinations; biopsy samples were taken from the non-LBC mucosa in the LBC-negative patients.

### Histological assessment

All biopsy specimens were immersed in formalin and then embedded in paraffin. Sections cut from the paraffin blocks were stained with haematoxylin and eosin, Alcian blue PAS and Giemsa. The histological findings were evaluated by two expert pathologists who specialised in GI pathology; the pathologists were unaware of the medical history of the patients and of the endoscopic findings regarding LBC appearance. Inflammation, atrophy, metaplasia and dysplasia were classified according to the updated Sydney system<sup>[17]</sup> and the revised Vienna classification<sup>[18]</sup>. Intestinal metaplasia was also expressed as the percentage of metaplastic glands on the entire antral or oxyntic mucosa specimens. Moreover, each sample was evaluated for the presence of *H. pylori*.

### Statistical analysis

Based on prior studies reporting a sensitivity of more than 80% and a specificity of more than 90%, we determined that a cohort of 100 patients was needed to detect an effect with a confidence interval of 95%, a sensitivity of 80% (interval width of 17%), and a specificity of 90% (interval width of 13%) (calculated with the Clopper-Pearson method using PASS 11; NCSS, LLC, Kaysville, Utah, United States; www.ncss.com). The sensitivity, specificity, positive and negative predictive values and accuracy were calculated and expressed as percentages and 95%CI. Pearson correlation analysis was used to compare the LBC grading with the percentage of histologically as-

**Table 2** Relationship between narrow-band-imaging with magnification endoscopy ranking and the percentage of gastric intestinal metaplasia detected by histological assessment

| Intestinal metaplasia | LBC ( $n$ ) |   |    |     | Total ( $n$ ) |
|-----------------------|-------------|---|----|-----|---------------|
|                       | Negative    | + | ++ | +++ |               |
| 0%                    | 59          | 6 | 0  | 0   | 65            |
| $\leq$ 5%             | 7           | 8 | 2  | 0   | 17            |
| 5%-45%                | 1           | 6 | 5  | 2   | 14            |
| 45%-80%               | 0           | 0 | 2  | 1   | 3             |
| $\geq$ 80%            | 0           | 0 | 0  | 1   | 1             |

LBC: Light blue crest.

sessed metaplasia.

## RESULTS

The study included 100 consecutive patients (58 F/42 M, mean age 67  $\pm$  12 years) who presented at our endoscopy unit between December 2010 and February 2012. The demographic and clinical characteristics of the patients are shown in Table 1. The patients were referred to our endoscopy unit mainly because of gastro-oesophageal reflux disease and/or dyspepsia symptoms (46%); 34% underwent the endoscopic procedure for cancer screening, anaemia, a positive faecal occult blood test or suspicion of a peptic ulcer; 9% for chronic liver disease; 6% for suspected celiac disease and the remaining patients for other indications. The prevalence of *H. pylori* infection on the biopsies was 31%; in addition, 67% of the patients used proton pump inhibitors, 16% were current smokers, and 25% of the patients were alcohol consumers.

### Endoscopic and histological findings

During the upper NBI-ME procedure, we observed LBC appearance in the gastric antrum of 33 patients (33%); 20 of the LBCs were classified as LBC+, 9 as LBC++, and 4 as LBC++++. Moreover, we detected LBC appearance in the gastric body in only 6 patients (6%), and 5 of the patients also had LBC appearance in the antrum; of these, 3 cases were classified as LBC+, 2 as LBC++, and 1 as LBC++++.

Intestinal metaplasia in the biopsy specimens (2 antrum, 1 angulus and 2 corpus) obtained during the endoscopy from both LBC-positive and -negative patients was classified as complete and incomplete based on special staining. Gastric IM was histologically detected in 35 (35%) patients, and 27 cases were LBC-positive. In five patients, histological IM was found in both the antrum and body, and this finding correlated with LBC appearance at both sites. A percentage of IM greater than 20% was observed in 13 (13%) of the cases.

### Accuracy of LBC presence for diagnosing gastric intestinal metaplasia

For the diagnosis of IM, compared to histological assessment, the NBI-ME findings had an accuracy of 93%

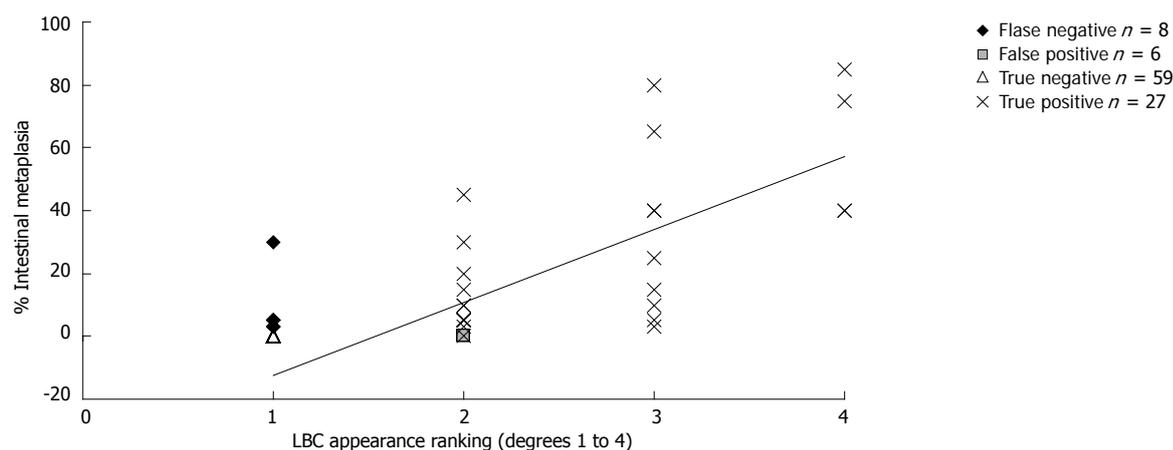


Figure 2 Graphic representation of the correlation between the degree of light blue crest appearance during narrow-band imaging with magnifying endoscopy examination and the percentage of gastric intestinal metaplasia detected by histological examination. LBC: Light blue crest.

(95%CI: 90-97), a sensitivity of 80% (95%CI: 67-92), a specificity of 96% (95%CI: 93-99), a positive predictive value of 84% (95%CI: 73-96), and a negative predictive value of 95% (95%CI: 92-98).

Interestingly, in our study, the NBI-ME examination overlooked the presence of gastric IM in 8 cases (false negative cases), and 7 out of the 8 cases had an IM that was less than 5%. Moreover, in the 6 false positive cases, the histological examination showed the presence of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases).

The semiquantitative correlation between the grades of LBC appearance and the percentage of histological IM is shown in Table 2 and Figure 2; the correlation index was 79% ( $P < 0.01$ ).

## DISCUSSION

Intestinal metaplasia has been widely studied as the main mucosal background for the development of oesophageal and gastric adenocarcinomas. However, to date, no endoscopic patterns that clearly define IM or dysplasia in the stomach have been found. Kaminishi *et al*<sup>[19]</sup> evaluated the accuracy of endoscopic findings for diagnosing chronic gastritis and found that ash-coloured nodular changes were highly specific (98%  $\pm$  99%) but had a very poor sensitivity (6%  $\pm$  12%) for detecting histological IM; the authors concluded that conventional endoscopy is not really useful for diagnosing gastric IM. Later, Dinis-Ribeiro *et al*<sup>[20]</sup> observed that magnifying chromoendoscopy with methylene blue staining had good sensitivity (76%) and specificity (87%) for detecting gastric IM. Moreover, methylene blue chromoendoscopy has been found to be useful for assessing the extent of IM<sup>[21]</sup>. However, this latter technique is time consuming and has some limitations, including the need for preparation with mucolytic agents, dye spraying and vigorous irrigation of the mucosal surface; furthermore, a recent report suggests that methylene blue chromoendoscopy induced questionable oxidative DNA damage in a patient with Barrett's

mucosa<sup>[22]</sup>. Therefore, collecting biopsies for subsequent histological evaluation remains the current gold standard for diagnosing IM. However, the fact that premalignant gastric lesions may occur multifocally represents a limitation of random biopsy sampling.

Several new imaging techniques have been developed recently to improve the diagnostic yield of endoscopy. The NBI-ME system has the practical advantage of not requiring complex preparation procedures, the use of any staining technique or a long duration. The additional value of NBI-ME in the detection of gastric pre-malignant lesions is still unclear, especially in countries with a low gastric cancer incidence<sup>[7]</sup>.

Recently, Capelle *et al*<sup>[23]</sup> compared the yield of NBI alone to that of white light endoscopy (WLE) in the surveillance of 43 patients with IM and dysplasia. The authors concluded that NBI alone considerably increased the detection of premalignant gastric lesions compared to routine WLE and appeared to be superior to WLE for the surveillance of patients with these advanced gastric lesions. However, a combination of NBI and magnification is likely to provide the best alternative to current endoscopic practice. Previous studies have already demonstrated a high correlation between the microvascular patterns found with NBI-ME and the diagnosis of gastric cancer<sup>[24,25]</sup>. Moreover, the combined optical technology of NBI with ME has been shown to identify with great accuracy the presence of gastric IM through the visualisation of LBC appearance on the epithelial surface<sup>[16]</sup>. However, the main limitations of the above-mentioned studies are that they were all conducted in patients with already known diagnoses of intestinal metaplasia/dysplasia/gastric cancer or in a population at a high risk for developing gastric cancer. Other limitations included the small sample sizes and the limited assessment of the gastric antrum and angulus with the exclusion of the gastric body.

Therefore, no prospective data are available on the diagnostic utility and accuracy of NBI-ME for detecting IM in an unselected population presenting at an endos-

copy unit in routine clinical practice. Our study showed that NBI-ME allowed us to detect gastric IM areas with an accuracy of 93%, a sensitivity of 80%, a specificity of 96%, a positive predictive value of 84% and a negative predictive value of 95%. These results are similar to those recently published by Pimentel-Nunes *et al.*<sup>[26]</sup>, who found a sensitivity of 68% and a specificity of 87% using the same method and a simplified classification system for NBI in the diagnosis of gastric lesions. In our investigation, NBI-ME underestimated the presence of IM in 8 patients; for 7 of these patients, metaplasia was histologically estimated to be less than 5%. The fact that NBI-ME has its major limitation in identifying gastric IM lower than 5% when performed by experienced endoscopists represents a new finding about the diagnostic yield of this technique; at this stage, we can assume that this value constitutes the sensitivity limit of this technique. The false positive cases (6 patients) presented with a histological diagnosis of reactive gastropathy (4 cases) or *H. pylori* active chronic gastritis (2 cases). Both of these conditions may sometimes represent a confounding factor because they show endoscopic features similar to those of gastric IM.

The prevalence of histologically observed IM in our cohort (35%) was slightly higher than the prevalence estimated in the general Western population (25%)<sup>[5-9]</sup> and in other studies on the Italian population (20%-30%)<sup>[5,8,11-13]</sup>. A possible explanation for our findings may be related to the higher mean age and prevalence of *H. pylori* infection in our patients, as it is well known that *H. pylori* infection, increased age and smoking (> 20 cigarettes/daily) are the main risk factors<sup>[27]</sup> for the development of premalignant gastric lesions. However, the prevalence of IM in the general population is reported to be highly variable. Additionally, the majority of data come from studies on patients with dyspeptic symptoms and/or suspected *H. pylori* infections, while information derived from unselected patients (*i.e.*, consecutive patients enrolled independently of GI symptoms or clinical status) is limited.

The semiquantitative evaluation of LBC appearance in our study and its correlation with the histological measurement of IM percentage shows that the agreement was high (79%). Previously, Uedo *et al.*<sup>[16]</sup> also found a significant correlation between the diffuse positivity of LBC (+++) mucosa and the presence of histological markers of IM, such as immunohistochemical staining for CD10 and Alcian blue/high iron diamine. However, these authors did not assess the correlation between LBC appearance and the percentage of histologic IM. Therefore, to the best of our knowledge, our study is the first to show a good correlation between the degree of IM detected by NBI-ME and the severity of IM identified by histological measurement in a series of consecutive patients without specific indications to perform an upper GI endoscopy.

In contrast to Uedo *et al.*<sup>[16]</sup> who used NBI-ME and Capelle *et al.*<sup>[23]</sup> who performed NBI alone, we opted to evaluate not only the antrum and the angulus of the stomach but also the body; including the body was re-

cently emphasised in the ESGE guidelines<sup>[28]</sup>, despite the significantly longer examination time. This measurement was included because our intention was to demonstrate the potential application of NBI-ME in a population-based screening and surveillance setting, and we thought that gastric assessment of only one region of the stomach was not adequate and fully informative. However, IM is predominantly found in the antrum, which was confirmed in our study. Therefore, limiting the endoscopic evaluation to the antrum permits an accurate observation and a shorter duration of the examination. Moreover, it has been shown that three biopsies (two antral and one angular) appear to be the best compromise between acceptable accuracy<sup>[29]</sup> and ease of use in the detection of IM by endoscopy.

Our study had some limitations. First, the study included only a single centre. Further research in larger multicentre prospective studies is necessary to evaluate whether NBI-ME yields adequate results in the detection and grading of gastric IM. Second, the endoscopic procedure of WLE and NBI-ME was performed by the same endoscopist; thus, detection of IM by NBI-ME could possibly be biased by the previous WLE observations. Third, as a learning curve must still be defined, regular training is mandatory to improve our findings. Indeed, despite our prolonged training, we obtained a value of sensitivity that was lower than the value obtained by Uedo *et al.*<sup>[16]</sup>. This difference was likely the result of differences in training between Japanese and Western gastroenterologists. Due to the higher gastric cancer incidence, Japanese endoscopists are trained to scrutinise gastric mucosal areas that are compatible with atrophy and early cancer and spend more time on a thorough mucosal examination than in Western countries. In addition, uniform criteria for this technique must be adopted in large controlled trials in Western or Eastern countries.

In conclusion, NBI-ME was shown to be a valid method for IM detection, except in those cases with IM lower than 5%, which may not be clinically relevant. In routine clinical practice, this technique can reliably target which patients should be biopsied to evaluate IM and those who do not need biopsies. Moreover, a semi-quantitative evaluation of LBC appearance was feasible as there was a good correlation with the histological assessment of IM percentage.

## COMMENTS

### Background

Gastric cancer is one of the most common neoplastic diseases in the Western world and has a poor prognosis and inconsistent signs and symptoms in the early phases. Therefore, adequate screening and prevention are needed to improve the diagnostic process and to identify this condition and its risk factors earlier. Recently, upper gastric intestinal endoscopy has been enhanced with the addition of the narrow band imaging with magnification endoscopy (NBI-ME) technique, which was found to identify precancerous lesions, such as gastric intestinal metaplasia (GIM).

### Research frontiers

Light blue crests (LBCs) are light-blue patches or lines visible on gastric mucosa by NBI endoscopy. Several studies have demonstrated that LBCs are a sign

of underlying intestinal metaplasia, with a good correlation between endoscopic findings and histological assessments. Therefore, authors have assessed the predictive values of the NBI-ME technique to identify and estimate the extension of GIM to biopsies of the antrum, angulus and corpus in the stomach. Authors also studied the correlation between the appearance of LBCs and the histological detection of GIM in prospectively recruited consecutive unselected patients.

### Innovations and breakthroughs

One hundred consecutive patients were studied. NBI-ME-aimed gastric bioptic samples were collected (2 in the antrum, 1 in the angulus, and 2 in the corpus) from each patient and compared with the presence or absence of LBCs as stated by skilled endoscopists; the biopsies were assessed according to the pathologists' expertise in the upper digestive tract. They determined a correlation of 79% between the appearance of LBCs and histologically assessed GIM. Moreover, authors observed that the appearance of LBCs correlated with the histological evidence of intestinal metaplasia, with a sensitivity of 80%, a specificity of 96%, a positive predictive value of 84%, a negative predictive value of 95% and an overall accuracy of 93%. Their results are similar, if not superior, to those of previously published studies in selected populations.

### Applications

NBI-ME is a good technique to identify areas suggestive of GIM in the stomach and can be used to take adequate biopsies from the stomach to provide the pathologist with a useful sample. This technique is easy to learn and provides an accuracy of greater than 90% for diagnosing GIM.

### Terminology

NBI-ME is an endoscopic technique that uses narrow band imaging to produce superior image contrast combined with a magnification of the image up to  $\times 115$ ; this technique enables a more accurate observation of the gastric epithelium. Gastric intestinal metaplasia is a condition characterised by the replacement of gastric epithelial cells with cells of intestinal morphology. GIM is a pre-cancerous condition that can progress towards gastric cancer, especially the intestinal-type according to the Lauren classification. This progress varies from 0% to 10% according to different studies, with a prevalence of more than 20% in Western countries, and is considered to be the "breaking point" in the Correa sequence of carcinogenesis. Identifying this condition could halt or reverse the neoplastic progression.

### Peer review

This research was performed by endoscopists who belong to single medical center in Italy. This article should be helpful for understanding the efficacy of NBI-magnifying endoscopy in detecting the presence of intestinal metaplasia of stomach.

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P- Reviewer Tohda G S- Editor Gou SX L- Editor A  
E- Editor Zhang DN



## Clinical effects of adalimumab treatment with concomitant azathioprine in Japanese Crohn's disease patients

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Received: December 20, 2012 Revised: January 17, 2013

Accepted: February 8, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To assess adalimumab's efficacy with concomitant azathioprine (AZA) for induction and maintenance of clinical remission in Japanese Crohn's disease (CD) patients.

**METHODS:** This retrospective, observational, single-center study enrolled 28 consecutive CD patients treated with adalimumab (ADA). Mean age and mean disease duration were  $38.1 \pm 11.8$  years and  $11.8 \pm 10.1$  years, respectively. The baseline mean Crohn's disease activity index (CDAI) and C-reactive protein were  $177.8 \pm 82.0$  and  $0.70 \pm 0.83$  mg/dL, respectively. Twelve of these patients also received a concomitant stable dose

of AZA. ADA was subcutaneously administered: 160 mg at week 0, 80 mg at week 2, followed by 40 mg every other week. Clinical response and remission rates were assessed *via* CDAI and C-reactive protein for 24 wk.

**RESULTS:** The mean CDAI at weeks 2, 4, 8, and 24 was 124.4, 120.2, 123.6, and 135.1, respectively. The CDAI was significantly decreased at weeks 2 and 4 with ADA and was significantly suppressed at 24 wk with ADA/AZA. Overall clinical remission rates at weeks 4 and 24 were 66.7% and 63.2%, respectively. Although no statistically significant difference in C-reactive protein was demonstrated, ADA with AZA resulted in a greater statistically significant improvement in CDAI at 24 wk, compared to ADA alone.

**CONCLUSION:** Scheduled ADA with concomitant AZA may be more effective for clinical remission achievement at 24 wk in Japanese Crohn's disease patients.

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**Key words:** Crohn's disease; Adalimumab; Immuno-modulator; Azathioprine; Inflammatory bowel disease

**Core tip:** In this study, the authors found that adalimumab (ADA) treatment with concomitant azathioprine usage significantly suppressed the Crohn's disease activity index and increased the remission rate at 24 wk after the initiation of therapy, compared with ADA treatment alone.

Ishida K, Inoue T, Fujiwara K, Sakanaka T, Narabayashi K, Nouda S, Okada T, Kakimoto K, Kuramoto T, Kawakami K, Abe Y, Takeuchi T, Murano M, Tokioka S, Umegaki E, Higuchi K. Clinical effects of adalimumab treatment with concomitant azathioprine in Japanese Crohn's disease patients. *World J Gastroenterol* 2013; 19(17): 2676-2682 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a proinflammatory cytokine implicated in the pathogenesis of inflammatory bowel disease (IBD)<sup>[1-3]</sup>. Thus far, a variety of therapeutic approaches have been used to inhibit TNF- $\alpha$  in patients with IBD, including infliximab (IFX), which was the first biologic agent approved for the treatment of IBD<sup>[4]</sup>. IFX is a mouse and human chimeric immunoglobulin G1 (IgG1) monoclonal antibody directed against TNF- $\alpha$ ; it binds to both the soluble and transmembrane forms of TNF- $\alpha$  and is effective as both an induction and maintenance therapy in patients with Crohn's disease (CD), including patients who have draining fistulas<sup>[5-8]</sup>. However, IFX has a 25% murine fraction that has potent immunogenicity and can cause the formation of human anti-chimeric antibodies (HACA)<sup>[9]</sup>. Furthermore, this potent immunogenicity may lead to the loss of efficacy, hypersensitivity reactions, or infusion reactions<sup>[10]</sup>.

Adalimumab (ADA) is the second biologic agent approved for use in the treatment of IBD. It is a subcutaneously administered, recombinant, fully human, IgG1 monoclonal antibody that binds with a high affinity and specificity to TNF- $\alpha$  and was found to be effective in refractory CD patients who were either naïve to or previously treated with IFX<sup>[11-15]</sup>. Combination therapy with IFX and an immunosuppressant demonstrated increased efficacy compared with nonconcomitant use of immunosuppressants<sup>[16,17]</sup>. Nonetheless, limited data are available for ADA. In the pivotal registration study of ADA (Crohn's trial of the fully Human antibody ADA for Remission Maintenance, CHARM), post-hoc analysis did not detect an impact of coadministration of immunosuppressants on the remission rates achieved at 1 year<sup>[12]</sup>. Ardizzone *et al.*<sup>[18]</sup> reported that ADA with concomitant immunosuppressant treatment was not associated with the loss of response or the need for dose escalation. However, Reenaers *et al.*<sup>[19]</sup> reported that there may be a benefit from using immunosuppressive drugs during the first semester of initiating ADA treatment, with a slight decrease in ADA failure and lower need for ADA dosage escalation.

In Japan, ADA was approved for the treatment of CD; however, only limited data have been collected on its optimal usage. Therefore, this study aimed to assess the impact of ADA with concomitant azathioprine (AZA) therapy on the rate of clinical remission during maintenance with ADA in CD patients at the Osaka Medical College Hospital during routine clinical practice.

## MATERIALS AND METHODS

### Study participants

Between November 2010 and March 2012, about 28 CD

patients who were diagnosed by clinical, endoscopic, and histological criteria and who received ADA at the Osaka Medical College Hospital, Osaka, Japan, were consecutively enrolled in this retrospective, observational, single-center study. The patients were informed about the potential risks and benefits of ADA therapy and were provided and signed informed consent forms before all procedures. The medical records of all participating patients were reviewed by two senior investigators (Ishida K and Inoue T) for the following information: demographics; disease duration; disease location; prior surgical history; perianal disease presence; smoking behavior; previous IFX treatment and response to IFX treatment; Crohn's disease activity index (CDAI); C-reactive protein (CRP) levels; and concomitant medications (corticosteroids, AZA, mesalazine, and elemental diet).

All patients received 160 mg of ADA subcutaneously at week 0, 80 mg at week 2, followed by 40 mg every other week. A clinical response was defined as a  $\Delta$ CDAI > 70 points, whereas a clinical remission was defined as a CDAI < 150 points. The loss of response to IFX was defined as a loss of therapeutic efficacy after > 2 IFX infusions.

### Statistical analysis

Categorical data were compared by the  $\chi^2$  test or the Fisher's exact test, and continuous variables were compared by the Student *t* test. The results were expressed as the mean  $\pm$  SD. *P* values less than 0.05 were considered to be statistically significant. All calculations were made using the StatView system (SAS Institute, Cary, NC, United States).

## RESULTS

### Patient characteristics

ADA was administered to 28 consecutive patients with CD from November 2011 to March 2012 at the Osaka Medical College Hospital (male-to-female ratio: 22/6; age at presentation:  $38.1 \pm 11.8$  years; and disease duration:  $11.8 \pm 10.1$  years). The patients' baseline characteristics and demographics are listed in Table 1. According to the Montreal classification, patients presented with CD in the following locations: about 10.7% (3/28) in an isolated ileal location; 17.9% (5/28) in an isolated colonic location; and 71.4% (20/28) in an ileocolonic location. Of the patients, 60.7% (17/28) had a history of bowel resection, 57.1% had perianal disease, and 17.9% had an associated fistulizing disease. Two patients (7.1%) were current smokers.

Seventeen (60.7%) patients had previously taken IFX, and 6 patients did not have a response to IFX, including no primary response in one patient and emergent allergic reaction in another patient. Twelve of the 28 patients were treated with ADA and a concomitant stable dose of AZA throughout the observational period. The mean CDAI and mean CRP at baseline were  $177.8 \pm 82.0$  and  $0.70 \pm 0.83$  mg/dL, respectively.

**Table 1** Baseline characteristics of all patients who received adalimumab for treatment of Crohn's disease *n* (%)

|  | Adalimumab<br><i>n</i> = 16 | Combination<br><i>n</i> = 12 | Total<br><i>n</i> = 28 |
|--|-----------------------------|------------------------------|------------------------|
| Gender   |                             |                              |                        |
| Male   | 13 (81.3)                   | 9 (75.0)                     | 22 (78.6)              |
| Female   | 3 (18.7)                    | 3 (25.0)                     | 6 (21.4)               |
| Age at presentation <sup>1</sup> (yr)                    | 43.1 ± 11.8                 | 31.4 ± 8.3 <sup>a</sup>      | 38.1 ± 11.8            |
| Disease duration <sup>1</sup> (yr)                       | 13.6 ± 11.5                 | 9.3 ± 7.6                    | 11.8 ± 10.1            |
| Location of disease                                      |                             |                              |                        |
| Isolated ileal   | 2 (12.5)                    | 1 (8.3)                      | 3 (10.7)               |
| Isolated colonic   | 4 (25.0)                    | 1 (8.3)                      | 5 (17.9)               |
| leocolonic   | 10 (62.5)                   | 10 (83.3)                    | 20 (71.4)              |
| Previous resection                                       | 10 (62.5)                   | 7 (58.3)                     | 17 (60.7)              |
| Perianal disease   | 10 (62.5)                   | 6 (50)                       | 16 (57.1)              |
| Current smokers  | 1 (6.3)                     | 1 (8.3)                      | 2 (7.1)                |
| Previous anti-infliximab treatment                       | 10 (62.5)                   | 7 (58.3)                     | 17 (60.7)              |
| Primary nonresponse                                      | 0 (0.0)                     | 1 (8.3)                      | 1 (3.6)                |
| Secondary loss of response                               | 8 (50.0)                    | 6 (50.0)                     | 14 (50.0)              |
| Allergic reaction  | 1 (6.3)                     | 0 (0.0)                      | 1 (3.6)                |
| Others   | 1 (6.3)                     | 0 (0.0)                      | 1 (3.6)                |
| CDAI at initiation of adalimumab therapy <sup>1</sup>    | 195.4 ± 89.2                | 152.2 ± 65.8                 | 177.8 ± 82.0           |
| CDAI > 150 at initiation of adalimumab therapy, <i>n</i> | 9                           | 7                            | 16                     |
| CRP at initiation of adalimumab therapy <sup>1</sup>     | 0.72 ± 0.92                 | 0.66 ± 0.73                  | 0.70 ± 0.83            |

<sup>1</sup>Data are expressed as mean ± SD. CDAI: Crohn's disease activity index.  
<sup>a</sup>*P* < 0.05 *vs* adalimumab.

### Clinical efficacy of ADA therapy

The overall clinical response and remission rates are shown in Table 2. The rates of clinical remission at weeks 2, 4, 8, and 24 were 60%, 66.7%, 69.6%, and 63.2%, respectively. The mean CDAI of all patients at weeks 2, 4, 8, and 24 was 124.4 ± 60.2, 120.0 ± 66.8, 123.6 ± 73.5, and 135.1 ± 74.4, respectively. The CDAI was significantly decreased commencing 2 wk after the initiation of ADA treatment and remained low for 24 wk (Table 2).

### Impact of ADA/AZA on the clinical remission compared to ADA maintenance

Regarding the concomitant use of immunosuppressive agents, 12 patients (42.9%) were treated with a stable dose of AZA (50.0 ± 21.3 mg/d, 1.0 ± 0.5 mg/kg/d) before the initiation of ADA. The concomitant use of AZA was well tolerated, with only minor side effects. Although there were no statistically significant differences seen until 12 wk into ADA and AZA treatment (compared with ADA treatment), combinational therapy with ADA and AZA significantly increased the clinical remission rate (Figure 1) and reduced the CDAI, compared with nonconcomitant use at week 24 (Figure 2A).

Sixteen of the 28 patients were found to have increased CRP serum levels (> 0.25 mg/dL) before starting ADA therapy. The mean serum CRP of all of the patients at weeks 2, 4, 8, and 24 were 0.27 ± 0.40, 0.28 ± 0.51, 0.46 ± 0.69, and 0.51 ± 0.94 mg/dL, respectively.

ADA significantly reduced the CRP levels at weeks 2 and 4 after the initiation of treatment. The concomitant use of AZA tended to maintain low CRP levels for 24 wk, even though the result was not statistically significant (CRP levels at weeks 0 and 24 were 0.66 ± 0.73 and 0.27 ± 0.44 mg/dL, respectively; *P* = 0.09, Figure 2B).

## DISCUSSION

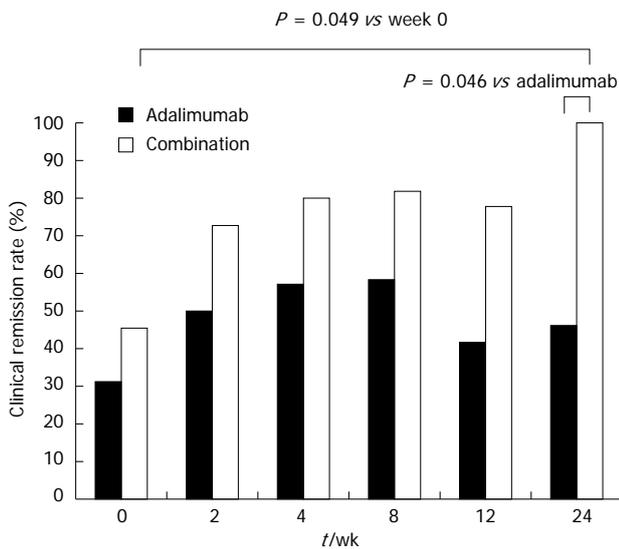
The effectiveness of AZA in CD patients treated with IFX has been clearly reported by many investigators. However, few reports have demonstrated the benefits of ADA and AZA combination therapy. In this study, we analyzed the use of ADA and AZA in patients with mild to moderate CD at our clinical practice and found that scheduled ADA with concomitant use of AZA was more effective in inducing and maintaining remission for 24 wk after the initiation of ADA treatment, compared with ADA monotherapy. Although ADA monotherapy was also able to induce and maintain clinical remission from 2 to 24 wk compared to the baseline, the effects of the combination therapy were better. Since previous IFX nonresponse and previous treatment with an anti-TNF agent were reported to be associated with a decreased clinical efficacy and a loss of response<sup>[20]</sup>, we also compared the CDAI between anti-TNF-naïve patients and anti-TNF-treated patients in this study, but there was no significant difference.

ADA is a human monoclonal IgG1 antibody that has demonstrated efficacy in the induction and maintenance of clinical remission in patients with moderate to severe CD and is useful in patients who have lost responsiveness to IFX<sup>[11-15]</sup>. IFX, a chimeric anti-TNF-α antibody, has potent immunogenicity; repeated administration of IFX could result in the development of antibodies that may lead to the loss of response<sup>[21]</sup>. Although no consensus has yet been established on the appropriateness of concomitant immunomodulators with anti-TNF-α antibody therapy for CD<sup>[22-24]</sup>, immunosuppressants were shown to inhibit the development of HACA<sup>[10]</sup>. Moreover, since the clearance of IFX is affected by the presence of HACA, IFX serum levels were significantly higher in patients with concomitant immunosuppressive therapy, as this concomitant usage reduces the frequency of HACA formation<sup>[4,25]</sup>. Sokol *et al.*<sup>[17]</sup> assessed the efficacy of immunosuppressants with scheduled IFX in patients with IBD and reported that IBD flares, perianal complications, and switching to ADA were less frequently observed in patients with combined immunosuppressant and biologic agent use than in those patients who were not concomitantly treated with immunosuppressants<sup>[22]</sup>. Furthermore, in the SONIC study, Colombel *et al.*<sup>[16]</sup> compared the efficacy and safety of IFX monotherapy and IFX plus AZA combination therapy for CD and showed that the combination therapy provided a significantly greater benefit than that of IFX monotherapy at weeks 26 and 50. These studies suggest

**Table 2 Clinical efficacy of adalimumab therapy**

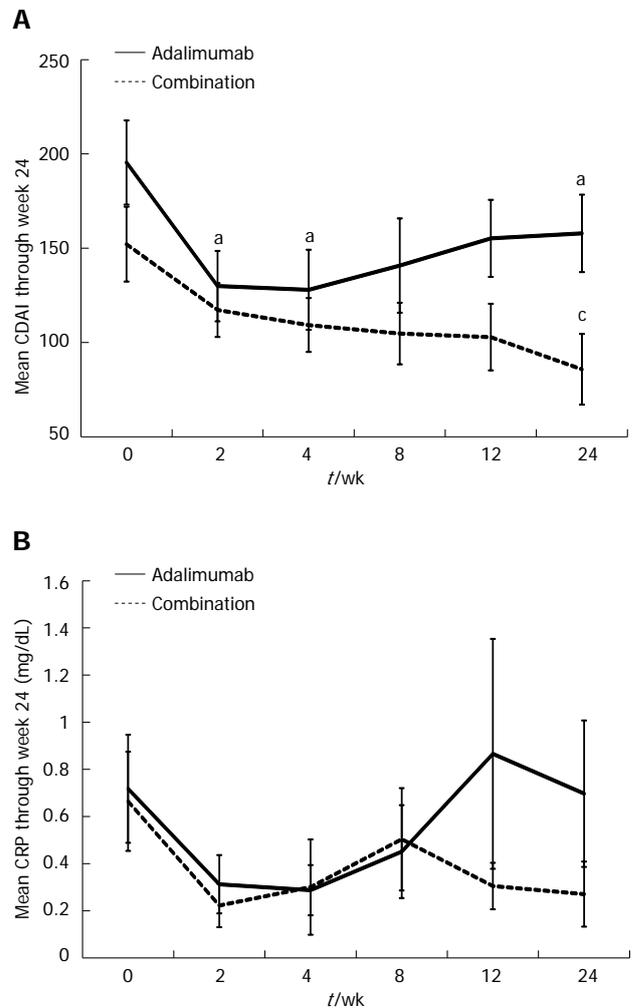
| Week      | 0            | 2                         | 4                         | 8                         | 24                        |
|-----------|--------------|---------------------------|---------------------------|---------------------------|---------------------------|
| <i>n</i>  | 27           | 25                        | 24                        | 23                        | 19                        |
| CDAI      | 177.8 ± 82.0 | 124.4 ± 60.2 <sup>a</sup> | 120.0 ± 66.8 <sup>a</sup> | 123.6 ± 73.5 <sup>a</sup> | 135.1 ± 74.4 <sup>a</sup> |
| Remission | 37%          | 60%                       | 66.70%                    | 69.60%                    | 63.20%                    |

Remission: Crohn's disease activity index (CDAI) < 150 points. <sup>a</sup>*P* < 0.05 vs week 0.



**Figure 1 Clinical remission rate.** Combinational therapy with adalimumab and azathioprine significantly increased the clinical remission rate vs nonconcomitant use at week 24.

that combination therapy with IFX and AZA may have an added benefit in maintaining the remission of CD. With regard to ADA, a substantial proportion of patients, including previously treated anti-TNF- $\alpha$  patients and anti-TNF- $\alpha$  treatment-naïve patients, needed dose escalation during ADA treatment<sup>[12,26]</sup>. However, concomitant immunosuppressive therapy was not associated with a loss of response or a need for dose escalation and did not influence the development of ADA antibodies<sup>[27-29]</sup>. Recently, Reenaers *et al.*<sup>[19]</sup> reported that usage of ADA with immunosuppressive drugs, such as AZA, during the first semester of initiating ADA demonstrated a slight decrease in ADA failure and a lower need for ADA dose escalation. Moreover, the presence of anti-ADA antibodies and a low serum ADA concentration have been reported to be associated with a diminished clinical response in rheumatoid arthritis patients, and the concomitant use of an immunomodulator significantly suppresses the concentration of anti-ADA antibodies<sup>[30,31]</sup>. Interestingly, in this study, AZA in the presence of ADA provides better efficacy in patients it has previously failed. However, some patients with IFX had not early received concomitant AZA. Therefore, we consider that ADA and AZA combination therapy may be more effective than ADA monotherapy via the inhibition of the development of anti-ADA antibodies. Prospective



**Figure 2 Mean Crohn's disease activity index and C-reactive protein through week 24.** A: Combinational therapy with adalimumab (ADA) and azathioprine (AZA) significantly reduced the Crohn's disease activity index (CDAI), compared with nonconcomitant use at week 24. <sup>a</sup>*P* < 0.05 vs week 0; <sup>c</sup>*P* < 0.05 vs adalimumab; B: The concomitant use of AZA tended to maintain low C-reactive protein (CRP) levels for 24 wk (CRP levels at weeks 0 and 24 were 0.66 ± 0.73 and 0.27 ± 0.44, respectively, *P* = 0.09).

studies, combined with anti-ADA antibody and ADA trough level measurements, are required<sup>[19]</sup>.

In this study, we found that ADA treatment with concomitant AZA usage significantly suppressed the CDAI and increased the remission rate at 24 weeks after the initiation of therapy, compared with ADA treatment alone. Furthermore, these results were not consistent with previous reports showing that the efficacy of ADA is independent of the use of concomitant immunosup-

pression<sup>[27-29]</sup>. One possible explanation for this discrepancy is that these previous studies assessed the efficacy of ADA in patients with moderate to severe CD, whereas the present study included mild to moderate CD patients. Notably, the CDAI at the beginning of this study was relatively low ( $177.8 \pm 82.0$ ). Since the CDAI scores were gradually increasing, some patients were switched from IFX treatment to ADA treatment, even though their CDAI scores were still in remission (CDAI < 150 at the initiation of ADA treatment). Indeed, Zheng *et al.*<sup>[32]</sup> evaluated the efficacy of AZA in controlling the relapse of disease in patients with CD for at least 6 months and showed that AZA treatment decreased the CDAI from  $187.3 \pm 23.4$  to  $96.1 \pm 13.5$ . Actually, the finding of a significant efficacy with ADA plus AZA combination therapy compared to ADA monotherapy is revealed only after week 24 in this study. Generally, thiopurines require a lag time of approximately 6 mo before expressing full activity. Taken together, we consider that the combination of ADA and AZA may have an added benefit in inducing long-term remission compared with ADA alone, even though ADA has shown less immunogenicity compared with IFX.

Our study had some major limitations. The patients were not randomized between ADA monotherapy and ADA plus AZA combination therapy. Also, the mean age was significantly different between these two groups due to the nature of retrospective study. Therefore, these two groups were not strictly comparable. In addition, the patient numbers were limited, as we only analyzed the data in our own hospital. Moreover, recent studies have revealed that Japanese and Caucasian CD patients genetically differ, as Japanese CD patients lack the polymorphism in the *NOD2* gene<sup>[33-36]</sup>. Genetic differences might have affected the results in this study. An additional, larger, long-term multicenter study should be conducted to determine the effect of combination therapy with ADA and AZA for patients with CD.

So far, there have been few reports demonstrating that CD patients could achieve a better clinical remission with ADA and immunomodulator combination therapy, compared with ADA monotherapy<sup>[19,37]</sup>. This study may help physicians decide whether to use combination therapy. In conclusion, scheduled ADA with concomitant AZA is more effective for clinical remission achievement at 24 wk in Japanese CD patients. Although further long-term studies evaluating the efficacy of combination therapy with ADA and AZA are required, our data suggest that ADA with concomitant AZA therapy may provide clinical benefit in inducing long-term remission.

## COMMENTS

### Background

The benefits of azathioprine (AZA) in Crohn's disease (CD) with infliximab as scheduled anti-tumor necrosis factor- $\alpha$  maintenance therapy have been established but remain unclear with adalimumab (ADA).

### Research frontiers

So far, there have been few reports demonstrating that CD patients could achieve better clinical remission with ADA and immunomodulator combination therapy, compared with ADA monotherapy.

### Innovations and breakthroughs

This is the first study reported in Asia on the effect of ADA with concomitant AZA for induction and maintenance of clinical remission in CD patients. The results of this study showed that ADA treatment with concomitant AZA usage significantly suppressed the Crohn's disease activity index and increased the remission rate at 24 wk after the initiation of therapy, compared with ADA treatment alone.

### Applications

Although further long-term studies evaluating the efficacy of combination therapy with ADA and AZA are required, this study suggests that ADA with concomitant AZA therapy may provide clinical benefit in inducing long-term remission. The results of this study may help physicians decide whether to use combination therapy.

### Peer review

This is an interesting small retrospective, observational, study assessing the effect of effects of ADA treatment with concomitant AZA in CD patients.

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**P- Reviewers** Actis GC, de Boer NKH, Chermesh I  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN



## Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study

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Supported by the Scientific Support Programs for Cancer Research, Grant-in-Aid for Scientific Research on Innovative Areas, the Ministry of Education, Culture, Sports, Science and Technol-

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Received: August 28, 2012 Revised: November 7, 2012

Accepted: December 27, 2012

Published online: May 7, 2013

### Abstract

**AIM:** To investigate the associations between dietary intake of polyphenols and colorectal cancer.

**METHODS:** The study subjects were derived from the Fukuoka colorectal cancer study, a community-based case-control study. The study subjects were 816 cases of colorectal cancer and 815 community-based controls. The consumption of 148 food items was assessed by a computer-assisted interview. We used the consumption of 97 food items to estimate dietary intakes of total, tea and coffee polyphenols. The Phenol-Explorer database was used for 92 food items. Of the 5 foods which were not listed in the Phenol-Explorer Database, polyphenol contents of 3 foods (sweet potatoes, satsumo and daikon) were based on a Japanese study and 2 foods (soybeans and fried potatoes) were estimated by ORAC-based polyphenol contents in the United States Department of Agriculture Database. Odds ratios (OR) and 95%CI of colorectal cancer risk according to quintile categories of intake were obtained by using logistic regression models with adjustment for age, sex, residential area, parental history of colorectal cancer, smoking, alcohol consumption, body mass index 10 years before, type of job, leisure-time physical activity and dietary intakes of calcium and n-3 polyunsaturated fatty acids.

**RESULTS:** There was no measurable difference in total or tea polyphenol intake between cases and controls, but intake of coffee polyphenols was lower in cases than in controls. The multivariate-adjusted OR of colorectal cancer according to quintile categories of coffee polyphenols (from the first to top quintile) were 1.00 (referent), 0.81 (95%CI: 0.60-1.10), 0.65 (95%CI: 0.47-0.89), 0.65 (95%CI: 0.46-0.89) and 0.82 (95%CI: 0.60-1.10), respectively ( $P_{\text{trend}} = 0.07$ ). Similar, but less pronounced, decreases in the OR were also noted for the third and fourth quintiles of total polyphenol intake. Tea polyphenols and non-coffee polyphenols showed no association with colorectal cancer risk. The site-specific analysis, based on 463 colon cancer cases and 340 rectal cancer cases, showed an inverse association between coffee polyphenols and colon cancer. The multivariate-adjusted OR of colon cancer for the first to top quintiles of coffee polyphenols were 1.00 (referent), 0.92 (95%CI: 0.64-1.31), 0.75 (95%CI: 0.52-1.08), 0.69 (95%CI: 0.47-1.01), and 0.68 (95%CI: 0.46-1.00), respectively ( $P_{\text{trend}} = 0.02$ ). Distal colon cancer showed a more evident inverse association with coffee polyphenols than proximal colon cancer. The association between coffee polyphenols and rectal cancer risk was U-shaped, with significant decreases in the OR at the second to fourth quintile categories. There was also a tendency that the OR of colon and rectal cancer decreased in the intermediate categories of total polyphenols. The decrease in the OR in the intermediate categories of total polyphenols was most pronounced for distal colon cancer. Intake of tea polyphenols was not associated with either colon or rectal cancer. The associations of coffee consumption with colorectal, colon and rectal cancers were almost the same as observed for coffee polyphenols. The trend of the association between coffee consumption and colorectal cancer was statistically significant.

**CONCLUSION:** The present findings suggest a decreased risk of colorectal cancer associated with coffee consumption.

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**Key words:** Colorectal cancer; Colon cancer; Rectal cancer; Polyphenols; Coffee; Tea

Wang ZJ, Ohnaka K, Morita M, Toyomura K, Kono S, Ueki T, Tanaka M, Kakeji Y, Maehara Y, Okamura T, Ikejiri K, Futami K, Maekawa T, Yasunami Y, Takenaka K, Ichimiya H, Terasaka R. Dietary polyphenols and colorectal cancer risk: The Fukuoka colorectal cancer study. *World J Gastroenterol* 2013; 19(17): 2683-2690 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2683.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2683>

## INTRODUCTION

Colorectal cancer is one of the most common cancers in

the world and the second most common cause of cancer death in developed countries<sup>[1]</sup>. In Japan, the incidence rate of colorectal cancer, especially of colon cancer, has increased dramatically until the 1990s<sup>[2]</sup>. Lifestyle factors such as physical inactivity, obesity and alcohol use are known to confer increased risk of colorectal cancer. Of dietary factors, high intake of red meat has been related to increased risk of colorectal cancer, and fiber-containing plant foods and calcium have been considered to be protective<sup>[3]</sup>.

Polyphenols are the most abundant antioxidants in foods and have drawn much interest as potentially anticarcinogenic compounds. The flavonoids have been identified as potential cancer-preventive components of fruits and vegetables<sup>[4]</sup>. Catechins and tea polyphenols inhibit tumorigenesis at initiation, promotion, and progression stages in animal experiments<sup>[5]</sup>. Coffee is the major source of polyphenol intake in populations in which coffee is commonly consumed<sup>[6]</sup>, and phenolic compounds in coffee such as chlorogenic acids also have anticarcinogenic effects in animal models<sup>[7]</sup>. Epidemiological studies have suggested that polyphenol-rich foods such as fruits and vegetables<sup>[3,8]</sup> and beverages such as tea<sup>[9-13]</sup> and coffee<sup>[14,15]</sup> may be protective against colorectal cancer. Experimental studies on colorectal cancer cell lines also support a protective role of polyphenols in carcinogenesis by exerting antioxidant and anti-proliferative effects and by inducing cell cycle arrest<sup>[16]</sup>. In the study reported here, we addressed the association of dietary intake of polyphenols with colorectal cancer risk using data from a community-based case-control study in Japan<sup>[17]</sup>.

## MATERIALS AND METHODS

The present data were derived from the Fukuoka colorectal cancer study, a community-based case-control study in Fukuoka, Japan. The research protocol was approved by the ethics committee of Kyushu University and collaborating hospitals. Details of the design and conduct of the study have been described elsewhere<sup>[17]</sup>.

### Study subjects

Cases were histologically confirmed incident cases of colorectal adenocarcinomas who were admitted for surgical treatment to one of the collaborating hospitals in Fukuoka City and three adjacent areas during the period from September 2000 to December 2003. Of 1053 eligible cases, a total of 840 cases (80%) participated in the interview; the eligible cases were aged between 20-74 years at the time of diagnosis; lived in the study area; had no prior history of partial or total removal of the colon, familial adenomatous polyposis or inflammatory bowel disease; and were mentally competent to give informed consent and to complete the interview. Research staff visited each hospital regularly, determined the eligibility of cases by referring to admission logs and medical records, and interviewed him/her if written informed consent was obtained.

Eligibility criteria for controls were the same as described for the cases except that they had no history of colorectal cancer. A total of 1500 control candidates were randomly selected by a two-stage random sampling on the basis of a resident registry with the frequency by sex and 10-year age class matched to expected sex- and age-specific frequencies of cases. Recruitment was initiated by a letter of invitation, which was followed by phone calls if available. After exclusion of 113 who were found to be ineligible and 5 who were diagnosed with colorectal cancer after the interview, 833 (60%) participated in the interview.

In the analysis, we excluded those who were in the top 1% or bottom 1% of total energy intake within each stratum of sex and age class (< 55, 55-64, and  $\geq$  65 years of age). A total of 816 cases and 815 controls remained.

### Lifestyle questionnaire

Cases and controls were interviewed in person regarding smoking, alcohol use, physical activity and other factors using a uniform questionnaire. The index date was taken as the date of onset of symptoms or the screening leading to the diagnosis of colorectal cancer for cases and the date of interview for controls. Anthropometric questions inquired about height (cm) and body weight (kg) at the time of interview and 10 years earlier. Body mass index ( $\text{kg}/\text{m}^2$ ) 10 years earlier was used in the analysis because the current body mass index was unrelated to the risk<sup>[18]</sup>. For 4 cases and 11 controls, body weight 10 years before was not ascertained, and the current body weight was used for substitution. The amount of alcohol was expressed using the conventional Japanese unit; one go (180 mL) of sake, one large bottle (633 mL) of beer and half a go (90 mL) of shochu were each expressed as one unit; and one drink (30 mL) of whisky or brandy and one glass (100 mL) of wine were each converted to half a unit. Regarding smoking, ever smokers reported years of smoking and the numbers of cigarettes per day for each decade of life, and we calculated the cumulative exposure to cigarette smoking until the beginning of the previous decade of age. Types of job and non-job physical activities 5 years before were ascertained. The amount of non-occupational physical activity was expressed as a sum of metabolic equivalents (MET) multiplied by the hours of weekly participation in each activity, *i.e.*, MET-hours per week. History of parental colorectal cancer also was obtained.

### Dietary assessment

Consumption frequencies and portion sizes of 148 food and beverage items were ascertained by a computer-assisted interview. Details of the dietary interview have been described elsewhere<sup>[19]</sup>. Participants were asked to report their usual consumption during the past 12 mo. Intakes of nutrients were calculated based on the food composition tables in Japan<sup>[20]</sup>. To estimate dietary intake of polyphenols, we used 97 food items, including cereals, soybeans, vegetables, fruits, beverages and condiments.

**Table 1** Characteristics of colorectal cancer cases and controls

| Variables                                 | Cases (n = 816) | Controls (n = 815) |
|---|-----------------|--------------------|
| Male                                      | 60%             | 62%                |
| Age (yr), mean $\pm$ SD                   | 60.5 $\pm$ 9.1  | 58.9 $\pm$ 10.7    |
| Dietary intake, median (IQR) <sup>1</sup> |                 |                    |
| Total polyphenols (mg/d)                  | 1025 (698-1487) | 1047 (736-1431)    |
| Tea polyphenols (mg/d) <sup>2</sup>       | 432 (226-576)   | 397 (215-509)      |
| Coffee polyphenols (mg/d) <sup>3</sup>    | 187 (0.2-619)   | 260 (39-643)       |

<sup>1</sup>Polyphenols and food intakes were energy-adjusted to 2000 kcal/d;

<sup>2</sup>Included green tea, black tea and oolong tea; <sup>3</sup>Included coffee infusion, instant coffee and coffee drinks. IQR: Interquartile range.

Polyphenols for 92 food items were derived from the Phenol-Explorer Database, which is a compilation of polyphenol contents of 452 daily foods and beverages based on 638 published analytical studies<sup>[21]</sup>. As for the remaining 5 foods which were not listed in the Phenol-Explorer Database, polyphenol contents of 3 foods (sweet potatoes, satoimo and daikon) were based on a Japanese study<sup>[22]</sup>, and 2 foods (soybeans and fried potatoes) were estimated by ORAC-based polyphenol contents in the United States Department of Agriculture Database<sup>[23]</sup>.

### Statistical analysis

Dietary intakes of the nutrients and polyphenols were transformed to a natural log-scale and were adjusted to a total energy intake of 2000 kcal/d by the regression residual method<sup>[24]</sup>. Subjects were divided into quintile categories according to intakes of polyphenols among controls. Logistic regression analysis was used to estimate odds ratios (OR) and 95%CI of colorectal cancer for each category with the lowest quintile category as the referent group. We also calculated the OR according to coffee consumption in terms of the number of cups per week or day, with an assumption that one cup corresponded to 150 g of coffee infusion.

Confounding variables under consideration were age, sex, residential area (Fukuoka City or others), parental history of colorectal cancer, smoking (0, 1-399, 400-799 or  $>$  800 cigarettes/year), alcohol consumption (0, 0.1-0.9, 1.0-1.9 or  $\geq$  2 units per day), body mass index 10 years before (< 22.5, 22.5-24.9, 25.0-27.4 or  $\geq$  27.5  $\text{kg}/\text{m}^2$ ), type of job (sedentary or non-sedentary), leisure-time physical activity (0, 1-5.9 or  $\geq$  6 MET-h/wk) and dietary intakes of calcium and n-3 polyunsaturated fatty acids (PUFA). Calcium and n-3 PUFA intakes were related to decreased risk of colorectal cancer in the study population<sup>[25-29]</sup>. Trends of the associations were assessed with ordinal scores assigned to quintile categories of intake. Statistical significance was declared with the two-sided  $P < 0.05$ . Statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, United States).

## RESULTS

Demographic and lifestyle characteristics of colorectal

**Table 2 Association of dietary polyphenol intakes with colorectal cancer risk**

| Nutrient (unit)                      | Quintile of intake |                   |                   |                   |                   | <i>P</i> <sub>trend</sub> |
|--------------------------------------|--------------------|-------------------|-------------------|-------------------|-------------------|---------------------------|
|                                      | Q1 (low)           | Q2                | Q3                | Q4                | Q5 (high)         |                           |
| <b>Total polyphenols</b>             |                    |                   |                   |                   |                   |                           |
| Median (mg/d)                        | 534                | 812               | 1047              | 1335              | 2104              |                           |
| Cases/controls                       | 190/163            | 170/163           | 142/163           | 138/163           | 176/163           |                           |
| OR (95%CI) <sup>1</sup>              | 1 (reference)      | 0.85 (0.62, 1.16) | 0.72 (0.52, 0.98) | 0.74 (0.54, 1.02) | 0.95 (0.70, 1.31) | 0.52                      |
| OR (95%CI) <sup>2</sup>              | 1 (reference)      | 0.85 (0.62, 1.16) | 0.72 (0.52, 1.00) | 0.74 (0.54, 1.03) | 0.97 (0.70, 1.33) | 0.59                      |
| <b>Tea polyphenols</b>               |                    |                   |                   |                   |                   |                           |
| Median (mg/d)                        | 65                 | 224               | 397               | 475               | 853               |                           |
| Cases/controls                       | 128/163            | 151/163           | 184/163           | 157/163           | 196/163           |                           |
| OR (95%CI) <sup>1</sup>              | 1 (reference)      | 1.14 (0.82, 1.58) | 1.37 (0.99, 1.89) | 1.13 (0.81, 1.58) | 1.37 (0.99, 1.90) | 0.09                      |
| OR (95%CI) <sup>2</sup>              | 1 (reference)      | 1.13 (0.81, 1.57) | 1.36 (0.98, 1.88) | 1.16 (0.83, 1.62) | 1.38 (0.99, 1.92) | 0.08                      |
| <b>Coffee polyphenols</b>            |                    |                   |                   |                   |                   |                           |
| Median (mg/d)                        | 0                  | 82                | 260               | 541               | 1287              |                           |
| Cases/controls                       | 220/163            | 167/163           | 133/163           | 129/163           | 167/163           |                           |
| OR (95%CI) <sup>1</sup>              | 1 (reference)      | 0.80 (0.59, 1.08) | 0.65 (0.48, 0.90) | 0.64 (0.46, 0.87) | 0.83 (0.60, 1.13) | 0.09                      |
| OR (95%CI) <sup>2</sup>              | 1 (reference)      | 0.81 (0.60, 1.10) | 0.65 (0.47, 0.89) | 0.65 (0.46, 0.89) | 0.82 (0.60, 1.12) | 0.07                      |
| <b>Polyphenols other than coffee</b> |                    |                   |                   |                   |                   |                           |
| Median (mg/d)                        | 280                | 466               | 631               | 790               | 1104              |                           |
| Cases/controls                       | 131/163            | 158/163           | 181/163           | 166/163           | 180/163           |                           |
| OR (95%CI) <sup>1</sup>              | 1 (reference)      | 1.17 (0.85, 1.63) | 1.29 (0.93, 1.79) | 1.13 (0.81, 1.58) | 1.23 (0.88, 1.72) | 0.35                      |
| OR (95%CI) <sup>2</sup>              | 1 (reference)      | 1.19 (0.86, 1.66) | 1.31 (0.94, 1.82) | 1.20 (0.85, 1.69) | 1.31 (0.93, 1.84) | 0.19                      |

<sup>1</sup>Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job and leisure-time physical activity; <sup>2</sup>Further adjusted for calcium and n-3 polyunsaturated fatty acids. OR: Odds ratio.

cancer cases and controls were previously described<sup>[25-29]</sup>. The two groups did not differ much with respect to sex, age, residence area and most other factors. Body mass index was greater and a history of parental colorectal cancer was slightly more frequent in the cases than in the controls. There was no measurable difference in total or tea polyphenol intake between cases and controls, but intake of coffee polyphenols was lower in cases than in controls (Table 1). In the whole sample, intake of total polyphenols derived from tea and coffee was 38% and 34%, respectively.

The associations of intakes of total, tea and coffee polyphenols with colorectal cancer are shown in Table 2. Statistically significant decreases in the OR were observed in those with high intake of coffee polyphenols (the third and fourth quintile categories) compared with the lowest quintile, but the OR for the top quintile did not decrease further, showing an upward tendency. Similar, but less pronounced, decreases in the OR were also noted for the third and fourth quintiles of total polyphenol intake. Tea polyphenols and non-coffee polyphenols showed no clear association with colorectal cancer risk regardless of adjustment for the dietary factors.

Multivariate-adjusted ORs in the subsite-specific analysis are summarized in Table 3. Cases of colon and rectal cancers numbered 463 and 340, respectively. There were 188 cases who had proximal colon cancer only and 272 cases with distal colon cancer alone. The OR of colon cancer decreased with increasing intakes of coffee polyphenols; although the individual OR for the third to fifth quintiles were not significantly different from unity, a decreasing trend was statistically significant. Distal colon cancer showed a more evident inverse association with coffee polyphenols than proximal colon cancer. The

association between coffee polyphenols and rectal cancer was U-shaped, with significant decreases in the OR at the second to fourth quintile categories. Again, there was a tendency for the OR of colon and rectal cancer to decrease in the intermediate categories of total polyphenols. The decrease in the OR in the intermediate categories of total polyphenols was most pronounced for distal colon cancer. Intake of tea polyphenols was not associated with either colon or rectal cancer.

Additionally, we analyzed the associations of coffee consumption with colorectal cancers (Table 4). The associations of coffee consumption with colorectal, colon, and rectal cancers were almost the same as observed for coffee polyphenols. Nonetheless, the trend of the association between coffee consumption and colorectal cancer was statistically significant. The trend of an inverse association with coffee was also statistically significant for distal colon cancer. The OR of proximal colon cancer tended to decrease with increasing consumption of coffee, but the trend failed to reach statistical significance.

## DISCUSSION

The present study showed protective associations of coffee polyphenols and coffee consumption with the risk of colorectal cancer, especially of colon cancer. Non-coffee polyphenols showed no measurable association with the overall or site-specific risk of colorectal cancer. Thus, a modestly decreased risk of colorectal cancer associated with total polyphenol intake was probably ascribed to coffee polyphenols.

The association between coffee consumption and colorectal cancer has been examined in many studies in different populations. A meta-analysis of 12 cohort stud-

**Table 3** Multivariate-adjusted odds ratio (95%CI) of colon and rectal cancers according to polyphenols intake

| Nutrient                | Quintile of intake |                   |                   |                   |                   | <i>P</i> <sub>trend</sub> |
|-------------------------|--------------------|-------------------|-------------------|-------------------|-------------------|---------------------------|
|                         | Q1 (low)           | Q2                | Q3                | Q4                | Q5 (high)         |                           |
| Total polyphenols       |                    |                   |                   |                   |                   |                           |
| Colon                   |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 111/163            | 110/163           | 76/163            | 80/163            | 86/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.90 (0.63, 1.29) | 0.66 (0.45, 0.96) | 0.73 (0.50, 1.07) | 0.81 (0.55, 1.18) | 0.13                      |
| Proximal colon          |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 40/163             | 45/163            | 34/163            | 38/163            | 31/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.91 (0.55, 1.52) | 0.78 (0.46, 1.33) | 0.91 (0.53, 1.54) | 0.79 (0.45, 1.38) | 0.44                      |
| Distal colon            |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 70/163             | 64/163            | 42/163            | 42/163            | 54/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.86 (0.56, 1.31) | 0.57 (0.36, 0.91) | 0.61 (0.38, 0.98) | 0.79 (0.50, 1.23) | 0.11                      |
| Rectum                  |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 77/163             | 60/163            | 62/163            | 56/163            | 85/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.73 (0.48, 1.10) | 0.73 (0.48, 1.11) | 0.70 (0.46, 1.07) | 1.08 (0.72, 1.62) | 0.74                      |
| Tea polyphenols         |                    |                   |                   |                   |                   |                           |
| Colon                   |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 64/163             | 92/163            | 103/163           | 98/163            | 106/163           |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 1.37 (0.91, 2.05) | 1.50 (1.00, 2.23) | 1.34 (0.89, 2.03) | 1.42 (0.95, 2.14) | 0.18                      |
| Proximal colon          |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 21/163             | 33/163            | 44/163            | 41/163            | 49/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 1.38 (0.75, 2.54) | 1.77 (0.98, 3.19) | 1.42 (0.77, 2.61) | 1.82 (1.01, 3.30) | 0.08                      |
| Distal colon            |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 43/163             | 58/163            | 58/163            | 56/163            | 57/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 1.36 (0.85, 2.19) | 1.32 (0.82, 2.12) | 1.26 (0.77, 2.06) | 1.18 (0.72, 1.92) | 0.74                      |
| Rectum                  |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 62/163             | 57/163            | 80/163            | 56/163            | 85/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.89 (0.57, 1.36) | 1.25 (0.83, 1.88) | 0.91 (0.59, 1.42) | 1.27 (0.84, 1.93) | 0.25                      |
| Coffee polyphenols      |                    |                   |                   |                   |                   |                           |
| Colon                   |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 128/163            | 103/163           | 82/163            | 75/163            | 75/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.92 (0.64, 1.31) | 0.75 (0.52, 1.08) | 0.69 (0.47, 1.01) | 0.68 (0.46, 1.00) | 0.02                      |
| Proximal colon          |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 52/163             | 41/163            | 34/163            | 32/163            | 29/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.89 (0.55, 1.44) | 0.75 (0.45, 1.25) | 0.76 (0.45, 1.27) | 0.70 (0.41, 1.20) | 0.15                      |
| Distal colon            |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 76/163             | 61/163            | 47/163            | 42/163            | 46/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.89 (0.58, 1.35) | 0.72 (0.46, 1.12) | 0.62 (0.39, 0.98) | 0.66 (0.42, 1.04) | 0.02                      |
| Rectum                  |                    |                   |                   |                   |                   |                           |
| Cases/controls          | 90/163             | 62/163            | 48/163            | 53/163            | 87/163            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)      | 0.65 (0.44, 0.98) | 0.50 (0.33, 0.77) | 0.55 (0.36, 0.84) | 0.93 (0.63, 1.37) | 0.51                      |

<sup>1</sup>Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job, leisure-time physical activity, calcium and n-3 polyunsaturated fatty acid. OR: Odds ratio.

ies found a relative risk (RR) of 0.91 (95%CI: 0.81-1.02) of colorectal cancer for the highest versus lowest coffee consumption<sup>[15]</sup>. A slightly pronounced decrease in the RR of colorectal cancer for the highest versus lowest consumption was noted in a combined analysis of 3 Japanese cohort studies (RR = 0.83, 95%CI: 0.62-1.10)<sup>[15]</sup>. On the other hand, a decrease in the risk of colorectal cancer associated with coffee use was more pronounced in a meta-analysis of 24 case-control studies<sup>[14]</sup>. The pooled OR for the highest versus non/low consumption was 0.70 (95%CI: 0.60-0.81) for colorectal cancer, 0.75 (95%CI: 0.60-0.81) for colon cancer and 0.87 (95%CI: 0.75-1.00) for rectal cancer<sup>[14]</sup>. A weaker association in the cohort studies is probably ascribed to a different time of exposure under consideration<sup>[14]</sup>. Coffee consumption in the recent past was assessed in most case-control studies. In the cohort studies, a follow-up shorter than 10 years was more likely to result in an inverse association between

coffee consumption and colorectal cancer risk than a longer follow-up<sup>[15]</sup>. In a recent large cohort study<sup>[30]</sup>, coffee consumption was associated with a decreased risk of proximal colon cancer, but not of distal colon cancer. The present study showed no such site-specific association with coffee.

Coffee polyphenolic compounds include chlorogenic, caffeic, ferulic and cumaric acids, and diterpenes (cafestol and kahweol) have been shown to possess anticarcinogenic properties<sup>[7]</sup>. Possible mechanisms by which coffee polyphenols are protective against colorectal cancer include reduction in bile acid secretion<sup>[31]</sup>, reduction in the synthesis of bile by down-regulation of the expression of bile acid homeostatic genes<sup>[32]</sup> and increase in colonic motility<sup>[33]</sup>.

The present study did not provide any evidence for a decreased risk of colorectal cancer associated with dietary intake of tea polyphenols. Tea polyphenols have been

**Table 4 Association of frequency of coffee consumption with colorectal cancer risk**

|                         | Frequency of coffee consumption |                   |                   |                   |                   | <i>P</i> <sub>trend</sub> |
|-------------------------|---------------------------------|-------------------|-------------------|-------------------|-------------------|---------------------------|
|                         | < 1 cup/wk                      | 1-3 cups/wk       | 4-6 cups/wk       | 1-3 cups/d        | ≥ 4 cups/d        |                           |
| Colorectal              |                                 |                   |                   |                   |                   |                           |
| Cases/controls          | 245/181                         | 93/83             | 66/78             | 265/317           | 147/156           |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)                   | 0.88 (0.61, 1.26) | 0.66 (0.45, 0.99) | 0.65 (0.50, 0.85) | 0.82 (0.59, 1.13) | 0.01                      |
| Colon                   |                                 |                   |                   |                   |                   |                           |
| Cases/controls          | 145/181                         | 62/83             | 40/78             | 141/317           | 75/156            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)                   | 1.04 (0.69, 1.57) | 0.75 (0.47, 1.18) | 0.64 (0.47, 0.87) | 0.78 (0.53, 1.14) | 0.01                      |
| Proximal colon          |                                 |                   |                   |                   |                   |                           |
| Cases/controls          | 60/181                          | 22/83             | 19/78             | 62/317            | 25/156            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)                   | 0.84 (0.47, 1.50) | 0.88 (0.48, 1.62) | 0.69 (0.45, 1.06) | 0.69 (0.39, 1.21) | 0.08                      |
| Distal colon            |                                 |                   |                   |                   |                   |                           |
| Cases/controls          | 84/181                          | 40/83             | 21/78             | 78/317            | 49/156            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)                   | 1.16 (0.72, 1.89) | 0.65 (0.37, 1.15) | 0.58 (0.40, 0.85) | 0.82 (0.52, 1.29) | 0.02                      |
| Rectum                  |                                 |                   |                   |                   |                   |                           |
| Cases/controls          | 98/181                          | 29/83             | 26/78             | 118/317           | 69/156            |                           |
| OR (95%CI) <sup>1</sup> | 1 (reference)                   | 0.63 (0.38, 1.04) | 0.55 (0.32, 0.93) | 0.63 (0.45, 0.88) | 0.82 (0.54, 1.23) | 0.10                      |

<sup>1</sup>Adjusted for age, sex, residence area, parental history of colorectal cancer, smoking, alcohol drinking, body mass index 10 years before, type of job, leisure-time physical activity, calcium and n-3 polyunsaturated fatty acid. OR: Odds ratio.

shown to have anti-cancer properties in numerous *in vitro* and *in vivo* studies<sup>[34,35]</sup>, but epidemiologic findings are inconsistent as regards tea and colorectal cancer risk. In China, regular green tea consumption was associated with a reduced risk of colorectal cancer in male non-smokers, but not in male smokers<sup>[9]</sup> and in women<sup>[10]</sup>. On the other hand, in cohort studies of Chinese in Singapore<sup>[11]</sup> and Japan<sup>[13]</sup>, green tea consumption was unrelated to colorectal cancer risk in men and women combined. A pooled analysis of 13 cohort studies in Western countries showed an increased risk associated with tea consumption; the RR for a consumption of > 900 g in liquid/d versus no consumption was 1.28 (95%CI: 1.02-1.61) in men and women combined<sup>[12]</sup>. A combined analysis of two Japanese prospective studies found no association between green tea consumption and colorectal cancer risk<sup>[13]</sup>.

We examined the associations with polyphenol and coffee for proximal and distal colon cancer separately, because these two cancers have distinct molecular mechanisms in carcinogenesis<sup>[36]</sup>. It was recently suggested that molecular changes in colorectal cancer were continuous, rather than dichotomous, from rectum to ascending colon<sup>[37]</sup>. The number of colorectal cancer cases was not large enough to perform a detailed subsite analysis in the present study. It should be noted that the difference in the molecular changes among the detailed subsites of proximal colon was less marked as compared with the difference between proximal and distal colon cancer.

In addition to a large sample size and the use of community controls, the detailed dietary assessment was a notable strength in the present study. The dietary interview was conducted as an in-person interview with typical dishes and serving sizes shown on a computer display. The present study had some weaknesses to be mentioned. The retrospective assessment of diet is a problem inherent to case-control studies. Dietary intakes in the past year may not have captured a habitual consumption

relevant to the development of colorectal cancer. Additionally, the participation rate was lower in the controls (60%) than in the cases (80%), and this may have caused a selection bias.

In conclusion, a case-control study in Japan showed protective associations of coffee polyphenols and coffee consumption with the risk of colorectal cancer, especially of colon cancer.

## ACKNOWLEDGMENTS

The authors acknowledge support from Emeritus Professors Keizo Sugimachi, Seiyo Ikeda, Sumitaka Arima, and Takayuki Shirakusa and from Drs. Motonori Saku, Yoichi Ikeda, Soichiro Maekawa, Kazuo Tanoue, Kinjiro Sumiyoshi, and Shoichiro Saito in conducting the survey of cases. The following physicians kindly supervised the survey of controls at their clinics: Drs. Hideaki Baba, Tomonori Endo, Hiroshi Hara, Yoichiro Hirokata, Motohisa Ikeda, Masayoshi Ishibashi, Fumiaki Itoh, Yasuhiro Iwanaga, Hideki Kaku, Shoshi Kaku, Minoru Kanazawa, Akira Kobayashi, Ryunosuke Kumashiro, Shinichi Matsumoto, Soukei Mioka, Umeji Miyakoda, Osamu Nakagaki, Nobuyoshi Nogawa (deceased), Nobuyuki Ogami, Toyooki Okabayashi, Hironao Okabe, Nishiki Saku, Masafumi Tanaka, Masahiro Ueda, Bunichi Ushio, and Koheisho Yasunaga.

## COMMENTS

### Background

Polyphenols are the most abundant antioxidants in foods and have drawn much interest as potentially anticarcinogenic compounds. Epidemiological studies have suggested that polyphenol-rich foods and beverages may be protective against colorectal cancer.

### Research frontiers

The Phenol-Explorer Database has enabled researchers to study the association between polyphenol intake and cancer risk. Evidence from meta-analyses

indicates that coffee may be protective against colorectal cancer.

### Innovations and breakthroughs

This was the first Japanese epidemiological study reporting that coffee polyphenol intake was associated with a decreased risk of colorectal cancer, particularly of colon cancer.

### Applications

Understanding the role for coffee polyphenols in colorectal carcinogenesis may advance a future strategy in the prevention of colorectal cancer.

### Terminology

The flavonoids are plant antioxidative compounds and possess anticarcinogenic properties. Tea catechins are a class of flavonoids, and are shown to inhibit tumorigenesis in animal experiments. Coffee is the major source of polyphenol intake such as chlorogenic and caffeic acids.

### Peer review

The present study is a very timely and well performed study that investigates the associations between intake of dietary polyphenols and colorectal cancer. The authors have done a tremendous job of designing and executing the project, and the data analysis and interpretations are very logical. I think these data are very important and provide another dimension and validity to the growing awareness of diet and its link to colorectal cancer.

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**P- Reviewers** Goel A, Ogino S **S- Editor** Zhai HH  
**L- Editor** O'Neill M **E- Editor** Zhang DN



## Coinfection with hepatitis C virus and schistosomiasis: Fibrosis and treatment response

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Supported by The Science and Technology Development Fund, No. 1708

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Received: October 31, 2012 Revised: January 29, 2013

Accepted: February 5, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To assess whether schistosomiasis coinfection with chronic hepatitis C virus (HCV) influences hepatic fibrosis and pegylated-interferon/ribavirin (PEG-IFN/RIB) therapy response.

**METHODS:** This study was designed as a retrospective analysis of 3596 chronic HCV patients enrolled in the Egyptian National Program for HCV treatment with PEG-IFN/RIB. All patients underwent liver biopsy and anti-schistosomal antibodies testing prior to HCV treatment. The serology results were used to categorize the patients into group A (positive schistosomal serology) or group B (negative schistosomal serology). Patients in group A were given oral antischistosomal treatment

(praziquantel, single dose) at four weeks prior to PEG-IFN/RIB. All patients received a 48-wk course of PEG-IFN (PEG-IFN $\alpha$ 2a or PEG-IFN $\alpha$ 2b)/RIB therapy. Clinical and laboratory follow-up examinations were carried out for 24 wk after cessation of therapy (to week 72). Correlations of positive schistosomal serology with fibrosis and treatment response were assessed by multiple regression analysis.

**RESULTS:** Schistosomal antibody was positive in 27.3% of patients (15.9% females and 84.1% males). The patients in group A were older ( $P = 0.008$ ) and had a higher proportion of males ( $P = 0.002$ ) than the patients in group B. There was no significant association between fibrosis stage and positive schistosomal serology ( $P = 0.703$ ). Early virological response was achieved in significantly more patients in group B than in group A (89.4% vs 86.5%,  $P = 0.015$ ). However, significantly more patients in group A experienced breakthrough at week 24 than patients in group B (36.3% vs 32.3%,  $P = 0.024$ ). End of treatment response was achieved in more patients in group B than in group A (62.0% vs 59.1%) but the difference did not reach statistical significance ( $P = 0.108$ ). Sustained virological response occurred in significantly more patients in group B than in group A (37.6% vs 27.7%,  $P = 0.000$ ). Multivariate logistic regression analysis of patient data at treatment weeks 48 and 72 showed that positive schistosomal serology was associated with failure of response to treatment at week 48 (OR = 1.3,  $P = 0.02$ ) and at week 72 (OR = 1.7,  $P < 0.01$ ).

**CONCLUSION:** Positive schistosomal serology has no effect on fibrosis staging but is significantly associated with failure of response to HCV treatment despite anti-schistosomal therapy.

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**Key words:** Hepatitis C virus; Schistosomiasis; Coinfection; Fibrosis; Treatment response

**Core tip:** Both hepatitis C virus (HCV) and schistosomiasis are highly endemic in Egypt and coinfection is frequently encountered. The effect of such coinfection on hepatic fibrosis and response to pegylated-interferon and ribavirin therapy (PEG-IFN/RIB) remains unclear. Our study aimed to assess the impact of schistosomiasis on hepatic fibrosis and response to PEG-IFN/RIB therapy in chronic HCV Egyptian patients. Antischistosomal antibody was positive in 27.3% of 3596 chronic HCV patients. Findings suggest positive schistosomal serology has no effect on fibrosis stage but is significantly associated with failure of response to HCV treatment despite antischistosomal therapy.

Abdel-Rahman M, El-Sayed M, El Raziky M, Elsharkawy A, El-Akel W, Ghoneim H, Khattab H, Esmat G. Coinfection with hepatitis C virus and schistosomiasis: Fibrosis and treatment response. *World J Gastroenterol* 2013; 19(17): 2691-2696 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2691.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2691>

## INTRODUCTION

The hepatitis C virus (HCV) is a major public health problem and a leading cause of chronic liver disease<sup>[1]</sup>. In fact, Egypt has the largest epidemic of HCV in the world with an overall serum positive prevalence of 14.7% as reported by the Egyptian demographic health survey<sup>[2]</sup>. It also is a major cause of liver fibrosis, which is associated with significant morbidity and mortality<sup>[3]</sup>.

Schistosomiasis is also of significant concern as it is endemic in Egypt<sup>[4,5]</sup>. *Schistosoma haematobium* is endemic in Upper Egypt (7.8% prevalence), while *Schistosoma mansoni* has greater prevalence in Lower Egypt (36.4%)<sup>[6]</sup>. The presence of both HCV and *Schistosoma* spp. is of significant concern as patients with coinfections have been shown to have higher HCV RNA titers, increased histological activity, greater incidence of cirrhosis/hepatocellular carcinoma, and higher mortality rates than patients suffering from single infections<sup>[7]</sup>. In addition, patients diagnosed with hepatosplenic schistosomiasis have increased opportunities for additional infections and medical abnormalities. These may include up to a 10-fold opportunity for coinfection with hepatitis B virus (HBV) (compared to healthy counterparts), chronic hepatitis on liver biopsy, persistent antigenemia, and increased frequency of liver failure<sup>[8]</sup>.

The aim of this study was to evaluate the impact of schistosomiasis on hepatic fibrosis and on response to pegylated-interferon combined with ribavirin (PEG-IFN/RIB) therapy in Egyptian patients with chronic HCV.

## MATERIALS AND METHODS

### Patient characteristics and study design

This retrospective study included 3596 Egyptian patients

with chronic HCV treated with PEG-IFN/RIB at Cairo-Fatemic Hospital (Cairo, Egypt). Study enrollment inclusion and exclusion criteria are listed in Table 1.

All patients received PEG-IFN $\alpha$ 2a (180  $\mu$ g/wk dose) or PEG-IFN $\alpha$ 2b (1.5  $\mu$ g/kg/wk dose) via subcutaneous injection and oral RIB (800-1200 mg/d) for 48 wk as genotype 4 causes approximately 90% of HCV infections in Egypt<sup>[9]</sup>. Patients were followed for 24 wk after cessation of therapy (to week 72).

The study was approved by the ethical committee of the Ministry of Health (Cairo, Egypt), and all patients consented to blood sampling and data usage in future research. Anti-schistosomal antibody testing was completed for all patients. Patients were stratified according to their schistosomal serological status; group A, HCV patients with positive schistosomal serology; group B, HCV patients with negative schistosomal serology. Study participants with positive schistosomal serology were given praziquantel (PZQ) therapy (oral, 40 mg/kg, single dose) at four weeks prior to initiation of the PEG-IFN/RIB therapy. Liver biopsies were performed for all patients to determine the grade of necroinflammation and stage of fibrosis (Table 2) (based on the METAVIR scoring system<sup>[10]</sup>).

Quantitative real-time reverse transcription-polymerase chain reaction was used to detect HCV RNA (detection limit  $\geq$  50 IU/mL) at baseline and weeks 12, 24, 48 and 72 after initiation of anti-viral therapy. Clinical and laboratory follow-up examinations were carried out to identify the presence of adverse side effects and treatment response. A total of 845 patients were lost to follow-up, so that 2751 participants completed the follow-up to week 72. Patients with early virologic response (EVR) continued follow-up to identify subsequent non-responders. Patients with detectable HCV-RNA at week 24 or those with less than 2 log decrease in viral load at week 12 were designated as treatment failure. HCV therapy was discontinued prematurely in those patients.

### Statistical analysis

Quantitative data were described by averaging and calculating the SD. Intergroup differences were assessed by the Student's *t*-test,  $\chi^2$  or Fisher's exact tests were used for comparisons when appropriate. Multivariate logistic regression was performed with failure of treatment set as the dependent variable. In all tests, a *P*-value of  $<$  0.05 was considered as the threshold for significance.

## RESULTS

No statistically significant differences were found in the baseline characteristics of the two groups of patients, with the exceptions of age and sex (Table 3). HCV patients with positive schistosomal serology were older than the negative schistosomal group ( $P = 0.008$ ) and showed a higher rate of males ( $P = 0.002$ ). Of the 27.3% of the patients with positive schistosomal serology, 15.9% were

| Table 1 Inclusion criteria and exclusion criteria   |  |
|---|--|
| Inclusion criteria  |  |
| Age ≥ 18 yr and ≤ 60 yr   |  |
| Positive HCV antibodies and detectable HCV RNA by PCR   |  |
| Positive liver biopsy for chronic hepatitis with F1 METAVIR score and elevated liver enzymes or F2/F3 METAVIR score   |  |
| Naïve to treatment with PEG-IFN and RIB   |  |
| Hepatitis B surface antigen negativity  |  |
| Normal complete blood count, normal thyroid function, prothrombin concentration ≥ 60%, normal bilirubin, α-fetoprotein < 100 (ng/mL) and antinuclear antibody titer < 1/160                                     |  |
| Exclusion criteria  |  |
| Serious co-morbid conditions such as severe arterial hypertension, heart failure, significant coronary heart disease, poorly controlled diabetes (hemoglobin A1C > 8.5%), chronic obstructive pulmonary disease |  |
| Major uncontrolled depressive illness   |  |
| Solid transplant organ (renal, heart, or lung)  |  |
| Untreated thyroid disease   |  |
| History of previous anti-HCV therapy  |  |
| Body mass index (BMI) > 35 kg/m <sup>2</sup>  |  |
| Known human immunodeficiency virus (HIV) coinfection  |  |
| Hypersensitivity to one of the two drugs (PEG-IFN, RIB)   |  |
| Concomitant liver disease other than hepatitis C (chronic hepatitis B, autoimmune hepatitis, alcoholic liver disease, hemochromatosis, α-1 antitrypsin deficiency, Wilson's disease)                            |  |
| Liver biopsy showing severe steatosis (> 66%) and steatohepatitis, decompensated cirrhosis, hepatocellular carcinoma or METAVIR score F4  |  |

HCV: Hepatitis C virus; PCR: Polymerase chain reaction; PEG-IFN: Pegylated-interferon; RIB: Ribavirin.

| Table 2 Necroinflammatory activity and fibrosis staging scale |  |
|---|--|
| Feature   |  |
| Grade A0  | No histologic necroinflammatory activity |
| Grade A1  | Mild activity                            |
| Grade A2  | Moderate activity                        |
| Grade A3  | Severe activity                          |
| Stage F0  | No fibrosis                              |
| Stage F1  | Portal fibrosis without septa            |
| Stage F2  | Portal fibrosis with rare septa          |
| Stage F3  | Numerous septa without cirrhosis         |
| Stage F4  | Cirrhosis                                |

Cited from reference 10. A: Activity; F: Fibrosis.

females and 84.1% were males.

There were no significant differences between groups regarding fibrosis staging (Figure 1) or end of treatment response (ETR) (Table 4). However, the EVR and virological response at week 24 were significantly higher in patients with negative schistosomal serology ( $P = 0.015$  and  $P = 0.024$ , respectively).

Of the 2751 patients that were followed-up to week 72, those with negative schistosomal serology had achieved a higher sustained virological response (SVR) than the other group (37.6% *vs* 27.7%,  $P = 0.000$ ).

Multivariate logistic regression analyses indicated that patients with positive schistosomal serology were more likely to fail treatment (OR = 1.3,  $P = 0.02$ ) at week 48 and (OR = 1.7,  $P < 0.01$ ) at week 72 (Table 5).

| Table 3 Baseline characteristics |                                       |                                     |                |
|----------------------------------|---------------------------------------|-------------------------------------|----------------|
|                                  | Schisto + ve<br>( <i>n</i> = 983)     | Schisto - ve<br>( <i>n</i> = 2613)  | <i>P</i> value |
| Age                              | 42.59 ± 9.21                          | 41.62 ± 9.81                        | 0.008          |
| Albumin<br>(ref.: 3.5-5.5 mg/dL) | 4.20 ± 0.47                           | 4.20 ± 0.47                         | 0.251          |
| AST<br>(ref.: 40 IU/L)           | 55.91 ± 33.91                         | 57.07 ± 46.00                       | 0.412          |
| ALT<br>(ref.: 40 IU/L)           | 63.19 ± 41.68                         | 63.38 ± 43.30                       | 0.908          |
| HCV RNA, IU/mL                   | $1.4 \times 10^6 \pm 6.9 \times 10^6$ | $9.5 \times 10^5 \pm 7 \times 10^6$ | 0.083          |
| BMI                              | 28.12 ± 4.09                          | 28.27 ± 4.4                         | 0.387          |
| Male/female                      | 5.3/1                                 | 3.88/1                              | 0.002          |

Data are expressed as mean ± SD. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; BMI: Body mass index.

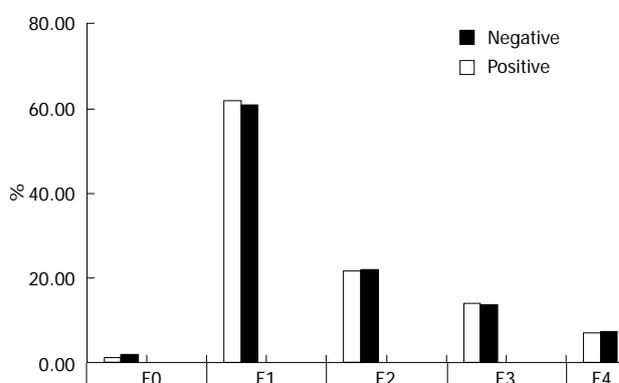


Figure 1 Fibrosis stages in relation to schistosomal serology.

## DISCUSSION

This study was undertaken to determine a correlation between HCV and schistosomiasis infection in relation to hepatic fibrosis stages and antiviral treatment response.

Our findings showed a correlation of positive schistosomal serology in reference to sex, with the predominance involving males. HCV patients with positive schistosomal serology were also found to be older than those with negative serology. This finding is suspected to be due to the reservoir of HCV infection in Egypt, for which intravenously administered tartar emetic was used as a primary treatment<sup>[11]</sup>.

While this study showed no significant difference between the two groups in terms of fibrosis staging, other studies report HCV/schistosomiasis coinfecting patients have more rapid progression of hepatic fibrosis than those with HCV mono-infection<sup>[12]</sup>, as evidenced by increased fibrosis scores for the liver biopsies taken at 96.0 ± 4.6 mo of follow-up. Moreover, another study demonstrated that serum transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) levels are higher in coinfecting groups<sup>[13]</sup>.

Ahmad *et al.*<sup>[14]</sup> showed that schistosomiasis coinfection with HCV and/or non-alcoholic steatohepatitis had no significant impact on fibrosis stage. Results involving differences in fibrosis stages may be explained by several

**Table 4** Virological response at weeks 12, 24, 48 in relation to schistosomal serology *n* (%)

|                             | Anti-schisto antibody          |                               |                             | <i>P</i> value |
|-----------------------------|--------------------------------|-------------------------------|-----------------------------|----------------|
|                             | Negative<br>( <i>n</i> = 2613) | Positive<br>( <i>n</i> = 983) | Total<br>( <i>n</i> = 3596) |                |
| Responders at week 12 (EVR) | 2335 (89.4)                    | 850 (86.5)                    | 3185 (88.6)                 | 0.015          |
| Responders at week 24       | 1768 (67.7)                    | 626 (63.7)                    | 2394 (66.6)                 | 0.024          |
| Responders at week 48 (ETR) | 1621 (62.0)                    | 581 (59.1)                    | 2202 (61.2)                 | 0.108          |

EVR: Early virologic response; ETR: End of treatment response.

factors. Genetic predisposition may play a role, whereby only a minority of the individuals infected with *Schistosoma mansoni* may develop hepatic fibrosis or be more sensitive to infection(s). Moreover, frequency of exposure is directly correlated with the presence and amount of fibrosis<sup>[15]</sup>. In addition, several clinical and pathological studies have shown that schistosomal hepatopathy is a reversible condition and that resolution of the schistosomiasis disease is accompanied by subsequent fibrosis resorption<sup>[16,17]</sup>.

Moreover, HCV patients with evidence of coinfection or previous exposure to schistosomiasis received oral antischistosomal treatment of PZQ prior to starting the anti-viral therapy. PZQ is believed to exert antifibrotic effects by affecting (decreasing) activation of hepatic stellate cells through inhibition of profibrotic gene expression<sup>[18]</sup>. Morcos *et al*<sup>[19]</sup> demonstrated that PZQ treatment could induce resolution of schistosoma-induced pathology, showing partial reversal of liver fibrosis in *Schistosoma mansoni* infected mice. Improvements and/or resolutions of schistosomal-induced periportal thickening/fibrosis in PZQ treated models have also been demonstrated by Berhe *et al*<sup>[20]</sup> and Frenzel *et al*<sup>[21]</sup>. It is theorized that the beneficial effects are likely related to the clearance of schistosomal worms and subsequent reduction of egg deposition.

Other limiting issues for the use of liver biopsy as a clinical tool for assessing fibrosis in schistosomiasis include ethical considerations and the risk of sampling errors, which may be especially evident for small-volume biopsies<sup>[22]</sup>.

In our current study, the EVR, virological response at week 24, and SVR were significantly higher in patients with negative schistosomal serology. This finding may be attributed to coinfecting patients with a down-regulated immune response to HCV leading to reduced IFN $\gamma$ , interleukin (IL)-4 and IL-10 secreted by HCV-specific T cells. Early reports by Kamal *et al*<sup>[23]</sup> using standard IFN in the treatment of chronic HCV patients reported that Egyptian patients with coinfections have higher HCV RNA titers, more advanced liver disease, more hepatic complications and greater mortality rates than those infected with HCV alone.

**Table 5** Multivariate logistic regression analysis in which the failure of treatment is the dependent variable at weeks 48 and 72

| Factors                                | OR   | <i>P</i> value | 95%CI |       |
|--|------|----------------|-------|-------|
|  |      |                | Lower | Upper |
| Week 48                                |      |                |       |       |
| Age of > 50 yr                         | 1.15 | 0.245          | 0.91  | 1.50  |
| Female                                 | 0.65 | 0.000          | 0.51  | 0.80  |
| Viremia, > 600 × 10 <sup>3</sup> IU/mL | 1.78 | 0.000          | 1.41  | 2.30  |
| IFN $\alpha$ 2b                        | 1.21 | 0.050          | 1.01  | 1.50  |
| Activity, A2, A3                       | 0.68 | 0.001          | 0.54  | 0.90  |
| Fibrosis, > F2                         | 1.69 | 0.000          | 1.35  | 2.10  |
| BMI, > 30 kg/m <sup>2</sup>            | 0.86 | 0.009          | 0.76  | 0.96  |
| Positive schisto status                | 1.29 | 0.015          | 1.10  | 1.60  |
| Week 72                                |      |                |       |       |
| Age of > 50 yr                         | 1.0  | 0.990          | 0.7   | 1.3   |
| Female                                 | 0.7  | 0.006          | 0.5   | 0.9   |
| Viremia, > 600 × 10 <sup>3</sup> IU/mL | 1.6  | 0.001          | 1.2   | 2.2   |
| Activity, A2, A3                       | 0.5  | < 0.01         | 0.4   | 0.7   |
| Fibrosis, > F2                         | 1.9  | < 0.01         | 1.5   | 2.5   |
| Positive schisto status                | 1.7  | < 0.01         | 1.3   | 2.1   |

IFN: Interferon; BMI: Body mass index.

In light of the previous real time PCR findings from Bahgat *et al*<sup>[24]</sup>, soluble egg antigen (SEA) should be considered as a potential stimulatory factor for HCV RNA that may have influenced the early detection of HCV RNA as SEA can stimulate viral replication. The higher morbidity that is observed in patients coinfecting with schistosomiasis and HCV is related, at least in part, to direct stimulation of viral replication by SEA<sup>[25]</sup>.

It is interesting to consider that Derbala *et al.* found no significant difference in the treatment responses of patients treated with and without bilharziasis<sup>[26]</sup>. This finding might be explained by phenotypic variations in Egyptian patients infected with HCV genotype 4, whereby some patients may mount HCV-specific T cell responses, both CD4+ and CD8+, despite the prevalence of concomitant schistosomiasis<sup>[27]</sup>.

A major limitation of this study was the need to diagnose schistosomiasis in patients by using an antischistosomal serology approach with a commercially available indirect hemagglutination test (IHAT). While the IHAT is sensitive in detecting bilharziasis, it cannot differentiate between past and current infection status nor between *Schistosoma mansoni* and *Schistosoma haematobium* species. While rectal snips are the preferred method of schistosomiasis diagnosis, this approach was not possible in our study population due to the large number of participants. Finally, genotyping for HCV was not performed on the patients in our study, since approximately 90% of infections in Egypt are due to genotype 4<sup>[9]</sup> and the Egyptian National Committee for Control of Viral Hepatitis does not recommend routine genotyping.

In conclusion, positive schistosomal serology has no effect on fibrosis stage but it is significantly associated with failure of response to HCV treatment despite antischistosomal therapy.

## COMMENTS

**Background**

Hepatitis C virus (HCV) is a major public health problem and is the primary cause of liver fibrosis and chronic liver disease worldwide. Both HCV and schistosomiasis are highly endemic in Egypt and cases of coinfection are frequently encountered. Intriguingly, HCV prevalence shows a direct correlation to the amount of intravenous tartar emetic used to control schistosomiasis in some geographic regions of Egypt. Moreover, patients with hepatosplenic schistosomiasis show a higher susceptibility to coinfection with hepatitis B virus and HCV than healthy individuals.

**Research frontiers**

HCV/Schistosomiasis coinfecting patients are characterized by higher HCV RNA titers, histological activity, incidence of cirrhosis and hepatocellular carcinoma, as well as higher mortality rates than monoinfected patients. This research aimed to assess the influence of schistosomiasis on hepatic fibrosis and to evaluate the response to pegylated-interferon/ribavirin (PEG-IFN/RIB) therapy in patients with chronic HCV infection.

**Innovations and breakthroughs**

Results showed that 27.3% of the patients had positive schistosomal serology, with a prevalence towards males (15.9% female and 84.1% male). The extent of fibrosis was not significantly different between patients with HCV/schistosomiasis coinfection and patients with chronic HCV mono-infection. Patients with HCV/schistosomiasis coinfection showed lower rates of early virologic response and virological response at week 24 of antiviral treatment, as well as lower rates of end-treatment response and sustained virological response. Schistosomiasis appears to be significantly associated with failure to respond to HCV treatment despite antischistosomal therapy.

**Applications**

Schistosomiasis appears to be significantly associated with failure to respond to HCV treatment. Authors propose that mandatory schistosomal serology should be considered for chronic HCV patients prior to initiating PEG-IFN/RIB therapy. In those patients with positive schistosomal serology, administration of anti-schistosomal therapy (praziquantel at 40 mg/kg single dose) four weeks prior to antiviral therapy (PEG-IFN/RIB) may decrease the effect of schistosomiasis, reduce subsequent complications and improve response to treatment.

**Terminology**

Early virological response is defined as a  $\geq 2$  log reduction or complete absence of serum HCV RNA at week 12 of therapy, as compared to the baseline level. End-of-treatment response is defined as an undetectable level of virus (by polymerase chain reaction) in serum at the end of a 48-wk course of therapy (for patients infected with HCV genotype 4). Sustained virological response is defined as an undetectable level of HCV RNA in serum at 24 wk after the discontinuation of therapy. Virological breakthrough refers to the reappearance of HCV RNA during the ongoing course of therapy.

**Peer review**

This study analyzes the impact of schistosomiasis on hepatic fibrosis and response to PEG-IFN/RIB therapy in patients with chronic HCV. The results suggest that positive schistosomal serology has no effect on fibrosis stage, but is significantly associated with failure to respond to HCV treatment.

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**P- Reviewers** Lonardo A, Aghakhani A, Antonelli A  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN



## Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma

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Received: September 27, 2012 Revised: October 17, 2012

Accepted: November 24, 2012

Published online: May 7, 2013

### Abstract

**AIM:** To investigate the expression of distal-less homeobox 2 (DLX2) in gastric adenocarcinoma and its clinicopathological significance.

**METHODS:** Gastric adenocarcinoma tissues were obtained from gastrectomy specimens of 129 patients from the Department of Surgery and Pathology, the Second Affiliated Hospital of Kunming Medical University. Sixty cases of normal gastric tissues were collected from gastrectomy specimens of adjacent gastric cancer margins greater than 5 cm. Patient diagnosis was established pathologically, and no patient had received chemotherapy or radiotherapy before surgery. All tissue specimens were formalin-fixed and paraffin-embedded. Immunohistochemistry was carried out to investigate the expression of DLX2 in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues. The immunos-

taining reaction was semiquantitatively evaluated based on the proportion of positive cells and the median staining intensity in normal gastric epithelial cells or tumor cells. All patients had follow-up records for more than 5 years. Correlations of DLX2 expression with clinicopathological features and prognosis of patients with gastric adenocarcinoma were analyzed. All statistical analyses were performed using the SPSS 17.0 software.

**RESULTS:** The positive expression of DLX2 was detected in 68 (52.7%) cases of 129 gastric adenocarcinoma tissues and 14 (23.3%) cases of 60 adjacent normal tissues. The difference in DLX2 expression between gastric adenocarcinoma tissues and adjacent normal tissues was statistically significant ( $\chi^2 = 14.391$ ,  $P < 0.001$ ). Moreover, high expression of DLX2 was detected in 48 (37.2%) cases of 129 human gastric cancer tissues, but not in adjacent normal tissues. The expression of DLX2 correlated with the size of tumor ( $P = 0.001$ ), depth of invasion ( $P = 0.008$ ), lymph node metastasis ( $P = 0.023$ ) and tumor-node-metastasis stages ( $P = 0.020$ ), but was not correlated with age, gender, histological differentiation and distant metastasis. The Kaplan-Meier survival analysis revealed that survival time of patients with high DLX2 expression was significantly shorter than that with low DLX2 expression. However, the multivariate analysis showed that invasion depth ( $P < 0.001$ ), lymph nodes metastasis ( $P = 0.001$ ) and distant metastasis ( $P < 0.001$ ) were independent prognostic factors for patients with gastric adenocarcinoma, but DLX2 expression, tumor location and tumor size were not.

**CONCLUSION:** These results suggest that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development.

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**Key words:** Gastric adenocarcinoma; Distal-less homeobox 2; Immunohistochemistry; Invasion; Metastasis; Prognosis

Tang P, Huang H, Chang J, Zhao GF, Lu ML, Wang Y. Increased expression of DLX2 correlates with advanced stage of gastric adenocarcinoma. *World J Gastroenterol* 2013; 19(17): 2697-2703 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2697.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2697>

## INTRODUCTION

Gastric cancer is one of the leading causes of cancer-related death worldwide due to its frequency, poor prognosis and limited treatment options<sup>[1]</sup>. Although the incidence of gastric cancer has been declining for several decades in most Western countries, it remains a crucial public health problem in developing countries<sup>[2,3]</sup>. In China, gastric cancer is the second most common malignancy and was the third leading cause of death from cancer in 2007, representing a major disease burden on health services<sup>[4]</sup>. Several studies have shown that various genetic and epigenetic alterations are involved in the course of carcinogenesis and progression of gastric cancer<sup>[5-8]</sup>. However, the molecular mechanism involved in the development of gastric cancer remains unclear.

The distal-less homeobox (*DLX*) gene family, a homolog of *Drosophila* distal-less, comprises six *DLX* genes in humans, of which three exist as bigene clusters: *DLX-1/DLX-2*, *DLX-3/DLX-4*, *DLX-5/DLX-6*<sup>[9]</sup>. The *DLX* gene family has crucial roles in regulating embryonic development, tissue homeostasis, lymphocyte development, cell cycle and apoptosis<sup>[10-14]</sup>. However, the role of the *DLX* gene family in tumor development has only recently been explored. As a member of *DLX* gene family, the abnormal expression of *DLX2* has been reported in many human hematological malignancies and solid tumors, including acute lymphoblastic leukemia, acute myeloid leukemia, melanoma, glioma, breast, lung, prostate, ovarian and colon cancer<sup>[9,14-17]</sup>. A recent study showed that the expression of *DLX2* plays a critical role in shifting transforming growth factor  $\beta$  (TGF- $\beta$ ) from its tumor suppressive to its tumor-promoting functions<sup>[13]</sup>. Moreover, abnormal TGF- $\beta$  expression is involved in tumor progression, metastasis, angiogenesis and poor survival of gastric cancer<sup>[18,19]</sup>. These studies led us to investigate the possible role of *DLX2* in the development of gastric adenocarcinoma.

In the present study, we assessed the expression of *DLX2* in gastric adenocarcinoma tissues and adjacent normal tissues by immunohistochemistry. Correlations of *DLX2* expression with clinicopathological features and survival of gastric adenocarcinoma patients were then analyzed.

## MATERIALS AND METHODS

### Patients and tissue samples

Gastric adenocarcinoma tissues were obtained from gastrectomy specimens of 129 patients from the Depart-

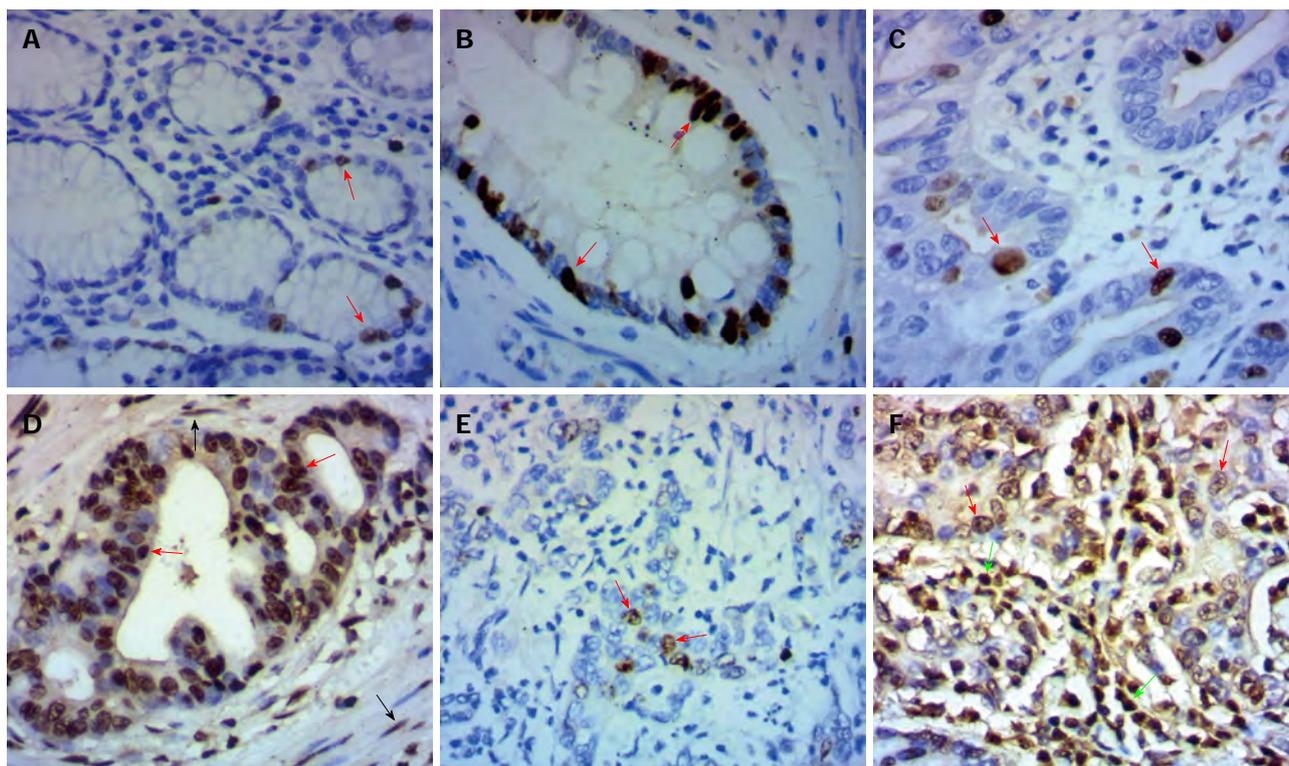
ment of Surgery and Pathology, the Second Affiliated Hospital of Kunming Medical University. Sixty samples of normal gastric tissues were collected from gastrectomy specimens of adjacent gastric cancer margins greater than 5 cm and served as controls. All operations were performed between January 2001 and June 2007. Patient diagnosis was established pathologically, and no patient had received chemotherapy or radiotherapy prior to surgery. All tissue specimens were formalin-fixed and paraffin-embedded. There were 87 males and 42 females (mean age, 57.6 years; range, 26-84 years). The age and gender of patients, tumor size, tumor location, histological differentiation, depth of invasion, status of lymph node metastasis and distant metastasis were obtained from histopathology records. The stage was determined according to the 7<sup>th</sup> edition of the AJCC Tumor Staging Manual and Japanese Classification 2011 in gastric cancer<sup>[20,21]</sup>. Forty-three cases were categorized as stage I, 43 were stage II, 34 were stage III and nine were stage IV. All patients had follow-up records for more than 5 years. The follow-up deadline was July 2012. The survival time was determined from the date of surgery to the follow-up deadline or date of death, which was mostly caused by recurrence or metastasis. The hospital's ethics committee approved this study.

### Immunohistochemistry

Immunohistochemical analysis was used to investigate *DLX2* expression in 129 cases of gastric adenocarcinoma tissues and 60 cases of adjacent normal tissues. According to protocol<sup>[22,23]</sup> for immunohistochemistry on paraffin-embedded tissue sections, paraffin-embedded blocks were sectioned at about 4  $\mu$ m thickness. Slides were baked at 60 °C for 2 h, deparaffinized with xylene and rehydrated using an alcohol gradient (100% alcohol, 95% alcohol, 80% alcohol, and 70% alcohol). After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, sections were treated with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Sections were incubated with 1% bovine serum albumin to block nonspecific binding, and then incubated overnight at 4 °C with the polyclonal antibody against *DLX2* (Epitomics, Inc., California, United States) at a dilution of 1:100. Phosphate buffer solution (PBS) was used as a negative control. After rinsing 3  $\times$  3 min with PBS, tissue sections were treated with peroxidase-linked secondary antibody (Maixin-Bio, Inc., Fuzhou, China) for 30 min at room temperature. Staining was carried out with diaminobenzidine chromogen (Maixin-Bio, Inc., Fuzhou, China) and counterstained with hematoxylin. All slides were then dehydrated using an alcohol gradient, and mounted with a coverslip.

### Scoring of immunohistochemical staining

The results of immunostaining were reviewed and scored independently by two observers in a blinded fashion without knowledge of clinical and pathological information. To avoid artifactual effects, the cells on the margins



**Figure 1** Immunohistochemical staining for distal-less homeobox 2 in gastric adenocarcinoma tissues and adjacent normal gastric tissues. A: Low expression of distal-less (DLX2) in normal gastric mucosa; B: High expression of DLX2 in intestinal metaplasia cells; C: Low expression of DLX2 in gastric adenocarcinoma tissue with well differentiation; D: High expression of DLX2 in gastric adenocarcinoma tissue with well differentiation; E: Low expression of DLX2 in gastric adenocarcinoma tissue with poor differentiation; F: High expression of DLX2 in gastric adenocarcinoma tissue with poor differentiation. DLX2 staining was detected mainly in nucleus of normal gastric epithelial cells (Figure 1A, red arrows) or tumor cells (Figure 1C-F, red arrows). Besides, increased expression of DLX2 was detected in intestinal metaplasia cells (Figure 1B, red arrows), fibroblasts (Figure 1D, black arrows) and inflammatory cells (Figure 1F, green arrows) around tumor cells. Original magnification,  $\times 200$ .

of sections and areas with poorly presented morphology were not counted. Five fields ( $\times 400$  magnification) per tissue section, chosen at random, were counted. The immunostaining reaction was semiquantitatively evaluated based on the proportion of positive cells and the median staining intensity in normal gastric epithelial cells or tumor cells. The proportion of positive cells was scored as follows: 0,  $\leq 5\%$ ; 1, 6%-25%; 2, 26%-50% and 3,  $\geq 51\%$ . The sections were considered to be positively stained when there were more than 5% of observed cells with immunostaining. Staining intensity was graded according to the following criteria: 0, no staining; 1, weak staining, light yellow; 2, moderate staining, yellow brown; and 3, strong staining, brown. The immunoreactive score was calculated based on the proportion score multiplied by the staining intensity score. All immunoreactive scores were less than 4 in adjacent normal tissues; therefore, the results of immunostaining in tumor tissues were divided into two groups, low expression (immunoreactive score  $\leq 3$ ) and high expression (immunoreactive score  $\geq 4$ ).

### Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software. Correlation of DLX2 expression with clinicopathological parameters was calculated by Pearson  $\chi^2$  test,  $\chi^2$  test with continuity correction and Spearman's rank

correlation test, respectively. Univariate survival analysis was assessed by the Kaplan-Meier method and the difference in survival curves was analyzed by the log-rank test. The Cox proportional hazards regression model was used to analyze independent prognostic factors. All reported *P* values were two-sided and *P* < 0.05 was considered statistically significant.

## RESULTS

### Expression of DLX2 in gastric cancer and adjacent normal tissues

In the present study, immunohistochemical analysis was carried out to investigate the DLX2 expression in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues. Positive expression of DLX2 was detected in 68 (52.7%) cases of 129 gastric cancer tissues and in 14 (23.3%) cases of 60 adjacent normal tissues. The difference of DLX2 expression between gastric cancer tissues and adjacent normal tissues was statistically significant ( $\chi^2 = 14.391$ , *P* < 0.001). Moreover, high expression of DLX2 was detected in 48 (37.2%) cases of 129 human gastric cancer tissues, but not in adjacent normal tissues. DLX2 staining was detected mainly in the nuclei of normal gastric epithelial cells (Figure 1A) or tumor cells (Figure 1C-F). In addition, increased expression of

**Table 1 Relationship of distal-less homeobox 2 expression with clinicopathological features of gastric adenocarcinoma *n* (%)**

| Clinicopathological features | Cases | DLX2 expression |           | $\chi^2$ test | <i>P</i> value |
|------------------------------|-------|-----------------|-----------|---------------|----------------|
|                              |       | Low             | High      |               |                |
| Age (yr)                     |       |                 |           | 0.954         | 0.329          |
| < 60                         | 69    | 46 (66.7)       | 23 (33.3) |               |                |
| ≥ 60                         | 60    | 35 (58.3)       | 25 (41.7) |               |                |
| Gender                       |       |                 |           | 1.989         | 0.158          |
| Female                       | 42    | 30 (71.4)       | 12 (28.6) |               |                |
| Male                         | 87    | 51 (58.6)       | 36 (41.4) |               |                |
| Tumor size (cm)              |       |                 |           | 11.518        | 0.001          |
| < 5                          | 68    | 52 (76.5)       | 16 (23.5) |               |                |
| ≥ 5                          | 61    | 29 (47.5)       | 32 (52.5) |               |                |
| Tumor location               |       |                 |           | 2.335         | 0.506          |
| Upper                        | 14    | 10 (71.4)       | 4 (28.6)  |               |                |
| Middle                       | 20    | 14 (70.0)       | 6 (30.0)  |               |                |
| Lower                        | 84    | 52 (61.9)       | 32 (38.1) |               |                |
| Diffuse                      | 11    | 5 (45.5)        | 6 (54.5)  |               |                |
| Histologic differentiation   |       |                 |           | 3.186         | 0.364          |
| Well                         | 16    | 11 (68.8)       | 5 (31.3)  |               |                |
| Moderately                   | 28    | 21 (75.0)       | 7 (25.0)  |               |                |
| Poorly                       | 74    | 42 (56.8)       | 32 (43.2) |               |                |
| Other                        | 11    | 7 (63.6)        | 4 (36.4)  |               |                |
| Depth of invasion            |       |                 |           | 11.940        | 0.008          |
| T1                           | 28    | 24 (85.7)       | 4 (14.3)  |               |                |
| T2                           | 24    | 16 (66.7)       | 8 (33.3)  |               |                |
| T3                           | 58    | 28 (48.3)       | 30 (51.7) |               |                |
| T4                           | 19    | 13 (68.4)       | 6 (31.6)  |               |                |
| Lymph node metastasis        |       |                 |           | 9.577         | 0.023          |
| N0                           | 69    | 50 (72.5)       | 19 (27.5) |               |                |
| N1                           | 17    | 12 (70.6)       | 5 (29.4)  |               |                |
| N2                           | 25    | 11 (44.0)       | 14 (56.0) |               |                |
| N3                           | 18    | 8 (44.4)        | 10 (55.6) |               |                |
| Distant metastasis           |       |                 |           | 0.012         | 0.914          |
| M0                           | 120   | 76 (63.3)       | 44 (36.7) |               |                |
| M1                           | 9     | 5 (55.6)        | 4 (44.4)  |               |                |
| TNM stages                   |       |                 |           | 9.849         | 0.020          |
| I                            | 43    | 35 (81.4)       | 8 (18.6)  |               |                |
| II                           | 43    | 24 (55.8)       | 19 (44.2) |               |                |
| III                          | 34    | 17 (50.0)       | 17 (50.0) |               |                |
| IV                           | 9     | 5 (55.6)        | 4 (44.4)  |               |                |

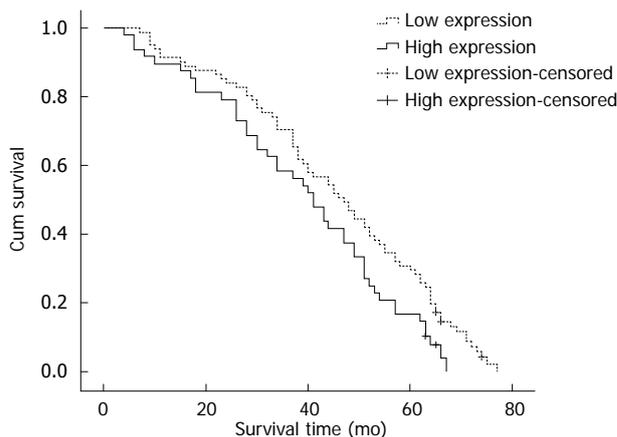
DLX2: Distal-less homeobox 2.

DLX2 was detected in intestinal metaplasia cells (Figure 1B), fibroblasts (Figure 1D) and inflammatory cells (Figure 1F) around tumor cells.

**Expression of DLX2 and clinicopathological features**

The expression of DLX2 in gastric adenocarcinoma was significantly correlated with tumor size (*P* = 0.001), depth of invasion (*P* = 0.008), lymph node metastasis (*P* = 0.023) and TNM stages (*P* = 0.020), but was not correlated with age, gender, histological differentiation and distant metastasis (*P* > 0.05, Table 1). Spearman’s rank correlation test showed that DLX2 expression was positively related to tumor size (*P* < 0.001), depth of invasion (*P* = 0.010), lymph node metastasis (*P* = 0.013) and TNM stages (*P* = 0.004).

To investigate whether the ranks of percentage or staining intensity of DLX2 expression was more prominent for the immunoreactive assessment, the statistical analysis of the correlation between clinicopathological parameters and the proportion score or staining intensity



**Figure 2 Kaplan-Meier curves with univariate analysis (log-rank) for patients with low distal-less homeobox 2 expression vs high distal-less homeobox 2 expression.**

score of DLX2 expression was calculated separately. As shown in Table 2, the proportion score of DLX2 expression was significantly correlated with tumor size (*P* = 0.002), depth of invasion (*P* = 0.016) and lymph node metastasis (*P* = 0.002). The staining intensity score was significantly correlated with tumor size (*P* = 0.029) and lymph node metastasis (*P* = 0.044). These results suggest that the ranks of percentage of DLX2 expression in gastric cancer tissues may be more prominent than staining intensity for the immunoreactive assessment.

**Correlation between DLX2 expression and patient prognosis**

The Kaplan-Meier survival analysis revealed that survival time of patients with high DLX2 expression was significantly shorter than for those with low DLX2 expression ( $\chi^2 = 4.986, P = 0.026$ ; Figure 2). The mean survival time of the former was only 39.216 mo (95%CI: 34.030-44.402), whereas the mean survival time of latter was 45.669 mo (95%CI: 41.426-49.912). For patients with high DLX2 expression, the cumulative 3- and 5-year survival rates were 58.3% and 16.7%, respectively, which was significantly lower than those for patients with low DLX2 expression (70.4% and 29.6%, respectively).

Additionally, the clinicopathological features for possible prognostic effects in gastric cancer were analyzed by Cox regression analysis. The following six clinicopathological features were selected for evaluation: tumor size, tumor location, depth of invasion, lymph nodes metastasis, distant metastasis and DLX2 expression (all *P* < 0.05 in univariate survival analysis). The multivariate analysis showed that invasion depth (*P* < 0.001), lymph nodes metastasis (*P* = 0.001) and distant metastasis (*P* < 0.001) were independent prognostic factors for patients with gastric adenocarcinoma, but DLX2 expression, tumor location and tumor size were not independent prognostic factors (Table 3).

**DISCUSSION**

In the present study, DLX2 expression levels were in-

**Table 2** Relationship of proportion score or staining intensity of distal-less homeobox 2 expression with clinicopathological features of gastric adenocarcinoma

| Clinicopathological features | Cases | Proportion score |    |      | $\chi^2$ test | P value | Staining intensity |      |    | $\chi^2$ test | P value |
|------------------------------|-------|------------------|----|------|---------------|---------|--------------------|------|----|---------------|---------|
|                              |       | 0                | 1  | 2, 3 |               |         | 0                  | 1, 2 | 3  |               |         |
| Age (yr)                     |       |                  |    |      | 5.637         | 0.060   |                    |      |    | 3.924         | 0.141   |
| < 60                         | 69    | 39               | 7  | 23   |               |         | 29                 | 26   | 14 |               |         |
| ≥ 60                         | 60    | 22               | 12 | 26   |               |         | 16                 | 25   | 19 |               |         |
| Gender                       |       |                  |    |      | 2.583         | 0.275   |                    |      |    | 1.785         | 0.410   |
| Female                       | 42    | 22               | 8  | 12   |               |         | 18                 | 15   | 9  |               |         |
| Male                         | 87    | 39               | 11 | 37   |               |         | 27                 | 36   | 24 |               |         |
| Tumor size (cm)              |       |                  |    |      | 12.975        | 0.002   |                    |      |    | 7.115         | 0.029   |
| < 5                          | 68    | 42               | 9  | 17   |               |         | 30                 | 26   | 12 |               |         |
| ≥ 5                          | 61    | 19               | 10 | 32   |               |         | 15                 | 25   | 21 |               |         |
| Histological differentiation |       |                  |    |      | 3.302         | 0.192   |                    |      |    | 0.286         | 0.867   |
| Well and moderately          | 44    | 24               | 8  | 12   |               |         | 16                 | 16   | 12 |               |         |
| Poorly and other             | 85    | 37               | 11 | 37   |               |         | 29                 | 35   | 21 |               |         |
| Invasion depth               |       |                  |    |      | 8.291         | 0.016   |                    |      |    | 3.724         | 0.155   |
| T1, T2                       | 52    | 31               | 9  | 12   |               |         | 22                 | 21   | 9  |               |         |
| T3, T4                       | 77    | 30               | 10 | 37   |               |         | 23                 | 30   | 24 |               |         |
| Lymph node metastasis        |       |                  |    |      | 17.511        | 0.002   |                    |      |    | 9.815         | 0.044   |
| N0                           | 69    | 39               | 11 | 19   |               |         | 30                 | 20   | 19 |               |         |
| N1                           | 17    | 6                | 6  | 5    |               |         | 4                  | 11   | 2  |               |         |
| N2, N3                       | 43    | 16               | 2  | 25   |               |         | 11                 | 20   | 12 |               |         |
| TNM stages                   |       |                  |    |      | 5.982         | 0.050   |                    |      |    | 0.753         | 0.686   |
| I, II                        | 86    | 43               | 16 | 27   |               |         | 32                 | 32   | 22 |               |         |
| III, IV                      | 43    | 18               | 3  | 22   |               |         | 13                 | 19   | 11 |               |         |

**Table 3** Multivariate analysis for disease-related deaths (Cox regression model)

| Variables                                       | B      | P value | Exp (B) | 95%CI for Exp (B) |
|---|--------|---------|---------|-------------------|
| Tumor location                                  | 0.145  | 0.261   | 1.156   | 0.898-1.488       |
| Tumor size (< 5 cm vs ≥ 5 cm)                   | 0.176  | 0.421   | 1.193   | 0.777-1.832       |
| Depth of invasion                               | 0.726  | < 0.001 | 2.067   | 1.570-2.722       |
| Lymph node metastasis                           | 0.303  | 0.001   | 1.354   | 1.126-1.629       |
| Distant metastasis (no vs yes)                  | 2.415  | < 0.001 | 11.185  | 4.187-29.878      |
| Distal-less homeobox 2 expression (low vs high) | -0.214 | 0.308   | 0.808   | 0.535-1.218       |

investigated in 129 gastric adenocarcinoma tissues and 60 adjacent normal tissues by immunohistochemistry. We showed that DLX2 expression was more frequent in gastric cancer tissues than in adjacent normal tissues. The expression of DLX2 in gastric cancer tissues was significantly associated with the size of the tumor, the depth of invasion, lymph node metastasis and TNM stages. Based on these results, we suggest that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma.

In several investigations, it has been shown that the abnormal expression of DLX2 in cancer cells is associated with tumor progression. However, the mechanism of DLX2's involvement in tumor progression is not clear. Yilmaz *et al*<sup>[4]</sup> showed that expression of DLX2 correlated significantly with advanced tumor progression and with the metastatic potential of melanoma, glioma, lung, and prostate cancers. In their research, they found that DLX2 counteracted TGF- $\beta$ -induced cell-cycle arrest and apoptosis in mammary epithelial cells, and DLX2 ex-

pression supported experimental tumor growth and metastasis of B16 melanoma cells. These results established that DLX2 has an important role in shifting TGF- $\beta$  from its tumor suppressive to its tumor-promoting functions. Additionally, Lee *et al*<sup>[16]</sup> found that DLX2 expression was higher in breast and ovarian cancer tissues compared with the adjacent normal tissues. Furthermore, DLX2 expression was related to poor differentiation grade of ovarian cancer. DLX2 short hairpin RNA inhibited the metabolic stress-induced increase in propidium iodide-positive cell population and high mobility group box 1 and lactate dehydrogenase release. They concluded that DLX2 might be involved in tumor progression *via* the regulation of metabolic stress-induced necrosis.

In our research, high expression of DLX2 was detected in intestinal metaplasia, which is a risk factor for development of gastric cancer<sup>[24,25]</sup>, indicating that increased expression of DLX2 might contribute to an early event of gastric cancer development. In addition, we found that increased expression of DLX2 was detected in inflammatory cells around tumor cells. Recent studies have expanded the concept that inflammation is a critical component of tumor progression<sup>[26]</sup>. Moreover, the mediators and cellular effectors of inflammation are important constituents of the local environment of tumors<sup>[27]</sup>. These results further support the hypothesis that DLX2 is involved in the development of gastric adenocarcinoma.

In 2010, Morini *et al*<sup>[9]</sup> found that expression of DLX2 was detected in 21.6% of the patients with breast cancer, and was significantly correlated with prolonged disease-free survival and reduced incidence of relapse. DLX5 expression was detected in 2.2% of all cases, displaying reduced disease-free survival and high incidence

of relapse. In all cases, they found mutually exclusive expression of DLX2 and DLX5. Their study suggested that *DLX* genes were involved in human breast cancer progression, and that *DLX2* and *DLX5* genes might serve as prognostic markers. In our research, the Kaplan-Meier survival analysis revealed that the survival times of gastric adenocarcinoma patients with high DLX2 expression were significantly shorter than those with low DLX2 expression. However, the multivariate analysis showed that DLX2 expression was not an independent prognostic factor in gastric adenocarcinoma. The multivariate analysis might mask DLX2's contribution to survival rate. Therefore, DLX2 expression might not be related with poor prognosis in patients with gastric adenocarcinoma.

In conclusion, our study demonstrates that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development. These findings further support the hypothesis that, as a key regulator of embryogenesis, DLX2 may also play a critical role in tumor development. Consequently, further investigation is necessary to clarify the role of DLX2 in the development of gastric adenocarcinoma.

## COMMENTS

### Background

Gastric cancer is one of the leading causes of cancer-related death worldwide because of its frequency, poor prognosis and limited treatment options. Although the incidence of gastric cancer has been declining for several decades in most Western countries, it remains a crucial public health problem in developing countries. Several studies have demonstrated that various genetic and epigenetic alterations are involved in the course of carcinogenesis and progression of gastric cancer.

### Research frontiers

The distal-less homeobox (*DLX*) gene family exerts an important role in regulating embryonic development, tissue homeostasis, lymphocyte development, cell cycle and apoptosis. However, the role of the *DLX* gene family in tumor development has only recently been explored. As a member of *DLX* gene family, the abnormal expression of distal-less homeobox 2 (*DLX2*) has also been reported in many human solid tumors and hematological malignancies.

### Innovations and breakthroughs

This is the first study attempt to explore the expression of DLX2 in gastric adenocarcinoma and its clinicopathological significance. This study suggests that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development.

### Applications

By understanding the expression of DLX2 and its correlation with clinicopathological features of gastric adenocarcinoma, this study will form the basis for further research to explore the mechanism of tumor development, and may represent a potential therapeutic target of gastric adenocarcinoma.

### Terminology

Homeobox genes encode transcription factors that play essential roles in controlling cell growth and differentiation during embryonic development. These genes are characterized by a highly conserved 61-amino acid homeodomain that binds DNA elements containing a TAAT core motif. Many homeobox genes are aberrantly expressed in a wide variety of solid tumors and hematological malignancies.

### Peer review

The authors investigated the expression of DLX2 in gastric adenocarcinoma tissues and adjacent normal tissues by immunohistochemistry. Correlations of DLX2 expression with clinicopathological features and prognosis of patients with gastric adenocarcinoma were then analyzed. This study has shown that

increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development. These results are interesting and may represent a novel molecular mechanism of gastric adenocarcinoma development.

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**P- Reviewers** Nagahara H, Singh SR

**S- Editor** Wen LL **L- Editor** Stewart GJ **E- Editor** Lu YJ



## Effects of medical adhesives in prevention of complications after endoscopic submucosal dissection

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Supported by Shanghai Key Laboratory of Pediatric Digestion and Nutrition, No. 11DZ2260500 and NO. 2010009

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Received: September 21, 2012 Revised: January 8, 2013

Accepted: February 5, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To evaluate the use of medical adhesive spray in endoscopic submucosal dissection (ESD).

**METHODS:** Patients who underwent ESD between January 2009 and June 2012 ( $n = 173$ ) were enrolled in the prospective randomized study. Two patients undergoing surgery due to severe intraoperative hemorrhage and failed hemostasis were excluded, and the remaining 171 patients were randomly divided into two groups: group A (medical adhesive group,  $n = 89$ ) and group B (control group,  $n = 82$ ). In group A, a medical adhesive spray was evenly applied after routine electrocoagulation and hemostasis using hemostatic clip after ESD. Patients in group B only treated with routine wound management. Intraoperative and postoperative data were collected and compared.

**RESULTS:** In all 171 patients, ESD was successfully

completed. There was no significant difference in the average treatment time between groups A and B (59.4 min vs 55.0 min, respectively). The average length of hospital stay was significantly different between group A and B (8.89 d vs 9.90 d, respectively). The incidence of intraoperative perforation was 10.1% in group A and 9.8% in group B, and was not significantly different between the two groups. In all cases, perforations were successfully managed endoscopically and with conservative treatment. The incidence of postoperative delayed bleeding in group A was significantly lower than that in group B (0.00% vs 4.88%, respectively).

**CONCLUSION:** ESD is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer. Medical adhesive spray is effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

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**Key words:** Endoscopic submucosal dissection; Medical adhesive; Early-stage gastrointestinal cancer; Postoperative delayed bleeding; Intraoperative hemorrhage

**Core tip:** This is the first report to use medical adhesive after endoscopic submucosal dissection (ESD), and results were exciting. Application of medical adhesive spray can prevent complications of ESD, especially the delayed bleeding, consequently reducing the average length of hospital stay, and avoiding additional health care expenditures.

Zhang Y, Chen Y, Qu CY, Zhou M, Ni QW, Xu LM. Effects of medical adhesives in prevention of complications after endoscopic submucosal dissection. *World J Gastroenterol* 2013; 19(17): 2704-2708 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2704.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2704>

## INTRODUCTION

In endoscopic submucosal dissection (ESD), special instrument and other ancillary equipment are used to resect and strip gastrointestinal precancerous lesions and early-stage cancer on the basis of endoscopic mucosal resection (EMR). ESD has been widely performed in clinical practice due to its advantages of complete resection and reduced recurrence rates. However, compared to EMR, ESD is associated with a high incidence of complications such as perforation and bleeding, which has limited the utility of ESD. Currently, ESD is only performed at some institutions in China. Medical adhesives (spray type) have been widely used in surgery and for the treatment of gastric varices because of their adhesive, reinforcement, and leak proofing functions<sup>[1,2]</sup>. However, there has been no report of their use in gastrointestinal (GI) endoscopy. This study investigated the use of medical spray adhesives for the prevention of complications in patients undergoing ESD.

## MATERIALS AND METHODS

### Patients

Patients who underwent ESD at the department of digestive endoscopy of our hospital between January 2009 and June 2012 ( $n = 173$ ) were enrolled. Two patients undergoing surgery due to severe intraoperative hemorrhage and inability to achieve adequate hemostasis were excluded. The remaining 171 patients completed the ESD treatment and were included in the analysis. There were 75 males and 96 females with an average age of  $57.21 \pm 12.22$  years (range, 18-82 years). There were 37 cases of esophageal lesions, 110 cases of gastric lesions, and 24 cases of colorectal lesions diagnosed by routine preoperative endoscopy, endoscopic ultrasonography, and histopathological examination of biopsy specimens. There were 50 cases of mucosal or submucosal lesions (early-stage cancer or precancerous lesions) and 121 cases of muscularis propria lesions (stromal tumors). The pathological examination revealed 92 cases of leiomyoma, 61 cases of stromal tumor and 18 cases of early-stage cancer. The average size of lesions was  $4.42 \pm 1.28$  cm.

### Instruments and adhesives

An Olympus GIF-Q260J electronic gastroscope and CF-Q260 colonoscopy system were used. In addition, an NM-4L-1 injection needle, triangle-tip knife, FD-1U-1 hot biopsy forceps, snare, Poko hemostatic clip, HX-610-135 hemostatic clip, and ERBE ICC-200 high-frequency electric cutting device were also employed. The medical adhesive (Compont Medical Adhesive) is a spray-type adhesive and the primary ingredient is butyl  $\alpha$ -cyanoacrylate. During ESD, a transparent cap was added at the end of the lens and carbon dioxide insufflation was adopted.

### ESD

After conventional therapeutic steps, patients were ran-

domly divided into group A (medical adhesive group,  $n = 89$ ) and group B (control group,  $n = 82$ ) according to a computer-generated random number table. There was no significant difference in age, gender, or lesion type between the two groups (Table 1). Patients receiving anticoagulant drugs such as aspirin underwent ESD 5-7 d after drug withdrawal.

All patients underwent the following operations under intubation anesthesia or intravenous anesthesia: (1) Staining: During endoscopy, the lesions were identified and stained with methylene blue. After staining, the lesion boundary was obvious; (2) Marking: A needle knife or argon plasma coagulation (APC) was used to mark at the lesion edges; (3) Injection: An epinephrine/saline solution (1:10000) containing a small amount of methylene blue was injected submucosally at multiple places lateral to the marked points at the lesion edges; (4) Pre-cut: A needle knife was used to cut open the mucosa at the marked points at the lesion edges; (5) Cut: The TT knife was used to make a circular incision on the lesion edge along the marker; (6) Stripping: The TT knife was used to cut open the submucosa layer by layer, and the lesion was peeled off. For the muscularis propria lesions, the lesions were completely stripped from the nearby tissue after the lesion was exposed. In some cases, when the lesion was almost completely peeled off, the snare was used to trap the lesion root so that the lesion could be resected completely; and (7) Wound treatment: After resection of the lesion, small visible blood vessels were treated with argon plasma coagulation (APC) or hot biopsy forceps. The perforated wound surface was closed with metal hemostatic clips. In addition, medical adhesive was sprayed onto the wound surface *via* a spray catheter in group A patients.

Patients with severe intraoperative bleeding or perforation who were unable to undergo endoscopic treatment received surgical treatment. All the patients receiving ESD were maintained *non per os* (NPO) postoperatively and received a nasogastric tube and low flow suction. Antacids and necessary hemostatic drugs (Ethamsylate, PAMBA) were administered, together with the prophylactic antibiotics. Abdominal signs such as abdominal pain and distension were monitored closely. Endoscopy was repeated 1, 3, and 6 mo after surgery to examine wound healing, residual lesions, and recurrence.

The postoperative abdominal signs, recurrence, length of hospital stay, and incidence of complications including delayed bleeding, perforation and infection were compared between the two groups.

### Statistical analysis

Categorical data were analyzed using the  $\chi^2$  test, and quantitative data were analyzed using the *t* test. SPSS 10.0 statistical software was used for analysis and the significance level  $\alpha$  was set at 0.05.

## RESULTS

A total of 173 patients underwent ESD treatment. There

**Table 1 Patient clinical data**

|                         | All patients<br>(n = 171) | Group A<br>(medical<br>adhesive group)<br>(n = 89) | Group B<br>(control<br>group)<br>(n = 82) | P value |
|-------------------------|---------------------------|--|---|---------|
| Age <sup>1</sup> (yr)   | 57.21 ± 12.22             | 56.47 ± 13.02                                      | 58.01 ± 11.31                             | NS      |
| Gender<br>(male/female) | 75/96                     | 39/50  | 36/46                                     | NS      |
| Lesion location         |                           |  |   |         |
| Esophageal              | 37                        | 21   | 16  | NS      |
| Gastric                 | 110                       | 58   | 52  | NS      |
| Colorectal              | 24                        | 10   | 14  | NS      |
| Depth of lesion         |                           |  |   |         |
| Mucosa or<br>submucosa  | 50                        | 23   | 27  | NS      |
| Muscularis<br>propria   | 121                       | 66   | 55  | NS      |

<sup>1</sup>Data are expressed as mean ± SD. NS: Not significant.

were two patients with muscularis propria lesions with a diameter of 4 or 5 cm, respectively, protruding toward the abdominal cavity. During ESD, the bleeding was difficult to control and these two patients received surgical treatment. The lesions were completely stripped off in the remaining 171 patients. Patient clinical data are shown in Table 1.

The average duration of ESD (from submucosal injection to complete stripping of lesions) was 59.44 min (range, 36-150 min) in group A and 55.00 min (range, 35-140 min) in group B and the difference was not statistically significant. The average length of hospital stay was 8.89 d (range, 6-15 d) in group A and 9.90 d (range, 5-21 d) in group B and the difference was statistically significant. In group B, four patients experienced delayed bleeding and were hospitalized for 19, 19, 20, and 21 d, respectively. Excluding those patients, the average length of hospital stay in group B was 9.40 d (ranged, 5-15 d), which was not significantly different compared with group A (Table 2).

All the 171 patients treated with ESD, with the exception of two patients who were lost to follow-up after treatment, underwent repeat endoscopy 1, 3, 6, and 12 mo after treatment. To date, no recurrence has been noted.

During ESD, bleeding (< 40 mL) occurred in all patients, and hemostasis was successful after electrocoagulation, APC, and application of the hemostatic clip. No delayed bleeding occurred in group A (0.00%, 0/89), whereas delayed bleeding occurred in four patients in group B (4.88%, 4/82), which was significantly different from that in group A. In group B, one patient with an esophageal lesion, two patients with gastric lesions, and one patient with colon lesions experienced bleeding 3, 5, 10 and 7 d after surgery. One patient had symptoms of shock including progressive drop in blood pressure and cold sweats, and the vital signs became stable after transfusion and active medical treatment. Gastric endoscopy and colonoscopy revealed active bleeding at the location of the ESD. After norepinephrine saline flush, APC, ap-

**Table 2 Comparison of treatment duration and length of hospital stay**

|   | Group A<br>(medical<br>adhesive group) | Group B<br>(control<br>group) | P value |
|---|--|-------------------------------|---------|
| Average duration of ESD treatment (min)   | 59.44 ± 18.46                          | 55.00 ± 21.00                 | 0.143   |
| Average length of hospital stay (d)   | 8.89 ± 2.33                            | 9.90 ± 3.30                   | 0.021   |
| Average length of hospital stay when patients with delayed bleeding were excluded (d) | 8.89 ± 2.33                            | 9.40 ± 2.47                   | 0.172   |

Data are expressed as mean ± SD. ESD: Endoscopic submucosal dissection.

plication of hemostatic clips, and the endoscopic application of medical spray adhesive, hemostasis was successful. No patients underwent surgical intervention for the control of bleeding.

Perforation occurred in nine of 89 (10.1%) patients in group A. Of these patients, one had an esophageal lesion, seven had gastric lesions, and one patient had colon lesions. Perforation occurred in 8 (9.8%) of 82 patients in group B. Of these patients, two had esophageal lesions and six had gastric lesions. The incidence of perforation was not significantly different between the two groups. Among the nine patients who experienced perforation in group A, seven patients had muscularis propria lesions, one patient had ulcer scars, and the remaining patient had non-ulcer scar lesions. All of the eight patients who experienced perforation in group B had muscularis propria lesions, and none had ulcer scar formation or non-ulcer scar lesions. The diameter of perforation ranged from 0.2 to 2.0 cm, and the wound surface was clipped using hemostatic clips in all the cases. Abdominal paracentesis was performed to release gas in patients with obvious abdominal distension. All perforations resolved after fasting, placement of an indwelling nasogastric tube and gastrointestinal decompression in patients who received gastric procedures, absolute bed rest, and treatment with antibiotics. No patient underwent surgical treatment.

After ESD, 12 patients in group A and 10 patients in group B experienced varying degrees of abdominal distension and abdominal pain, and the incidence was 13.5% and 12.2%, respectively (*P* > 0.05). Eight of the 12 patients with abdominal distension and abdominal pain in group A experienced intraoperative perforation, and the remaining four patients had no intraoperative perforation. Six of the 10 patients with abdominal distension and abdominal pain in group B experienced intraoperative perforation, and the remaining four patients did not. The symptoms resolved in all patients within 1-3 d after conservative treatment including gastrointestinal decompression, fasting, antacids, and antibiotics. Anal pain during defecation occurred in two patients (2.25%) in group A 8-10 d after treatment, which was followed by the discharge of solid medical adhesive. A summary of complications is presented in Table 3.

Table 3 Complications

|                                      | Group A<br>(medical<br>adhesive group) | Group B<br>(control<br>group) | P value |
|--------------------------------------|--|-------------------------------|---------|
| Incidence of delayed bleeding        | 0.00%                                  | 4.88%                         | 0.035   |
| Incidence of perforation             | 10.10%                                 | 9.80%                         | 0.938   |
| Location of perforation (n)          |  |                               |         |
| Esophageal                           | 1                                      | 2                             |         |
| Gastric                              | 7                                      | 6                             |         |
| Colorectal                           | 1                                      | 0                             |         |
| Perforated lesions                   |  |                               |         |
| Muscularis propria                   | 7                                      | 8                             |         |
| Scar formation                       | 1                                      | 0                             |         |
| Non-scar forming lesion              | 1                                      | 0                             |         |
| Incidence of abdominal<br>distension | 13.50%                                 | 12.20%                        | 0.802   |
| With perforation                     | 8                                      | 6                             |         |
| Without perforation                  | 4                                      | 4                             |         |
| Difficulty excreting adhesive        | 2.25%                                  | 0.00%                         |         |

## DISCUSSION

ESD is a technique that uses special instruments and other equipment to resect and strip gastrointestinal precancerous lesions and early-stage tumors on the basis of EMR. ESD can be used for the one-time complete resection of lesions with a diameter greater than 2 cm, and the high *en bloc* resection rate can reduce residual lesions and the chances of recurrence, thus achieving a radical cure<sup>[3-6]</sup>. The indications for ESD are still controversial; however, some scholars believe that as long as there is no lymphatic and blood vessel invasion or metastasis, the lesion can be resected using ESD regardless of lesion location and size<sup>[7]</sup>. With improvement in the management of endoscopic complications, the depth of lesions treated with ESD has gradually increased and ESD has been used to treat some muscularis propria lesions and stromal tumors. Some authors have even proposed a concept of ESE<sup>[8,9]</sup>. However, the incidence of major complications with ESD, including bleeding and perforation, is still relatively high which has limited the generalization of ESD to a larger extent.

The main ingredient of the medical adhesive used in this study is butyl  $\alpha$ -cyanoacrylate. As an adhesive with special biomedical function, it has biomedical functions in addition to the common gluing function and mechanical function. It has been confirmed that butyl  $\alpha$ -cyanoacrylate is non-toxic to the human body and not mutagenic, teratogenic, or carcinogenic, and will not cause an irritant reaction. Medical adhesives are widely used in surgery, and can rapidly solidify in the presence of anionic substances such as tissue fluid and blood. The adhesive used has a similar strength as tissues, and therefore is not likely to fall off during extension and flexion movement. The adhesive strength is greater than the physiological strength of human body; therefore, its tissue adhesive function is reliable<sup>[1,2]</sup>.

Manner *et al*<sup>[10]</sup> reported an incidence of delayed bleeding after ESD of 6.5%, and Sugimoto *et al*<sup>[11]</sup> in a

multi-center study reported an incidence of 3.7%. In our study, the incidence of delayed bleeding in the control group was 4.88%, similar to that previously reported. However, no delayed bleeding occurred in the medical adhesive group, and this was significantly different from the incidence in the control group.

Ono *et al*<sup>[12]</sup> reported a perforation rate of 5% among 906 patients who were treated with ESD. Sugimoto *et al*<sup>[11]</sup> reported a perforation rate of 10.3% in patients with scar lesions and 3.5% in patients without scar lesions. In our study, the perforation rates in groups A and B were 10.1% and 9.8%, and the difference was not statistically different. The incidence of perforation in our study was slightly higher than that in the report by Ono *et al*<sup>[12]</sup>, but it was similar to the incidence of perforation in patients with scar lesions in the study by Sugimoto *et al*<sup>[11]</sup>. We analyzed the depth of the perforated lesions, and found that among the 17 cases of perforation in the two groups, there were 15 cases of muscularis propria lesions, one case of scar formation, and one case of a non-scar forming lesion. Therefore, the relatively high incidence of perforation in our study was due to relatively deep lesions. Generally, perforation was managed successfully with conservative treatment including fasting and antibiotics<sup>[13]</sup>.

As for other complications of ESD, 12 patients in group A (medical adhesive group) and 10 patients in group B (control group) experienced varying degrees of abdominal distension, and the incidence was 13.5% (12/89) and 12.2% (10/82), respectively, and was not different between the groups. Anal pain during defecation occurred in two patients in group A 8-10 d after treatment, which was followed by discharge of solid medical adhesive. Medical adhesive was not used in group B, so no difficulty in excreting the medical adhesive occurred.

The average duration of ESD in group A was 59.44 min (range, 36-150 min) and in group B was 55.00 min (range, 35-140 min) and the times were not statistically different. However, the average treatment time in group A was slightly more than that in group B, and we believe this is because of the extra time needed to apply the medical adhesive. Our results are consistent with a treatment time of 35-180 min reported by Sano *et al*<sup>[4]</sup>. With improvement of techniques and accumulation of experience, the operation time will be gradually shortened, while treating larger and deeper lesions may increase the operation time.

The average length of hospital stay was 8.89 d (range, 6-15 d) in group A and 9.90 d (range, 5-21 d) in group B, and the differences were statistically significant. In group B, four patients experienced delayed bleeding and were hospitalized for 19, 19, 20 and 21 d, respectively. Excluding those patients, the average length of hospital stay in group B was 9.40 d (ranged, 5-15 d), and this was not significantly different compared with group A, indicating that delayed bleeding may significantly increase the length of hospital stay.

In summary, application of spray-type medical adhe-

sive may cause difficulty in excreting the medical adhesive in a few patients, but it does not affect the overall therapeutic effects and prognosis. Use of a spray-type medical adhesive can prevent complications of ESD, especially delayed bleeding. Application of a spray-type medical adhesive can reduce the average length of hospital stay, thereby avoiding additional health care expenditures.

## COMMENTS

### Background

Endoscopic submucosal dissection (ESD) is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer. Medical adhesive spray can be effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

### Research frontiers

ESD is associated with a high incidence of complications such as perforation and bleeding, which has limited the utility of ESD. Medical adhesives (spray type) have been widely adopted in surgery and for the treatment of gastric varices because of their adhesive, reinforcement, and leak proofing functions.

### Innovations and breakthroughs

This is the first report to use medical adhesive after ESD, and results were exciting. Use of a spray-type medical adhesive can prevent complications of ESD, especially delayed bleeding. Application of a spray-type medical adhesive can reduce the average length of hospital stay, thereby avoiding additional health care expenditures.

### Applications

Medical adhesive spray can be effective in preventing delayed bleeding after ESD, and can thus reduce the average length of hospital stay.

### Terminology

ESD is an effective minimally invasive treatment for gastrointestinal precancerous lesions or early-stage gastrointestinal cancer.

### Peer review

This is a good prospective randomized study in which authors analyzed the effect of medical adhesive spray in preventing delayed bleeding after ESD. The results are interesting and suggest that the use of medical adhesive spray may be a useful therapeutic approach in the prevention of delayed bleeding after ESD.

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P- Reviewer Cerwenka HR S- Editor Huang XZ  
L- Editor Ma JY E- Editor Zhang DN



## Difference in *DRB1*\* gene polymorphisms between Han and Uyghur ulcerative colitis patients in China

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Supported by Natural Science Foundation of Xinjiang Uyghur Autonomous Region of China, No. 2009211A26

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Received: November 18, 2012 Revised: February 28, 2013

Accepted: March 6, 2013

Published online: May 7, 2013

### Abstract

**AIM:** To evaluate the association between *HLA-DRB1* alleles and Han and Uyghur ulcerative colitis (UC) patients residing in the Xinjiang Uyghur Autonomous Region of China.

**METHODS:** In this study, 102 UC patients (53 Han including 22 men and 31 women, and 49 Uyghur patients including 25 men and 24 women; aged  $48.07 \pm 15.83$  years) and 310 age- and sex-matched healthy controls were enrolled in the Department of Gastroenterology, Xinjiang People's Hospital of China from January 2010 to May 2011. UC was diagnosed based on the clinical, endoscopic and histological findings following Lennard-Jones criteria. Blood samples were collected and genomic DNA was extracted by routine laboratory methods, and both polymerase chain reaction and gene sequencing were used to identify *HLA-DRB1* allele variants. The potential association between genetic varia-

tion and UC in Han and Uyghur patients was examined. There were no statistical differences in *HLA-DRB1* allele frequencies in Han UC patients.

**RESULTS:** There was no significant difference in the sex ratio between the controls and UC patients ( $P = 0.740$ ). In Han patients with UC ( $n = 53$ ), *HLA-DRB1*\*03, \*13 allele frequencies were lower than in healthy controls ( $n = 161$ ), but not statistically significant, and *HLA-DRB1*\*04\*11\*14 allele frequencies were higher than in healthy controls, but without statistical significance. Differences between Uyghur UC patients and the control group were observed for *HLA-DRB1*\*04 and *HLA-DRB1*\*13, both showed a greater frequency in UC patients (10.21% vs 2.69%,  $P = 0.043$ ; 14.29% vs 4.03%,  $P = 0.019$ ). *HLA-DRB1*\*14 also showed a greater frequency in UC patients (14.29% vs 2.69%,  $P = 0.006$ ). The frequencies of *DRB1*\*04, \*13\*14 alleles were increased in Uyghur UC patients compared with normal controls. The frequency of *DRB1*\*08 was decreased in Uyghur UC patients compared with normal controls. *HLA-DRB1* alleles showed no association with UC in Han patients. There were no statistical differences in *HLA-DRB1* allele frequencies in Han UC patients. The frequencies of *DRB1*\*04, \*13\*14 alleles were increased in Uyghur UC patients compared with normal controls. The frequency of *DRB1*\*08 was decreased in Uyghur UC patients compared with normal controls. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in China.

**CONCLUSION:** *HLA-DRB1*\*04\*13\*14 and *DRB1*\*08 may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients.

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**Key words:** Ulcerative colitis; *DRB1*\* gene polymorphisms; Han and Uyghur

**Core tip:** This study evaluated the association between

*HLA-DRB1* alleles and Han and Uyghur ulcerative colitis (UC) patients residing in the Xinjiang Uyghur Autonomous Region of China. The authors found that polymorphism of the *HLA-DRB1*\* gene differed between the Han and Uyghur patients with UC. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in North-West China.

Aheman A, Gao F, Kuerbanjiang A, Li YX, Abuduhadeer M. Difference in *DRB1*\* gene polymorphisms between Han and Uyghur ulcerative colitis patients in China. *World J Gastroenterol* 2013; 19(17): 2709-2713 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2709.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2709>

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease are often grouped together as inflammatory bowel disease (IBD). IBD is the term used to describe idiopathic disorders associated with chronic inflammation of the gastrointestinal tract<sup>[1,2]</sup>. Clinical features common to both disorders include abdominal pain, diarrhea, weight loss, and increased risk of developing colorectal cancer<sup>[3,4]</sup>. The etiology of UC is still not known. However, underlying genetic, environmental, and lifestyle issues can affect an individual's predisposition to these diseases<sup>[5,6]</sup>. Genetic factors involved in the regulation of the immune system are thought to play a significant role in the pathogenesis of UC<sup>[7]</sup>. Human leukocyte antigens (HLA), located on chromosome 6, play an important role in the immune response and several immune-mediated diseases. Several studies have shown that HLA alleles are associated with UC<sup>[8]</sup>. We previously compared the clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China. We showed some differences between the Uyghur and Han populations living in the same region, which included a higher prevalence of UC, a younger age of onset, an increased prevalence of the chronic persistent and acute outbreak type, more moderate and severe forms, a higher complication rate, and an increased frequency of positive antineutrophilic cytoplasmic antibodies (ANCA) in the Uyghur population<sup>[9]</sup>. However, to date, hardly a research shows that there is association between *HLA-DRB1* alleles and UC in the Uyghur population. It would be interesting to know what causes the clinical heterogeneity of UC between Han and Uyghur UC patients in China.

## MATERIALS AND METHODS

### Patients and controls

Consecutive patients with UC were recruited from the Department of Gastroenterology, Xinjiang People's Hospital of China. The diagnosis of UC was made based on clinical, endoscopic, and histological findings in accordance with Lennard-Jones criteria<sup>[10]</sup>. The extent of

**Table 1** The 5' amplification primer mix included the following primers

|                                   |
|-----------------------------------|
| 5'-CCACAGCACGTTTCTTGGAGTACTCTA-3' |
| 5'-CCAGTTTCTTGTGGCAGCTTAAGTT-3'   |
| 5'-TCGTTCTGTGGCAGGGTAAGTATA-3'    |
| 5'-AGCCGTTTCTGAAGCAGGATAAGTT-3'   |
| 5'-CCAAGCACGTTTCTTGGAGGAGG-3'     |
| 5'-TCGTTCTGTGGCAGCTAAGA-3'        |
| 5'-AGCCGTTTCTTGGAGCAGGTTAAAC-3'   |

disease was assessed by colonoscopy at initial diagnosis and at follow-up. Extensive colitis was defined as lesions located beyond the splenic flexure. Distal colitis was defined as lesions limited to the region distal to the splenic flexure<sup>[11,12]</sup>. Age- and sex-matched healthy controls were also recruited from the Health Examination Center of Xinjiang People's Hospital. The study protocol was approved by the Ethics Committee of Xinjiang Uyghur Autonomous Region of China, and individuals selected from the populations sampled were Chinese and Uyghur UC patients from Urumqi, Xinjiang. Informed consent was obtained from all patients.

Genomic DNA was isolated using a QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (Qiagen, Germany). DNA samples were quantified by *ultraviolet* measurements at  $A_{260}$ .

### Polymerase chain reaction amplification

Polymerase chain reaction (PCR) amplifications were performed in a volume of 20  $\mu$ L consisting of 20 ng of genomic DNA, PCR buffer (1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L Tris-HCl pH 8.4, 50 mmol/L KCl, 0.1 mg/mL of Gelatin, 0.02% of NP-40), 200 mmol/L of each dNTP (Life Technologies, Rockville, MD, United States) and 1 unit of Taq Platinum polymerase (Life Technologies, Grand Island, NY, United States). Seven sense primers and 1 antisense primer were used to amplify *HLA-DRB1* alleles (Table 1).

The 3' amplification primer was 5'-CTGTTACCTC-GCCACTGCAC-3'. PCR amplification was performed in an ABI 9700. The DNA was amplified following initial denaturation at 95 °C for 120 s followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s. PCR amplification was confirmed following electrophoresis on a 2.0% agarose gel. Samples were electrophoresed at 10 V/cm for 36 min. PCR products were purified using the High Pure PCR Purification Kit (Roche Diagnostics, Basel, Switzerland).

### DNA sequencing and analysis

PCR products were sequenced using BigDye Terminator v3.1 kits (ABI, United States) and automated ABI 3730XL DNA sequencers. The 5' sequence primer was 5'-TTGCAATTCTTCAATGGGAC-3' and the 3' primer was 5'-ACCACCCGGTAGTTGTGTC-3'. The DNA was sequenced following initial denaturation at 95 °C for 120 s followed by 25 cycles at 95 °C for 10 s, 50 °C for 5 s and 60 °C for 60 s. The sequence within the 5' primer sites was included in the analysis to prevent the mistyping of

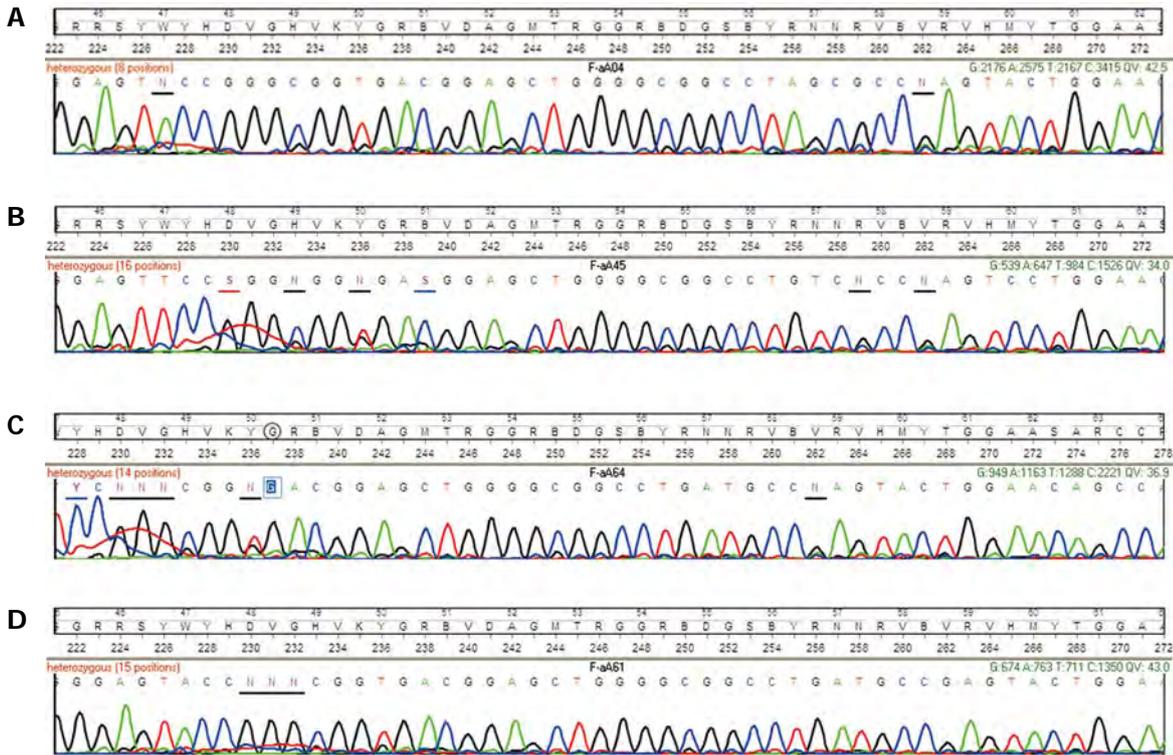


Figure 1 DRB1 genotyping was assigned using Qiagen SBTengine human leukocyte antigens typing software that compares an unknown sequence against a library of allele sequences. A: HLADRB1\*08 in a Han patient; B: HLADRB1\*12 in a Uyghur patient; C: HLADRB1\*13 in a Han patient; D: HLADRB1\*14 in a Uyghur patient.

| Characteristics                | UC patients   | Controls      | P value |
|--------------------------------|---------------|---------------|---------|
| Total number (n)               | 102           | 310           |         |
| Han                            | 53            | 161           | 0.998   |
| Uyghur                         | 49            | 149           | 0.998   |
| Gender, M/F                    | 47/55         | 137/173       | 0.740   |
| Age of onset <sup>1</sup> , yr | 48.07 ± 15.83 | 47.37 ± 14.49 | 0.801   |
| Extensive colitis (Han)        | 20 (38)       |               |         |
| Extensive colitis (Uyghur)     | 30 (61)       |               |         |
| Distal colitis (Han)           | 33 (62)       |               |         |
| Mild and intermediate          | 47 (89)       |               |         |
| Severe                         | 6 (11)        |               |         |
| Distal colitis (Uyghur)        | 19 (39)       |               |         |
| Mild and intermediate          | 40 (82)       |               |         |
| Severe                         | 9 (18)        |               |         |

<sup>1</sup>Data are expressed as mean ± SD. UC: Ulcerative colitis.

novel alleles that may have arisen due to recombination within the first variable region. DRB1 genotyping was assigned using Qiagen SBTengine HLA Typing software that compares an unknown sequence against a library of allele sequences (Figure 1).

**Statistical analysis**

Hardy-Weinberg disequilibrium was assessed with  $\chi^2$  tests and clinical records including age were analyzed with *t* tests.  $\chi^2$  tests were used to compare genotype and allele frequencies between patients and normal controls. Fisher exact tests with 95%CI were used when the number of samples was less than 5. *P* values less than 0.05 were con-

sidered statistically significant.

**RESULTS**

**Clinical characteristics of UC patients**

DNA was obtained from 102 consecutive UC patients and 310 healthy individuals well matched for sex and age. The main clinical characteristics of the UC patients are summarized in Table 2. UC patients included 47 males and 55 females and the controls included 137 males and 173 females. The average age of UC patients and controls was 48.07 ± 15.83 years and 47.37 ± 14.49 years, respectively. There was no significant difference in the sex ratio between the controls and UC patients (*P* = 0.740, Table 2).

**Comparison of DRB1\*genotype variants between UC patients and healthy controls in the Han population**

The HLA-DRB1 allele frequencies in the patients and controls are shown in Table 3. In Han patients with UC (*n* = 53), HLA-DRB1\*03, \*13 allele frequencies were lower than in healthy controls (*n* = 161), but not statistically significant (*P* > 0.05), and HLA-DRB1\*04\*11\*14 allele frequencies were higher than in the healthy controls, but without statistical significance (*P* > 0.05) (Figure 2).

**Comparison of DRB1\*genotype variants between the UC patients and healthy controls in the Uyghur population**

Differences between Uyghur UC patients and the control group were observed for HLA-DRB1\*04 and HLA-

**Table 3** Genotypic association between *DRB1*\* variants and ulcerative colitis in the Han and Uyghur population

|                     | Han UC (n = 53) |                      | Controls (n = 161) |                      | $\chi^2$ | P value | Uyghur UC (n = 49) |                      | Controls (n = 149) |                      | $\chi^2$ | P value |
|---------------------|-----------------|----------------------|--------------------|----------------------|----------|---------|--------------------|----------------------|--------------------|----------------------|----------|---------|
|                     | n (%)           | Genotype frequencies | n (%)              | Genotype frequencies |          |         | n (%)              | Genotype frequencies | n (%)              | Genotype frequencies |          |         |
| <i>HLA-DRB1</i> *01 | 0               | 0                    | 0                  | 0                    |          |         | 1 (2.04)           | 0.010                | 0                  | 0                    | 3.056    | 0.247   |
| <i>HLA-DRB1</i> *03 | 6 (11.32)       | 0.058                | 36 (22.36)         | 0.119                | 3.081    | 0.110   | 8 (16.33)          | 0.085                | 30 (20.13)         | 0.106                | 0.345    | 0.678   |
| <i>HLA-DRB1</i> *04 | 4 (7.55)        | 0.039                | 4 (2.49)           | 0.013                | 2.840    | 0.106   | 5 (10.21)          | 0.052                | 4 (2.69)           | 0.014                | 4.805    | 0.043   |
| <i>HLA-DRB1</i> *08 | 3 (5.66)        | 0.029                | 9 (5.59)           | 0.028                | 0.000    | 1.000   | 0                  | 0                    | 15 (10.07)         | 0.052                | 5.337    | 0.024   |
| <i>HLA-DRB1</i> *11 | 11 (20.76)      | 0.110                | 18 (11.18)         | 0.058                | 3.120    | 0.103   | 8 (16.33)          | 0.085                | 39 (26.18)         | 0.141                | 1.975    | 0.180   |
| <i>HLA-DRB1</i> *12 | 22 (41.51)      | 0.235                | 78 (48.45)         | 0.282                | 0.771    | 0.429   | 13 (26.53)         | 0.143                | 51 (34.23)         | 0.189                | 0.999    | 0.380   |
| <i>HLA-DRB1</i> *13 | 1 (1.89)        | 0.009                | 9 (5.59)           | 0.028                | 1.228    | 0.457   | 7 (14.29)          | 0.074                | 6 (4.03)           | 0.022                | 6.326    | 0.019   |
| <i>HLA-DRB1</i> *14 | 6 (11.32)       | 0.058                | 7 (4.35)           | 0.023                | 3.398    | 0.093   | 7 (14.29)          | 0.074                | 4 (2.69)           | 0.014                | 9.458    | 0.006   |

UC: Ulcerative colitis.

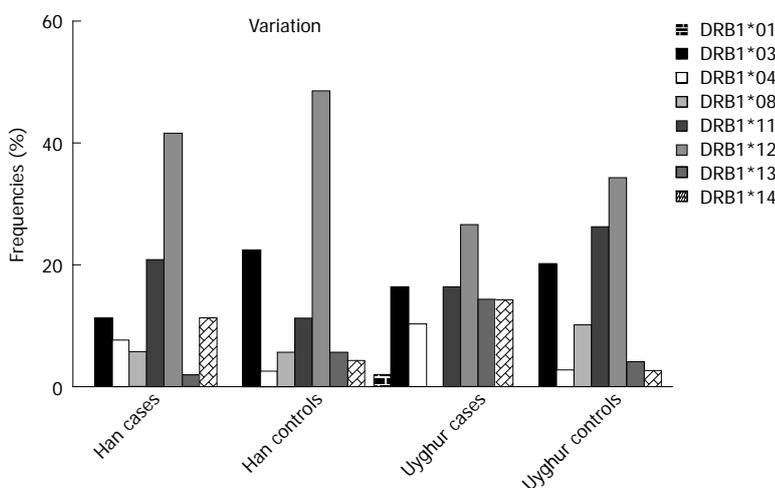


Figure 2 Comparison of the *DRB1*\* genotype variants between ulcerative.

*DRB1*\*13, both showed a greater frequency in UC patients (10.21% vs 2.69%,  $P = 0.043$ ; 14.29% vs 4.03%,  $P = 0.019$ ). *HLA-DRB1*\*14 also showed a greater frequency in UC patients (14.29% vs 2.69%,  $P = 0.006$ ), however, *HLA-DRB1*\*08 showed a lower frequency in UC patients than in the controls (0% vs 10.07%,  $P = 0.024$ ), Table 3.

## DISCUSSION

UC was previously uncommon in China, however, in the last 10 years more cases have been identified and the incidence of this disease has increased<sup>[13]</sup>. The clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China were shown to be different<sup>[14]</sup>. The genetic factors possibly associated with UC and its clinical characteristics in these two ethnic groups are unknown. A number of *HLA* alleles have been shown to be associated with variations in immune response diseases (e.g., celiac disease<sup>[15]</sup>, longitudinally extensive transverse myelitis<sup>[16]</sup>, and Behçet's disease<sup>[17]</sup>). Several genome-wide scans have shown that the susceptibility locus of UC and Crohn's disease is on chromosome 16q (IBD1)<sup>[18]</sup>. In the present study, we evaluated the association between the *HLA-DRB1* alleles and UC in Han and Uyghur populations from north-west China. We did

not find any association between *HLA-DRB1* alleles and UC in the Han population, and these results were consistent with previous Chinese studies. Lü *et al*<sup>[19]</sup> showed that polymorphism of the *HLA-DRB1* gene did not have a strong association with UC in Chinese patients. Lee *et al*<sup>[20]</sup> found that *HLA-DQA1c*, but not *HLA-DRB1* alleles, was associated with ANCA-positive UC in southern China.

We found differences between Uyghur UC patients and healthy controls, in whom *HLA-DRB1*\*04, *HLA-DRB1*\*13 and *HLA-DRB1*\*14 showed a greater frequency in UC patients than in controls. These results were consistent with previous Japanese studies<sup>[21]</sup>. In contrast to previous Chinese studies *HLA-DRB1*\*08 showed a lower frequency in UC patients than in controls<sup>[22]</sup>. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in China. The results in the present study were different probably due to the statistical methods used and the genetic heterogeneity of the ethnic populations. In future studies, we will continue to explore other *HLA* genes and amplify our sample size to identify the genes associated with UC in different ethnic groups in China and to provide more evidence for the genetic susceptibility of UC.

## COMMENTS

**Background**

Previous studies have shown that human leukocyte antigens (HLA) alleles are associated with ulcerative colitis (UC). The clinical characteristics of UC in the Han and Uyghur populations residing in the Xinjiang Uyghur Autonomous Region of China were shown to be different. In this study, the authors examined whether polymorphism of the *HLA-DRB1\** gene differed between Han and Uyghur patients with UC.

**Research frontiers**

Studies have found that disease distribution and phenotypic appearance differ significantly between ethnic groups and even within populations. The genetic and clinical heterogeneity of UC between the two ethnic groups, Chinese Han and Uyghur, were investigated.

**Innovations and breakthroughs**

This study found that polymorphism of the *HLA-DRB1\** gene differed between Han and Uyghur patients with UC. Polymorphism of the *HLA-DRB1* gene may contribute to the clinical heterogeneity of UC between Han and Uyghur UC patients in north-west China.

**Applications**

The effects of different genotypes in normal controls and patients with UC are still unknown and likely represent a potentially productive area for future research to better understand the pathogenesis and treatment of UC.

**Terminology**

UC is a form of *inflammatory bowel disease*. It is a refractory, chronic, and non-specific disease which usually occurs in the rectum and the entire colon. HLA, located on chromosome 6, play an important role in the immune response and several immune-mediated diseases.

**Peer review**

The authors investigated the contribution of *DRB1\** gene polymorphism to the genetic susceptibility and clinical heterogeneity of UC between Han and Uyghur patients. This manuscript contains potentially interesting findings.

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P-Reviewer Stemmler MP S-Editor Gou SX L-Editor A  
E-Editor Zhang DN



## Isolated arterioportal fistula presenting with variceal hemorrhage

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Received: September 15, 2012 Revised: December 26, 2012

Accepted: January 11, 2013

Published online: May 7, 2013

### Abstract

We report a case of life-threatening hematemesis due to portal hypertension caused by an isolated arterioportal fistula (APF). Intrahepatic APFs are extremely rare and are a cause of presinusoidal portal hypertension. Etiologies for APFs are comprised of precipitating trauma, malignancy, and hereditary hemorrhagic telangiectasia, but these were not the case in our patient. Idiopathic APFs are usually due to congenital vascular abnormalities and thus usually present in the pediatric

setting. This is one of the first cases of adult-onset isolated APF who presented with portal hypertension and was successfully managed through endoscopic hemostasis and subsequent interventional radiological embolization.

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**Key words:** Arterioportal fistula; Pre-sinusoidal portal hypertension; Hepatic vein pressure gradient; Hepatic artery embolization

Nookala A, Saberi B, Ter-Oganesyan R, Kanel G, Duong P, Saito T. Isolated arterioportal fistula presenting with variceal hemorrhage. *World J Gastroenterol* 2013; 19(17): 2714-2717 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2714.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2714>

### INTRODUCTION

Portal hypertension is defined as the increase in porto-systemic resistance and/or flow<sup>[1]</sup>. The causes of portal hypertension can be divided into pre-, intra-, and posthepatic causes. Moreover, intrahepatic portal hypertension can be further categorized into three groups: pre-, intra-, and postsinusoidal causes. The most common etiology of portal hypertension is due to liver cirrhosis which accounts for 90% of the cases in the United States<sup>[2]</sup>. It is a cause of intrasinusoidal portal hypertension due to distortion of the hepatic lobular architecture and results in hyperdynamic splanchnic circulation. The second major cause of portal hypertension is due to extrahepatic portal vein thrombosis, accounting for 7% of cases. The remaining 3% of causes of portal hypertension encompasses a variety of rare etiologies, including our case of intrahepatic presinusoidal portal hypertension<sup>[2]</sup>. While infrequent, the causes for presinusoidal portal hypertension comprise of schistosomiasis, myeloproliferative diseases, sarcoidosis, hepatic portal fibrosis, primary biliary cirrho-

sis, arsenic toxicity, idiopathic portal hypertension<sup>[3]</sup>.

## CASE REPORT

The patient is a 30 year old male who presented to Los Angeles County-University of Southern California Medical Center with a two day history of hematemesis. The physical examination on arrival showed vital signs of a blood pressure of 93/61 mmHg, a heart rate of 135 beats per minute with exam findings significant for splenomegaly and left femoral artery bruit, but otherwise negative for shifting dullness, spider angiomas, palmar erythema, asterix, and jaundice. Pertinent laboratory values were as follows: alkaline phosphatase 175 units/L, total protein 6.3 g/dL, albumin 3.9 g/dL, total bilirubin 0.6 mg/dL, aspartate aminotransferase 39 units/L, alanine transaminase 72 units/L, prothrombin 14.5 s, INR 1.16, hemoglobin 13.5 g/dL, white blood cells 8.0 k/cumm, and a platelet count of  $79 \times 10^3$ /cumm. Further testing was done and showed all viral hepatitis, autoimmune, and metabolic liver disease markers to be negative. No history of alcohol intake and no history of prescribed, over the counter or supplement medications. His family history was negative for liver disease.

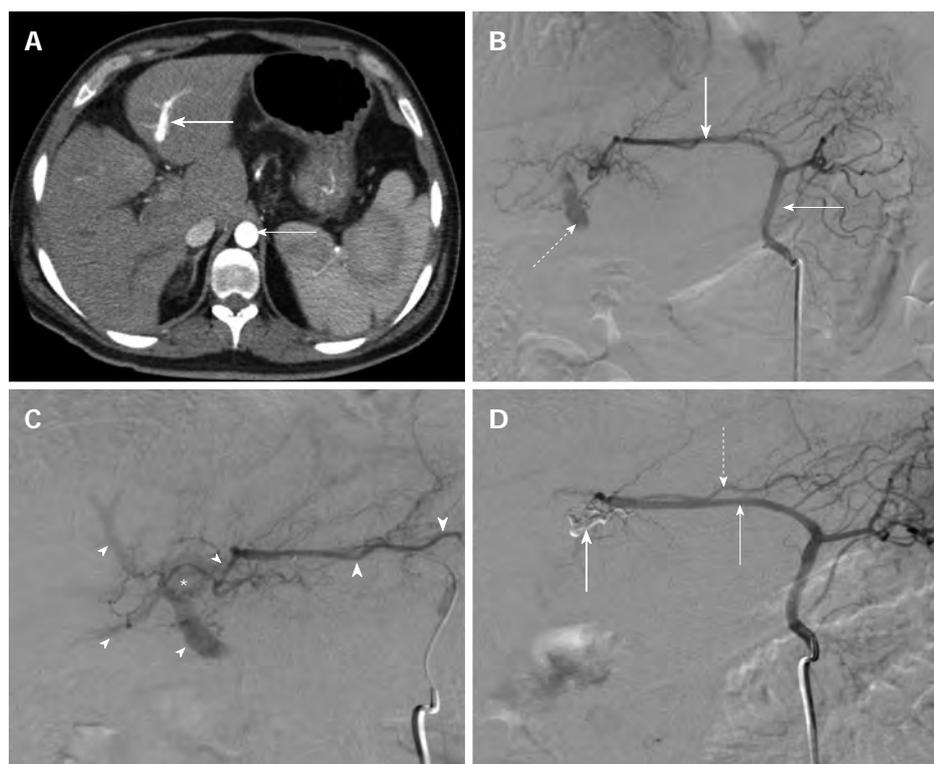
Upon presentation, the patient underwent an emergent esophagogastroduodenoscopy, which showed four columns of esophageal varices with active bleeding. Band ligation of the varices was performed successfully. Subsequent abdominal ultrasound demonstrated splenomegaly (length 14.8 cm) but a normal size, smooth liver surface and homogeneous liver parenchyma, suggesting a non-cirrhotic etiology for the portal hypertension. A multi-phase computed tomography (CT) examination of the liver demonstrated the left portal vein was highlighted at the arterial phase, strongly suggesting the existence of a hepatic arteriportal communication (Figure 1A). Based upon this finding, it was speculated that the overflowing of the portal vein due to the shunt between the hepatic artery to the portal vein was causing presinusoidal portal hypertension. In order to further confirm this, a hepatic venous pressure gradient (HVPG) measurement was performed and indicated normal value; 2 mmHg (< 5 mmHg is normal). Along with the signatures of portal hypertension-variceal bleeding and splenomegaly-the normal HVPG was consistent with pre-sinusoidal portal hypertension. To further confirm this, an angiogram was performed. A brisk contrast opacification of the left portal vein was noted upon contrast injection into the left hepatic artery, which branched from left gastric artery, confirming arteriportal shunting (Figure 1B). The shunting was determined not to be direct, but passing through a network of small capillary-like “fuzz” prior to brisk drainage into the portal system (Figure 1C). Furthermore, angiography showed the right superficial femoral artery with pseudoaneurysm and fistula and this additional vascular abnormality support our diagnosis of arteriportal fistulas (APFs) of congenital etiology. Taken all together, it was concluded that the arteriportal fistula is the cause

of this patient’s non-cirrhotic portal hypertension. The arteriportal and femoral artery fistulas were also closed with catheter directed embolization utilizing the liquid embolic agent Onyx<sup>®</sup> (ev3 Endovascular Inc., Plymouth, MN, United States). Following the embolization, no further early portal vein enhancement was seen during left hepatic arteriogram (Figure 1D). Finally, a liver biopsy was performed showing focal portal venule dilatation with dilated outflow vessels, but was otherwise normal, compatible with the clinical diagnosis of presinusoidal portal hypertension secondary to the hepatic artery and portal vein shunt (Figure 2). Post-embolization recovery of the patient has been uneventful, and no additional episodes of upper gastrointestinal bleeding have been reported by the patient on two subsequent clinic visits during the last four months.

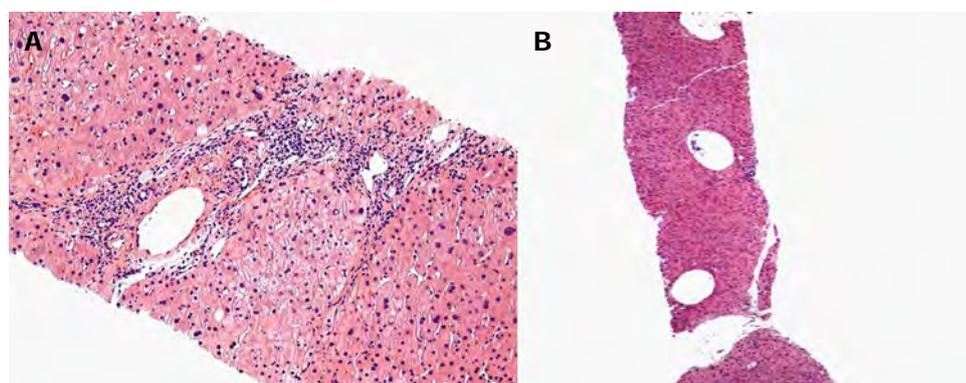
## DISCUSSION

HVPG measurements often provide valuable information in identification of the site or cause of the portal hypertension<sup>[4]</sup>. The HVPG is the difference between the wedged and free hepatic venous pressures, which has been shown to correlate well with actual portal vein pressure<sup>[5]</sup>. Its increase is consistent with sinusoidal portal hypertension and is known to be a predictor of development of varices and ascites<sup>[6]</sup>. Upon visualization of the hepatic artery and portal vein communication through CT, the measurement of HVPG was conducted. The classic pattern of the prehepatic or intrahepatic presinusoidal portal hypertension shows normal HVPG and free hepatic vein pressure (FHVP) along with the stigmata of portal hypertension. The pathophysiology consists of a presinusoidal block preventing the transmission of the elevated portal pressure to the sinusoid, thus resulting in a normal wedged hepatic vein pressure<sup>[5]</sup>. In our case, the HVPG value was consistent to the pathophysiology of presinusoidal portal hypertension.

Most of the literature regarding APFs is focused on the following etiologies: precipitating trauma, hepatocellular carcinoma (HCC), congenital, and in the context of hereditary hemorrhagic telangiectasia (HHT)<sup>[7]</sup>. Clinical presentation of APFs varies from asymptomatic to symptoms related to congestive heart failure (40%-60%), portal hypertension (20%-40%), and diarrhea with abdominal pain secondary to a “steal” phenomenon (20%)<sup>[8]</sup>. Hepatic trauma is a common cause for APFs. It may be from penetrating trauma such as gunshot wounds, a complication of liver transplantation or from previous liver biopsies<sup>[9,10]</sup>. HCC can cause APFs and two studies have shown that APFs may occur in up to 60% of patients with HCC<sup>[10,11]</sup>. Congenital APFs are associated with portal hypertension, failure to thrive, and gastrointestinal hemorrhage in infancy or early childhood<sup>[7,12]</sup>. In general, it is noted that less than 10% of all APFs are congenital<sup>[13]</sup>. As for HHT, hepatic involvement has been reported in 8%-78% of cases in retrospective and prospective studies<sup>[14]</sup>. Our patient did not have the any his-



**Figure 1** Intrahepatic communication between hepatic artery and portal vein. A: Contrast enhanced axial computed tomography of the abdomen demonstrating similar contrast opacification of the aorta (thin arrow) and the left portal vein (thick arrow); B: Left gastric arteriogram demonstrates gastrohepatic trunk (thin arrow) giving rise to aberrant left hepatic artery (thick arrow). Note early opacification of the portal vein (dashed arrow); C: Selective arteriogram with coaxial microcatheter in the left hepatic artery demonstrates medial branch of the left hepatic artery (large arrowheads) contributing to a parenchymal blush (star) and leading to opacification of the portal veins (small arrowheads). Note the normal appearance of the lateral branch of the left hepatic artery (thick arrow); D: Post-embolization arteriogram in the gastrohepatic trunk demonstrates opacification of the left hepatic artery (thin arrow). Onyx cast (thick arrow) is seen occupying the previously seen medial branch of the left hepatic artery. No further opacification of the portal veins is seen. Note the preserved lateral branch of the left hepatic artery (dashed arrow).



**Figure 2** The hepatic histology of arterioportal fistula. A: This low power image shows a small portal tract in the center of the field that has a markedly dilated portal venule. The terminal hepatic (central) venules above and below the portal tract are markedly dilated; B: The portal venule is slightly dilated and there are increased numbers of small portal venous radicals.

tory suggestive of congenital APF or signatures of HHT. Furthermore, he had no abdominal trauma or surgery, malignancy and no previous liver biopsies. Moreover the incidental finding of the femoral arteriovenous fistula and unique variation of hepatic artery further imply the congenital etiology. Therefore, we believe that this is a case of adult onset congenital APF that likely did not present in infancy or childhood because his fistula is through a capillary connection and probably took multiple decades

to develop clinical manifestation. Based our intensive literature search, this is one of the first cases reported to be adult onset portal hypertension due to congenital APFs.

Treatment of the APFs through shunt reduction is either surgical or minimally invasive though interventional radiology (IR) techniques. In the past, surgical ligation of the supplying artery was performed, however with the advancement of interventional radiology techniques, the trend has now shifted to endovascular catheter directed

therapy<sup>[15]</sup>. IR directed therapy offers many advantages over the conventional surgical treatment and is now the preferred technique for treatment. These advantages include decreased morbidity and mortality, reduced risk of subsequent complications, and significant reduction in the time required for recovery. IR directed therapy is accomplished through hepatic artery embolization (HAE)<sup>[16]</sup>. Embolization is usually performed with metal coils, detachable balloons, or gelfoam<sup>[17,18]</sup>. However at selected centers such as ours, liquid embolic agents are also utilized. Types of liquid embolic agents include ethylene vinyl alcohol (Onyx<sup>®</sup>) and *N*-butyl cyanoacrylate, both of which are predominantly used in congenital APFs per the literature<sup>[17,18]</sup>. HAE complications do occur, and include non-target embolization, hepatic infarction, and ischemic cholangitis. In a series of 15 patients studied by Chavan *et al*<sup>[19]</sup> ischemic cholangitis and/or cholecystitis occurred in three patients and one patient died of hepatic necrosis leading to multi-organ failure. Nevertheless, due to the dual blood supply of the liver (hepatic artery and portal vein), infarctions are rare<sup>[16]</sup>. In our limited experience with IR therapy of hepatic APFs, no such complications have occurred.

In summary, we described a case of lethal variceal bleeding due to an adult onset congenital APF. The patient was successfully treated through radiological interventional therapy and is being monitored regularly in our outpatient clinic without any further episodes of bleeding.

## ACKNOWLEDGMENTS

We would like to acknowledge Dr. Neil Kaplowitz for critical review of the manuscript.

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**P- Reviewers** Diamantis I, Grattagliano I **S- Editor** Huang XZ  
**L- Editor** A **E- Editor** Zhang DN



## Therapeutic efficacy of the Qing Dai in patients with intractable ulcerative colitis

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Received: December 29, 2012 Revised: January 28, 2013

Accepted: February 28, 2013

Published online: May 7, 2013

### Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease that may become intractable when treated with conventional medications such as aminosalicylates, corticosteroids, and azathioprine. The herbal medicine Qing Dai has traditionally been used in Chinese medicine to treat UC patients, but there is a lack of published data on the efficacy of Qing Dai in UC treatment. We report several cases of patients with intractable UC who take Qing Dai in a retrospective observational study. Furthermore, we explore the mechanisms of action of Qing Dai. Nine patients with active UC who received conventional medications but wished to receive Qing Dai as an alternative medication were included in our analysis. The UC severity level was determined based on the clinical activity index (CAI). Additionally, 5 of the 9 patients were endoscopically evaluated according to the Matts grading system. Each patient received

2 g/d of Qing Dai orally and continued taking other medications for UC as prescribed. Electron spin resonance was applied to explore the mechanisms of action of Qing Dai. After 4 mo of treatment with Qing Dai, the CAI score decreased from  $8.3 \pm 2.4$  to  $2.4 \pm 3.4$  (mean  $\pm$  SD;  $P < 0.001$ ). Similarly, the endoscopic Matts grade decreased from  $3.4 \pm 0.5$  to  $2.2 \pm 0.8$  ( $P = 0.02$ ). Six of 7 patients who were on prednisolone upon enrollment in the study were able to discontinue this corticosteroid. Electron spin resonance revealed that Qing Dai possesses strong hydroxyl radical scavenging activity. Qing Dai showed significant clinical and endoscopic efficacy in patients who failed to respond to conventional medications. Scavenging of hydroxyl radicals appears to be a potential mechanism through which Qing Dai acts, but the significance of the scavenging ability of Qing Dai with respect to the anti-inflammatory effect in UC patients warrants further investigation.

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**Key words:** Qing Dai; Herbal medicine; Ulcerative colitis; Hydroxyl radical; Electron spin resonance

**Core tip:** Nine intractable ulcerative colitis patients using the herbal medicine Qing Dai were observed in a retrospective observational study. After 4 mo of treatment, clinical activity and endoscopic findings improved dramatically in most patients. Electron spin resonance revealed that Qing Dai has a strong radical scavenging effect.

Suzuki H, Kaneko T, Mizokami Y, Narasaka T, Endo S, Matsui H, Yanaka A, Hirayama A, Hyodo I. Therapeutic efficacy of the Qing Dai in patients with intractable ulcerative colitis. *World J Gastroenterol* 2013; 19(17): 2718-2722 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2718.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2718>

## INTRODUCTION

The prevalence of ulcerative colitis (UC) is increasing worldwide and has reached a total of 130000 cases in Japan. Clinical practice guidelines issued by the Japanese Ministry of Health, Labor and Welfare recommend aminosalicylates, sulfasalazine, or 5-aminosalicylic acid (5-ASA) followed by corticosteroids [prednisolone (PSL)] or azathioprine (AZA) as the standard treatment for UC patients. Recently, infliximab (IFX) and tacrolimus have become available and are used clinically as novel medications for patients with UC. However, some patients do not respond to these pharmacological interventions; UC in such patients is called refractory or intractable. The herbal medicine Qing Dai is also known as Indigo naturalis and is extracted from plants such as *Strobilanthes cusia* and *Isatis tinctoria*. Qing Dai contains natural ingredients such as indigo, indirubin, isoindirubin, and nimboesterol. In China, Qing Dai has been traditionally used as an antipyretic, an antiphlogistic, and as a hemostatic remedy. Qing Dai enemas have been used for ulcerative proctitis and are currently described in Chinese medical guidelines for the treatment of UC. However, until now, no report has been published describing the efficacy of orally administered Qing Dai in UC patients.

## CASE REPORT

### Cases

Nine UC patients (7 men and 2 women, listed in Table 1) who voluntarily received Qing Dai between 2008 and 2011 were retrospectively evaluated. Patients who did not respond to 5-ASA and PSL or IFX were defined as “intractable”. One patient who was not intractable but refused to add PSL or IFX was included in the analysis. The average age of the patients was 34 years with a range of 16–75 years. All patients purchased Qing Dai from Seishinshoyakudo (Tokyo, Japan), a company that imports Qing Dai from China and sells it at a price of 4200 Japanese yen (US \$50) per 500 g. Qing Dai was provided as powder, and each patient took 1 g of Qing Dai orally twice a day without wrapping, according to the manufacturer’s recommendations. All 9 patients were on 5-ASA, and 7 patients were also on PSL. 5-ASA and PSL were taken at the recommended dose for at least 8 wk prior to the initiation of Qing Dai; however, unique cases include patient D, whose pharmacological regimen was changed from 3 g/d of Pentasa (Ferring Pharmaceuticals, Malmö, Sweden) to 3.6 g/d of Asacol (Warner Chilcott, Dublin, Ireland) 4 d before the start of Qing Dai and patient F, whose prescription was changed from 4 g/d of Pentasa to 3.6 g/d of Asacol 3 d before taking Qing Dai. The clinical activity index (CAI)<sup>[1]</sup> of the patients before taking Qing Dai and 1, 2, and 4 mo after initially taking Qing Dai and the endoscopic Matts grade<sup>[2]</sup> before and after treatment with Qing Dai were retrospectively collected from clinical records because those data had been routinely recorded for all UC patients.

**Table 1 Patient characteristics including lesion type and ulcerative colitis duration and treatment**

| Patient | Sex | Age (yr) | Type of UC Lesion  | Duration of UC (mo) | Ongoing treatment at time of Qing Dai initiation |
|---------|-----|----------|--------------------|---------------------|--|
| A       | M   | 16       | Proctitis          | 18                  | 5-ASA, PSL                                       |
| B       | M   | 36       | Left-sided colitis | 65                  | 5-ASA, IFX                                       |
| C       | M   | 35       | Proctitis          | 96                  | 5-ASA, PSL, CAP                                  |
| D       | M   | 33       | Pancolitis         | 60                  | 5-ASA, PSL, AZA, IFX                             |
| E       | M   | 31       | Left-sided colitis | 40                  | 5-ASA, PSL, AZA                                  |
| F       | M   | 27       | Left-sided colitis | 32                  | 5-ASA, PSL, CAP, IFX                             |
| G       | F   | 26       | Proctitis          | 6                   | 5-ASA  |
| H       | F   | 29       | Left-sided colitis | 8                   | 5-ASA, PSL                                       |
| I       | M   | 75       | Left-sided colitis | 74                  | 5-ASA, PSL, AZA                                  |

Nine UC patients (7 men and 2 women) were included in this study. The type of colitis lesion, the duration of UC and the ongoing treatment at the time of Qing Dai initiation are shown. UC: Ulcerative colitis; M: Male; F: Female; 5-ASA: 5-aminosalicylates; PSL: Prednisolone; IFX: Infliximab; CAP: Cytopheresis; AZA: Azathioprine.

### Electron spin resonance

To investigate the mechanisms of action of Qing Dai, we performed electron spin resonance (ESR) spectroscopy by using the spin-trapping reagent 5-(2,2-dimethyl-1,3-propoxy cyclophosphoryl)-5-methyl-1-pyrroline *N*-oxide (CYPMPO)<sup>[3]</sup>. Hydroxyl radicals were produced in an aqueous solution containing 50  $\mu$ L of 2 mmol/L H<sub>2</sub>O<sub>2</sub> dissolved in 0.1 mol/L phosphate buffer. Fifty microliters of 8.9 mmol/L DMPO with or without 2.5, 25, or 250  $\mu$ g/mL Qing Dai was incubated for 60 s after the addition of 50  $\mu$ L of 0.2 mmol/L FeSO<sub>4</sub>, and results were obtained using a JEOL-TE X-Band spectrometer (JEOL, Tokyo, Japan).

### Ethical consideration

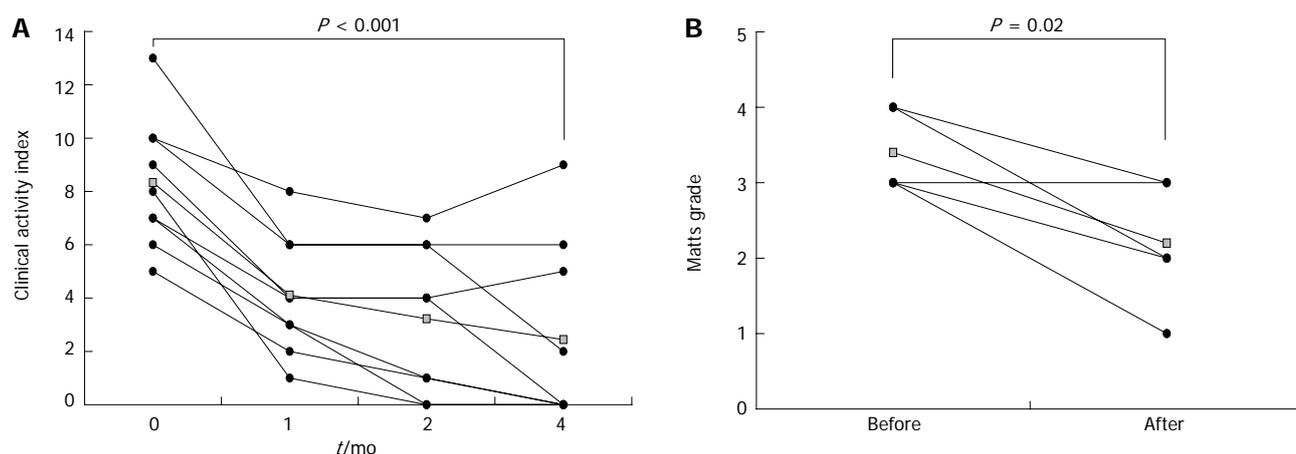
All 9 patients began taking Qing Dai voluntarily. Nonetheless, for this paper, written informed consent was obtained from all of the patients included in the analysis.

### Statistical analysis

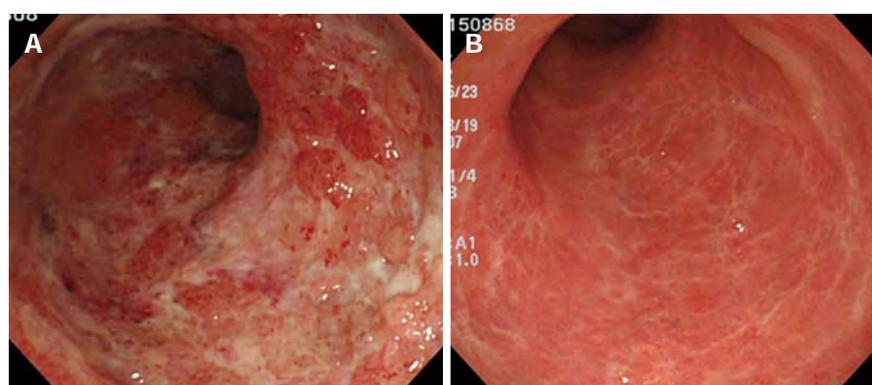
The data for CAI and Matts grade score were analyzed by a paired *t*-test using Excel 2010 (Microsoft, United States) with the add-in software Statcel 3 (OMS publishing Co., Saitama, Japan). Differences corresponding to a *P* value < 0.05 were considered statistically significant.

### Clinical and endoscopic efficacy of Qing Dai

The average CAI score (mean  $\pm$  SD) decreased from 8.3  $\pm$  2.4 to 2.4  $\pm$  3.4 (*P* < 0.001) (Figure 1A). This effect persisted unless the patient withdrew from Qing Dai treatment. Patients A and C showed recurrence of UC after cessation of Qing Dai. However, their CAI score immediately recovered upon re-administration of Qing Dai.



**Figure 1** The clinical activity index score and endoscopic Matts grade before and after Qing Dai initiation. A: The score markedly improved from  $8.3 \pm 2.4$  to  $2.4 \pm 3.4$  (mean  $\pm$  SD; *t*-test,  $P < 0.001$ ) after the initiation of Qing Dai. The line plotted in gray shows the average; B: The Matts grade significantly improved from  $3.4 \pm 0.5$  to  $2.2 \pm 0.8$  (mean  $\pm$  SD; *t*-test,  $P = 0.02$ ) after the initiation of Qing Dai treatment. The line plotted in gray shows the average.



**Figure 2** Endoscopic finding before and after treatment. A: Before the initiation of Qing Dai, endoscopic examination showed severe ulcers and erosions in the rectum; B: After 3 mo of Qing Dai treatment, the mucosal damage completely disappeared.

Five patients, including 3 patients with left-sided colitis, 1 patient with pancolitis and 1 patient with proctitis, were monitored by endoscopy. The average time interval between endoscopies was 7 mo (range 2-10 mo). The endoscopic Matts grade (Figure 1B) also decreased from  $3.4 \pm 0.5$  to  $2.2 \pm 0.8$  ( $P = 0.02$ ). Six of the 7 patients (86%) who were also taking PSL were able to discontinue this corticosteroid.

Patient E, a 28-year-old man who presented with hematochezia was diagnosed as having left-sided UC and demonstrated remarkable benefit from Qing Dai therapy. Initially, patient E was prescribed 4 g/d sulfasalazine and 40 mg/d PSL for the treatment of UC, and the hematochezia rapidly disappeared. However, the hematochezia recurred as the patient reduced his intake of PSL. Thus, patient E required the dose of PSL to be increased several times, and treatment with AZA and cytapheresis failed to provide long-term improvement. Patient E met our criteria for refractory UC. After 3 years of treatment, patient E began to use Qing Dai of his own volition. One month after treatment with Qing Dai, hematochezia in patient E was resolved, and the CAI score decreased from 9 to 4. The serum C-reactive

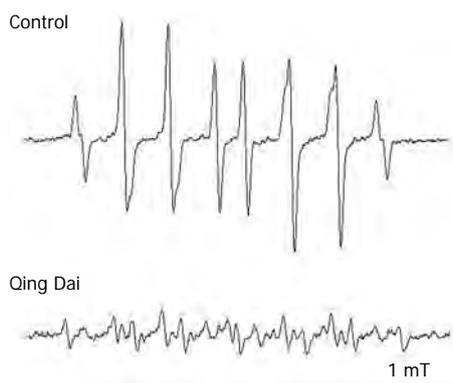
protein level also decreased from 1.33 mg/dL to 0.11 mg/dL. The CAI score fell to 0 after 3 mo of treatment with Qing Dai, and patient E was withdrawn from PSL and AZA after 8 mo of Qing Dai treatment. Notably, this effect of Qing Dai therapy persisted for more than 2 years. The endoscopic findings are shown in Figure 2A and B. The ulcers completely disappeared and only erythema was observed. In contrast, patient D did not respond to Qing Dai, and required the administration of conventional medications.

**Safety**

Although no serious adverse side effects were reported in patients consuming Qing Dai, 2 patients developed mild headaches while taking Qing Dai. The headaches disappeared upon reduction of the dose of Qing Dai by half.

**Hydroxyl radical scavenging effect of Qing Dai**

As shown in Figure 3, without Qing Dai, the ESR spectrum agreed with CYPMPO-OH, a hydroxyl radical adduct trapped by CYPMPO. The reduction in the signal intensity of CYPMPO-OH reflected the hydroxyl radical scavenging ability of Qing Dai, and the results of treat-



**Figure 3** Electron spin resonance of Qing Dai. Representative electron spin resonance spectra of CYPMPPO-OH (for hydroxyl radical determination) obtained by the addition of solvent control or an ethanol extract of Qing Dai at a concentration of 25  $\mu\text{g}/\text{mL}$ . The test was repeated in five independent trials, and a representative result is presented.

ment with 25  $\mu\text{g}/\text{mL}$  Qing Dai are shown in Figure 3. The 250 and 2.5  $\mu\text{g}/\text{mL}$  concentrations of Qing Dai showed a similar hydroxyl radical scavenging effect in a dose-dependent manner (data not shown).

## DISCUSSION

Approximately 21% of patients with inflammatory bowel disease reportedly use alternative treatment outside of the medications typically prescribed to treat inflammatory bowel disease in a clinical setting<sup>[4]</sup>. Consistent with this statistic, several herbal medications have shown promising efficacy and safety in patients with UC, including preparations that contain a sulfhydryl group in their structure<sup>[5]</sup>. Evidence of the clinical efficacy of Qing Dai and its constituent substances has been previously reported. Yuan *et al*<sup>[6]</sup>, reported that Qing Dai enemas are associated with significant clinical efficacy in the treatment of chronic hemorrhagic radiation proctitis. Likewise, Xilei-san, which is a major ingredient in Qing Dai, has traditionally been used for the treatment of UC in China and, more recently, in Japan<sup>[7,8]</sup>. Accordingly, in a double blind, randomized clinical trial setting, Fukunaga *et al*<sup>[9]</sup> reported that suppositories of Xilei-san showed significant efficacy in patients with ulcerative proctitis refractory to conventional medications. In the current study, we found a good clinical response to Qing Dai with no serious adverse side effect in patients with intractable UC. Ferber has also reported very low toxicity for Qing Dai<sup>[10]</sup>.

There are few reports on the pharmacokinetics of Qing Dai. Because Qing Dai appears in the stool without significant digestion, the mucosal healing effect could be associated with the ability of Qing Dai to provide a protective coating to the injured mucosa. Qing Dai has been reported to have anti-inflammatory effects on human neutrophils based on its ability to suppress superoxide generation<sup>[11]</sup>. The same authors reported efficacy of Qing Dai in patients with recalcitrant psoriasis in a randomized study<sup>[12]</sup>. Indirubin, a constituent of Qing Dai, has been reported to produce anti-inflamma-

tory effects by suppressing interferon- $\alpha$ , interleukin-6<sup>[13]</sup>, and nuclear factor  $\kappa\text{B}$  (NF- $\kappa\text{B}$ ) production<sup>[14]</sup>. Xilei-san was also reported to decrease the expression of toll-like receptor 4, NF- $\kappa\text{B}$ , and tumor necrosis factor- $\alpha$  in mice with oxazolone-induced colitis<sup>[15]</sup>. Interestingly, cytaferesis, which has been used in patients with active UC as a highly effective therapeutic, possesses a similar mechanism of action by decreasing reactive oxygen-producing neutrophils and interleukin-6 secretion<sup>[16]</sup>. In the present investigation, although we observed a strong hydroxyl radical scavenging effect in Qing Dai, further studies are warranted to fully understand the mechanisms of action in UC patients.

This study has several limitations. First, this was not a double-blind placebo-controlled trial. Second, basic experiments using animal models were not conducted. In light of these limitations, we are planning an *in vivo* study and a prospective multicenter double-blind placebo-controlled trial to investigate the effects of Qing Dai in patients with inflammatory bowel disease.

In conclusion, this investigation showed that oral Qing Dai is associated with significant clinical and endoscopic improvement of UC in patients who maintain active disease despite receiving conventional medications. The hydroxyl radical scavenging effect appears to be one mode of action of Qing Dai. Our results suggest that Qing Dai may represent a clinically relevant intervention for patients with inflammatory bowel disease. Additional controlled trials using larger cohorts of patients should be conducted to verify the findings of this investigation.

## ACKNOWLEDGMENTS

We would like to thank Dr. Hiroyuki Hanai for providing advice regarding the improvement of this manuscript.

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**P- Reviewers** Rosen, MJ, Leitman M, Lakatos PL, Sipos F  
**S- Editor** Song XX **L- Editor** A **E- Editor** Zhang DN



## Accurate hemostasis with a new endoscopic overtube for emergency endoscopy

Hirohito Mori, Hideki Kobara, Shintaro Fujihara, Noriko Nishiyama, Makoto Oryu, Kazi Rafiq, Tsutomu Masaki

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Author contributions: Mori H was responsible for conception of the research and for drafting the article; Kobara H, Fujihara S, Nishiyama N, Oryu M, and Rafiq K participated equally in the work; Masaki T provided critical revision of the manuscript for intellectual content and was responsible for final approval of the manuscript.

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Received: February 16, 2013 Revised: March 8, 2013

Accepted: March 21, 2013

Published online: May 7, 2013

posed vessels resulted in success of hemostasis.

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**Key words:** Emergency endoscopic hemostasis; Right lateral decubitus position; Identification of exposed vessel; Newly developed inverted overtube; Clear endoscopic view

**Core tip:** The inverted overtube helped us obtain a clear view in patients who were laid in the right lateral position. Rapid identification of exposed vessels resulted in success of hemostasis.

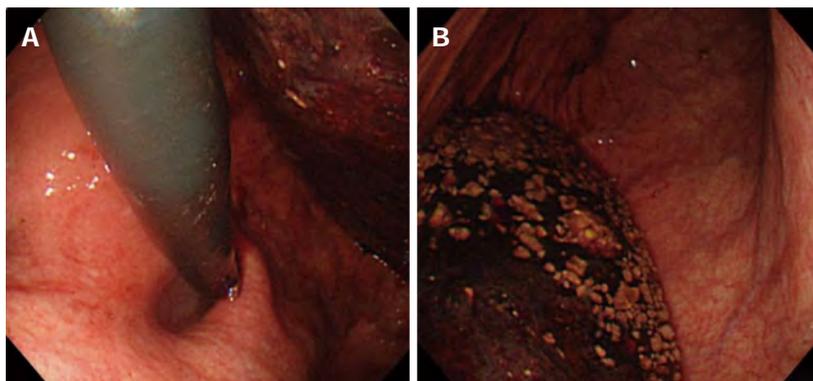
Mori H, Kobara H, Fujihara S, Nishiyama N, Oryu M, Rafiq K, Masaki T. Accurate hemostasis with a new endoscopic overtube for emergency endoscopy. *World J Gastroenterol* 2013; 19(17): 2723-2726 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i17/2723.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2723>

### Abstract

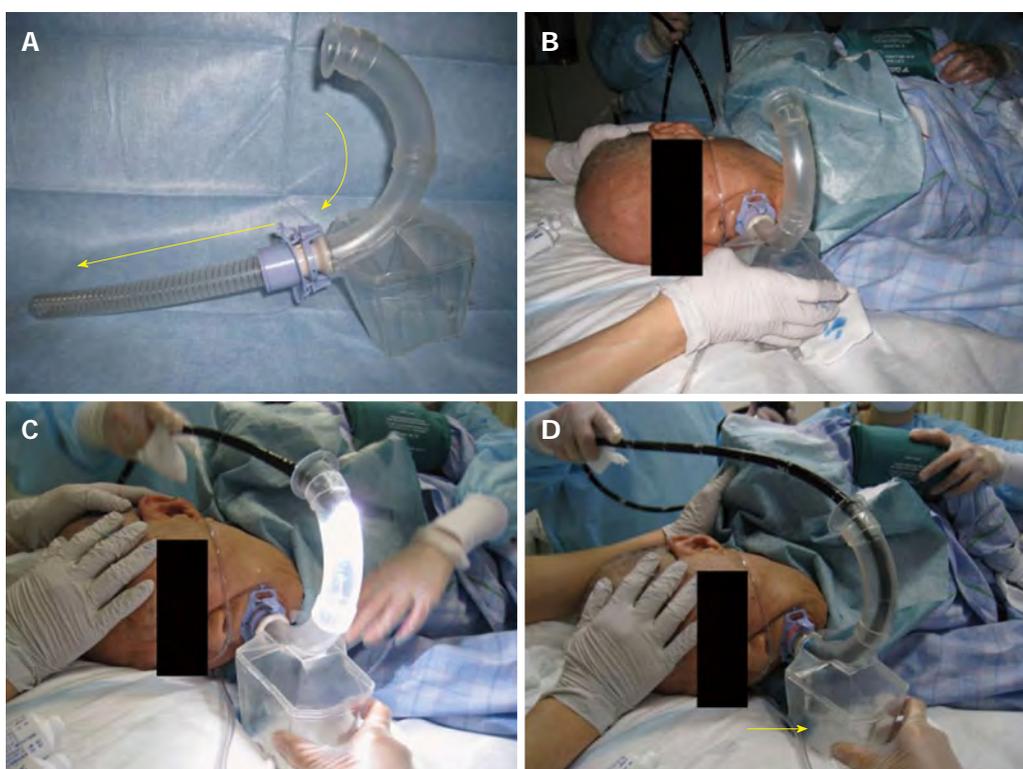
Endoscopic hemostasis performed in the emergency room is difficult due to the presence of blood clots and food residue that makes obtaining a clear view of the bleeding vessel difficult. We experienced the efficacy of a newly developed inverted overtube to shorten the hemostatic time and obtain a clear endoscopic view with upper gastrointestinal bleeding patient who were transferred by ambulance car and required emergency endoscopy. The technique improved the endoscopic views and enabled us to perform the hemostatic procedures from the conventional standing position while freely and easily changing the patient's position. The presence of blood clots and food residue in the gastric fornix or upper gastric body makes identifying a bleeding exposed vessel impossible. This set-up significantly shortened the procedure time. The inverted overtube helped us obtain a clear view in patients who were laid in the right lateral position. Rapid identification of ex-

### INTRODUCTION

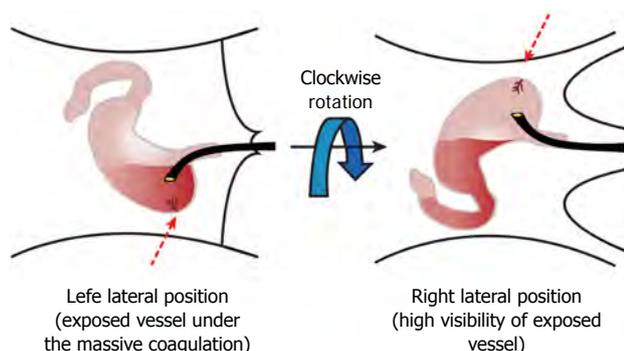
Patients with upper gastrointestinal bleeding who are transferred by an ambulance commonly present in emergency rooms and require an emergency endoscopy to achieve hemostasis. Although the significance, indication and timing of emergency endoscopies are controversial<sup>[1,2]</sup>, in the case of esophageal or gastric varix or Dieulafoy's ulcer, exposed vessels with spurting bleeding require prompt hemostasis, and failure to achieve hemostasis may lead to a serious condition. Endoscopists are under pressure to perform these emergency endoscopic treatments, especially in patients with cardiovascular or cerebrovascular diseases who are being treated with antiplatelet and anticoagulant agents<sup>[3]</sup> or in seriously ill patients who require rapid and reliable hemostasis<sup>[4]</sup>. In almost all cases, once the bleeding site is visually identified



**Figure 1** Emergency endoscopic view. A: The presence of large amounts of blood clots; B: The presence of food residue in the stomach complicated the observation of the region from the gastric fornix to the upper corpus.



**Figure 2** Outer appearance and procedures of using the inverted overtube. A: Outer appearance of the inverted overtube; B: Switching the patient from the conventional left lateral decubitus position to the right lateral decubitus position using the inverted overtube to dislodge blood clots during emergency endoscopic hemostasis; C: Insertion of the endoscope into the stomach through the inverted overtube in the right lateral decubitus position; D: Continuous aspiration from the bottom of the box (yellow arrow) enabled massive blood clots to be eliminated from the overtube, which maintained a clear endoscopic view during hemostasis.



**Figure 3** The schema of the visibility differences between 2 positions. We clockwise rotated the patient's position to a right lateral decubitus position to dislodge any massive clots and food residue.



**Figure 4** Obtaining a clear view of the gastric fornix and exposed vessels of a Dieulafoy's ulcer. Obtaining a clear view of the gastric fornix and upper gastric corpus, with all of the blood clots and food residue dislodged into the gastric antrum and duodenum. The exposed vessels of a Dieulafoy's ulcer were revealed (yellow arrows). The schema of the inverted overtube is shown in the center.

by endoscopy, hemostasis can be achieved using current endoscopic techniques and hemostatic forceps<sup>[5]</sup>; however, if it cannot be visually identified, a surgical procedure is required<sup>[6-8]</sup>. The presence of blood clots and food residue in the gastric fornix or upper gastric body makes identifying a bleeding exposed vessel impossible. In the case of gastric variceal bleeding, erythromycin or somatostatin has a beneficial effect on upper gastrointestinal bleeding by inducing rapid gastric emptying<sup>[9,10]</sup>. However, even with such drugs, obtaining a clear endoscopic view to rapidly identify the bleeding exposed vessels in an emergency situation is still difficult. This case series demonstrates a method for shortening the hemostasis time and reducing stress for both patients and endoscopists.

## CASE REPORT

A 55-year-old woman who was transported to our emergency room in shock after feeling nauseous and experiencing sudden massive hematemesis in June 2012. Although her family informed us that a blood test 6 mo prior had shown no signs of anemia, a blood examination revealed severe anemia (hemoglobin, 5.5 g/dL). Emergency endoscopic hemostasis and a blood transfusion were performed. Large amounts of blood and food residue were observed in the stomach. Because the patient appeared drowsy, an emergency endoscopy to detect the bleeding vessel was immediately performed in the areas that could be observed in the left lateral decubitus position. The bleeding vessel could not be identified in the duodenum or gastric antrum. As the amount of fresh blood increased, obtaining a clear endoscopic view to identify the bleeding vessel gradually increased in difficulty (Figure 1). Assuming that the bleeding vessel was located in either the upper gastric body or gastric fornix, which was not visible due to the blood clot, we immediately switched from the conventional observation position to the right lateral decubitus position using newly developed inverted overtube (Figure 2). The inverted overtube was approved by the Institutional Ethics Committee of Kagawa University Hospital, Kagawa, Japan. Additionally, the inverted overtube was approved by the

Japanese Pharmaceutical Law.

The blood clot and food residue in the gastric corpus and fornix were immediately dislodged to the right into the duodenum by gravity (Figure 3), allowing for the visual identification of an exposed blood vessel in the fornix (Figure 4). Up to this point, no spurting bleeding had been observed due to the decreased blood pressure. However, massive spurting bleeding occurred immediately after the exposed blood vessel was pinched with hemostatic forceps. The pinched vessel was then completely cauterized by coagulation mode to achieve hemostasis. No bleeding was observed thereafter.

## DISCUSSION

Despite the dramatic progress made in endoscopic hemostatic techniques<sup>[1,4]</sup>, hematemesis from an esophageal or gastric varix or Dieulafoy's ulcer can lead to serious consequences if hemostasis is not achieved<sup>[8]</sup>. Achieving accurate and reliable hemostasis is difficult without a clear view of the bleeding vessel. Although hemostasis *via* a laparotomy can be performed as a last resort, surgery in a patient with a poor systemic condition carries a high risk. Thus, emergency endoscopic hemostasis remains the first-line treatment of choice<sup>[2,6]</sup>.

During endoscopic hemostatic procedures performed in the emergency room, where pretreatment is not performed, the presence of blood clots and food residue makes obtaining a clear view of the bleeding vessel difficult<sup>[5]</sup>. The removal of blood clots from the stomach has conventionally been achieved by gastric suction with a gastric tube and/or by manual removal of the clot with grasping forceps. However, the use of gastric suction and/or grasping forceps currently requires a great deal of time. Thus, endoscopists have adopted a procedure in which the patient's posture is rotated to the right lateral decubitus position to dislodge the blood clots and enable the identification of the bleeding vessel. Because most endoscopists perform conventional endoscopic examinations and treatment procedures with the patient lying in the left lateral decubitus position, they find that performing accurate hemostatic procedures from the opposite

side, with the patient lying in the right lateral decubitus position, to be difficult. Thus, the use of the inverted overtube is the best method to help endoscopists perform an emergency endoscopy with less stress because they are in their conventional standing position relative to patients who are rotated to the right lateral decubitus position, without changing the positions of the endoscopy unit and light source. This technique is the most effective way to dislodge blood clots and food residue by gravity in these patients. The present technique dramatically improved the clarity of the endoscopic views and enabled the endoscopist to perform the hemostatic procedures from the conventional standing position while freely and easily changing the patient's position. This set-up significantly shortened the procedure time. The inverted overtube, with its very simple structure, helped the endoscopist acquire a clear view in patients who were laid in the right lateral position. This clear view contributed to the rapid identification of the bleeding vessels and the subsequent rapid achievement of hemostasis.

## ACKNOWLEDGMENTS

We thank Professor Yasuyuki Suzuki for technical and editorial assistance.

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**P- Reviewers** Lin CH, Kikuyama M **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Zhang DN



## Meckel's diverticulum bleeding diagnosed with magnetic resonance enterography: A case report

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Author contributions: Zhou FR designed the study, performed the operation and wrote the manuscript; Huang LY and Xie HZ performed the operation; all authors have read and approved the final version for publication.

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Received: February 6, 2013 Revised: March 26, 2013

Accepted: March 28, 2013

Published online: May 7, 2013

### Abstract

Although the introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, definite diagnosis of Meckel's diverticulum far from the ileocecal valve is still impossible in most cases. We explored the role of magnetic resonance (MR) enterography in detecting bleeding from Meckel's diverticulum that can not be confirmed *via* double-balloon enteroscopy. This study describes a case of male patient with bleeding from Meckel's diverticulum diagnosed with MR enterography of the small intestine. No bleeding lesion was found *via* colonoscopy, anal enteroscopy, or oral colonoscopy. MR enterography of the small intestine revealed an occupying lesion of 3.0 cm in the lower segment of the ileum. The patient was transferred to the Department of Abdominal Surgery of our hospital for surgical treatment. During surgery, a mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. The patient underwent partial resection of the small intestine. Postopera-

tive pathology showed Meckel's diverticulum containing pancreatic tissues. He was cured and discharged 7 d after operation. We conclude that MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

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**Key words:** Meckel's diverticulum; Double-balloon enteroscopy; Magnetic resonance enterography

**Core tip:** This study describes a case of male patient with bleeding from Meckel's diverticulum diagnosed with magnetic resonance (MR) enterography of the small intestine. MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

Zhou FR, Huang LY, Xie HZ. Meckel's diverticulum bleeding diagnosed with magnetic resonance enterography: A case report. *World J Gastroenterol* 2013; 19(17): 2727-2730 Available from: <http://www.wjgnet.com/1007-9327/full/v19/i17/2727.htm>  
DOI: <http://dx.doi.org/10.3748/wjg.v19.i17.2727>

### INTRODUCTION

Gastrointestinal bleeding of unknown cause is mostly a result of lesions located in the small intestine, for which the diagnosis and treatment can be challenging<sup>[1,2]</sup>. Meckel's diverticulum is a common congenital malformation of the small intestine, with gastrointestinal bleeding being the most common symptom. Preoperative diagnosis of this condition is difficult. Although the introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, definite

diagnosis of Meckel's diverticulum far from the ileocecal valve is still impossible in most cases. This study describes a case of bleeding from Meckel's diverticulum diagnosed with magnetic resonance (MR) enterography of the small intestine in our department.

## CASE REPORT

A 25-year-old man was first admitted to the hospital for lower abdominal pain on March 25, 2011. No abnormality was found through enhanced computed tomography (CT) scan of the whole abdomen on admission. No positive finding was reported on anal enteroscopy. Barium meal examination of the entire digestive tract showed multiple fluid levels in segments 4 and 5 of the small intestine. His condition improved with anti-inflammatory therapy, intestinal spasmolysis, and nutritional support. He was then discharged on April 3, 2011. After that, he was diagnosed as having "appendicitis" and underwent appendectomy in a local hospital. Encapsulated fluid was present in the right lower quadrant, which was improved after puncture and drainage. Later, he was again admitted to our hospital on February 17, 2012 due to melena for two days after recurrent abdominal pain for nearly a year. Examinations on admission showed a positive fecal occult blood test, stool red blood cell (RBC) +++/HP, C-reactive protein: 30.9 mg/L; abdominal CT revealed a small amount of fluid in the pelvic cavity. Routine examination of the ascites fluid collected by ultrasound-guided puncture showed red fluid, specific gravity > 1.018, positive Rivalta test, RBC ++++/HP, and nucleated cell count of  $1.8 \times 10^9/L$ . Ascites smears revealed no acid-fast bacilli. Bacterial culture indicated *Streptococcus sanguis*. No bleeding lesion was found *via* colonoscopy, anal enteroscopy, or oral colonoscopy. His condition improved after hemostatic, anti-infective and symptomatic treatment, and he was discharged on March 4, 2012. His condition remained stable after discharge until three days prior to admission when he experienced lower abdominal pain after eating spicy food, and had dark red bloody stools, 2-3 times a day. He was admitted for a third time on April 24, 2012. Blood tests upon admission showed hemoglobin (HGB) of 96 g/L; MR enterography of the small intestine showed an occupying lesion of 3.0 cm in the lower segment of the ileum. He was transferred to the Department of Abdominal Surgery for surgical treatment. During surgery, a mass of 3 cm  $\times$  2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues. The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation (Figure 1).

## DISCUSSION

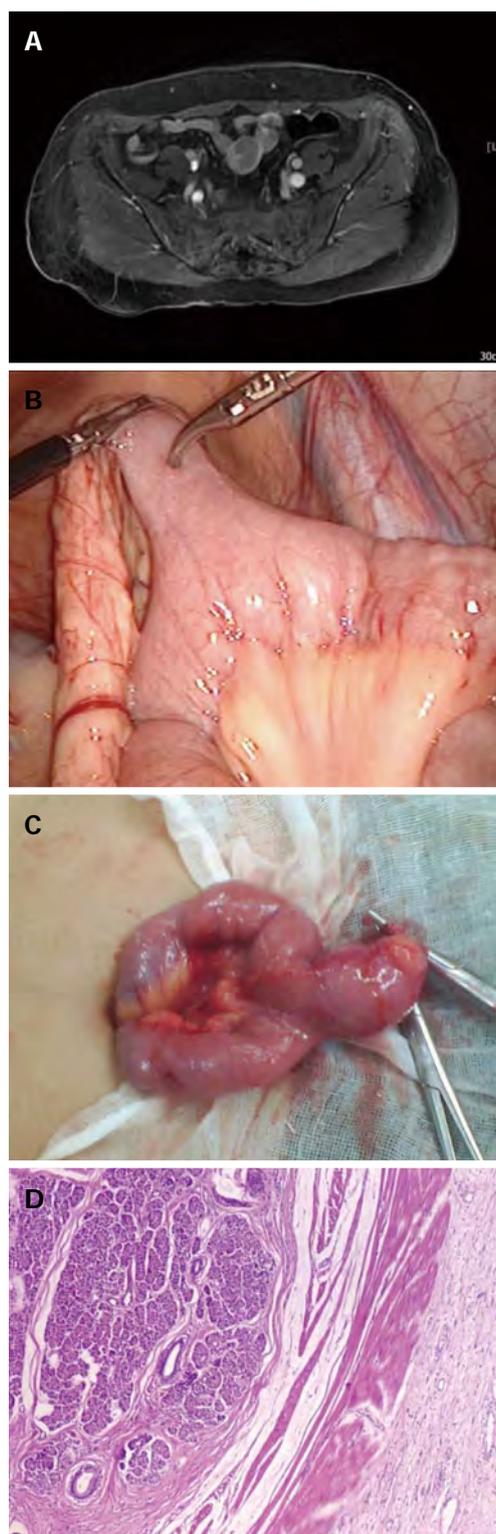
Meckel's diverticulum is the most common congenital

malformation of the small intestine, with an incidence of 1% to 4%<sup>[3]</sup>. It usually occurs in the ileum within 100 cm of the ileocecal valve<sup>[4]</sup>, and is a true diverticulum consisting of all layers of the bowel wall. Meckel's diverticulum harbors various ectopic tissues, with gastric mucosa being the most common (about 50%), followed by pancreatic tissues (about 5%)<sup>[5]</sup>. The majority of people afflicted with Meckel's diverticulum are asymptomatic, and 4% to 6% of the patients are detected due to symptoms of related complications<sup>[6-10]</sup>. Gastrointestinal bleeding is the most common symptom of Meckel's diverticulum<sup>[11]</sup>, followed by intussusception, and intestinal obstruction.

Such bleeding occurs from larger vessels invaded by erosion and ulceration of normal intestinal mucosa in the diverticulum, as a result of the secretion of gastric acid or alkaline pancreatic fluid from the ectopic mucosa<sup>[12-15]</sup>. Consisting mainly of fundic glands, where parietal cells secrete gastric acid and chief cells secrete pepsin, the ectopic gastric mucosa is closely related to gastrointestinal bleeding, as the acid does not only directly cause corrosion of the diverticulum and intestinal mucosa, but also promotes the conversion of pepsinogen secreted by ectopic chief cells into pepsin, which in turn induces tissue digestion and leads to ulceration and bleeding.

Preoperative diagnosis is difficult when Meckel's diverticulum is complicated by small intestinal bleeding<sup>[15]</sup>. Gastroscopy, colonoscopy, barium meal and ultrasound lack specificity, but they can be used to exclude lower gastrointestinal bleeding from other sites. Due to its high-density resolution, CT scanning can detect and accurately locate stones of low density in Meckel's diverticulum<sup>[16,17]</sup>. CT enterography of the small intestine has been proven to be more useful than conventional CT in patients suspected of having small bowel lesions, as it not only maximizes the visibility of the mucosa and intestine structure with the aid of contrast agents, but also detects parenteral abnormalities<sup>[18-22]</sup>. MR enterography is a radiation-free technique developed based on CT enterography, which allows evaluation of intestinal functionalities using MR fluoroscopy.

MR enterography is a novel technique that combines the advantages of both the conventional enterography and the morphological imaging of magnetic resonance imaging, thus making it an examination for both the functions and the morphologies of small intestine. Before MR enterography is done, the bowel must be completely cleansed and the intestinal canal distended. Therefore, it is critically important to select a contrast agent that can thoroughly distend the intestinal canal and clearly demonstrate the enteric cavity and intestinal wall, and does not produce artifacts or pose a hazard to human health. Although the double-contrast imaging of the small intestine is helpful for observing the early mucosal changes, it cannot visualize the lesions around the intestinal wall or in the mesentery. In contrast, MR enterography, with three-dimensional imaging capabilities and excellent soft-tissue contrast, can be used to observe the mucosa and analyze the changes around the intestinal canal<sup>[23-25]</sup>.



**Figure 1** The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation. A: Magnetic resonance enterography of the small intestine. An occupying lesion of 3.0 cm was found in the lower segment of the ileum; B: A mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve during surgery; C: Removed lesion during surgery; D: Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues.

The introduction of capsule endoscopy and double-balloon enteroscopy has made observation of the entire

small bowel mucosa possible, and the latter technique also enables biopsy for pathological diagnosis and endoscopic treatment<sup>[26-28]</sup>. Compared with traditional push enteroscopy (90-150 cm) and retrograde terminal ileoscopy (50-80 cm), double-balloon enteroscopy has a longer average depth (240-360 cm orally and 102-140 cm anally); compared with capsule endoscopy, it enables more pathological and endoscopic applications, such as hemostasis, polypectomy, dilatation treatment, and removal of foreign substances<sup>[29,30]</sup>. Heine *et al*<sup>[31]</sup> demonstrated in their study that double-balloon enteroscopy has a diagnosis rate of 63% for obscure gastrointestinal bleeding, and complete small bowel examination is achievable for about 1/3 of these cases.

Although double-balloon enteroscopy has a high diagnosis rate for unexplained gastrointestinal bleeding, since a complete small bowel examination can be done in only a few patients, the causes of bleeding still remain unknown in many other patients before surgery. In the present case, the lesion was up to 150 cm from the ileocecal valve, which was undetectable by anal colonoscopy with an average insertion depth of about 102-140 cm, therefore, no definite diagnosis could be made. In view of this, MR enterography of the small intestine was performed and an occupying lesion of 3.0 cm was found in the lower segment of the ileum, with clear boundaries, showing mixed signals. He was transferred to the Department of Abdominal Surgery for surgical treatment. During surgery, a mass of 3 cm × 2 cm was found 150 cm from the ileocecal valve, in conjunction with congestion and edema of the corresponding mesangium. Intraoperative diagnosis was small bowel diverticulum with bleeding. Postoperative pathology showed Meckel's diverticulum containing pancreatic tissues. The patient underwent partial resection of the small intestine, and was cured and discharged 7 d after operation.

In summary, Meckel's diverticulum is the most common congenital malformation of the small intestine that is easily complicated by small intestinal bleeding, for which preoperative diagnosis is difficult. The introduction of double-balloon enteroscopy has greatly improved the diagnostic rate, but definite diagnosis is still challenging in some patients, especially when the lesion is far from the ileocecal valve. MR enterography is a radiation-free technique developed based on CT enterography, which maximizes the visibility of the mucosa and intestine structure with the aid of contrast agents. MR enterography of the small intestine has greatly improved the diagnosis rate of Meckel's diverticulum, particularly in those patients with the disease which can not be confirmed *via* double-balloon enteroscopy.

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L- Editor A E- Editor Zhang DN



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*World Journal of Gastroenterology*

### ISSN

ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

### Launch date

October 1, 1995

### Frequency

Weekly

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*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 PMID: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 Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 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]

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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

*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. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.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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*Chow JYC, Li ZJ, Kei WK, Cho CH*

**FIELD OF VISION**            2736    Relationships of CDXs and apical sodium-dependent bile acid transporter in Barrett's esophagus  
*Zhao J, Gregersen H*

**REVIEW**                        2740    Persistent hypertransaminasemia in asymptomatic children: A stepwise approach  
*Vajro P, Maddaluno S, Veropalumbo C*

**ORIGINAL ARTICLE**           2752    Efficacy and safety of over-the-scope clip: Including complications after endoscopic submucosal dissection  
*Nishiyama N, Mori H, Kobara H, Rafiq K, Fujihara S, Kobayashi M, Oryu M, Masaki T*

2761    Lipoic acid suppresses portal endotoxemia-induced steatohepatitis and pancreatic inflammation in rats  
*Tian YF, He CT, Chen YT, Hsieh PS*

2772    Oncogene *GAEC1* regulates *CAPN10* expression which predicts survival in esophageal squamous cell carcinoma  
*Chan D, Tsoi MYT, Liu CD, Chan SH, Law SYK, Chan KW, Chan YP, Gopalan V, Lam AKY, Tang JCO*

2781    MAWBP and MAWD inhibit proliferation and invasion in gastric cancer  
*Li DM, Zhang J, Li WM, Cui JT, Pan YM, Liu SQ, Xing R, Lu YY*

**BRIEF ARTICLE**                2793    Sustained virological response: A milestone in the treatment of chronic hepatitis C  
*Morisco F, Granata R, Stroffolini T, Guarino M, Donnarumma L, Gaeta L, Loperto I, Gentile I, Auriemma F, Caporaso N*

2799    Long-term efficacy of endoscopic coagulation for different types of gastric vascular ectasia  
*Imai Y, Mizuno Y, Yoshino K, Watanabe K, Sugawara K, Motoya D, Oka M, Mochida S*

- 2806** Extremely high prevalence of *Helicobacter pylori* infection in Bhutan  
*Vilaichone R, Mahachai V, Shiota S, Uchida T, Ratanachu-ek T, Tshering L, Tung NL, Fujioka T, Moriyama M, Yamaoka Y*
- 2811** Prognostic value of preoperative mean corpuscular volume in esophageal squamous cell carcinoma  
*Zheng YZ, Dai SQ, Li W, Cao X, Li Y, Zhang LJ, Fu JH, Wang JY*
- 2818** Increased CD163 expression is associated with acute-on-chronic hepatitis B liver failure  
*Ye H, Wang LY, Zhao J, Wang K*

## CASE REPORT

- 2826** Massive hepatic necrosis with toxic liver syndrome following portal vein ligation  
*Dupré A, Gagnière J, Tixier L, Ines DD, Perbet S, Pezet D, Buc E*
- 2830** Polyarteritis nodosa diagnosed by surgically resected jejunal necrosis following acute abdomen  
*Hiraike Y, Kodaira M, Sano M, Terazawa Y, Yamagata S, Terada S, Ohura M, Kuriki K*
- 2835** Two case reports of gastroendoscopy-associated *Acinetobacter baumannii* bacteremia  
*Chen CH, Wu SS, Huang CC*

**APPENDIX I-VI** Instructions to authors

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**INDEXING/ABSTRACTING** *World Journal of Gastroenterology* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16951 (7/74); Current Articles: 677 (1/74); and Eigenfactor® Score: 0.06035 (5/74).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*  
**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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**PUBLISHER**  
 Baishideng Publishing Group Co., Limited  
 Flat C, 25/F, Lucky Plaza,  
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**PUBLICATION DATE**  
May 14, 2013

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## Cathelicidin a potential therapeutic peptide for gastrointestinal inflammation and cancer

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Received: February 22, 2013 Revised: March 19, 2013

Accepted: April 3, 2013

Published online: May 14, 2013

### Abstract

Cathelicidins, are host defense peptides synthesized and stored in circulating leukocytes and numerous types of epithelial tissues in particular the gastrointestinal (GI) tract and skin. They have been known for their antimicrobial activities against a variety of microbes. Recently it was discovered that they have other significant biological functions and produce appealing pharmacological actions against inflammation and cancer in the GI tract through defined mechanisms. Experimental evidence shows that these actions could be tissue and disease specific and concentration dependent. This article reviews some of the physiological functions of cathelicidins and also their therapeutic potential in the treatment of inflammation and cancer and also the delivery system for this peptide as targeted therapy for various disorders in the GI tract both in animals and humans.

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**Key words:** Cathelicidin; Gastrointestinal tract; Cancer; Inflammation; Ulcer repair



### Biography

Professor Chi Hin Cho received his under- and post-graduate trainings in Taiwan, Canada, United States and Hong Kong and obtained his PhD in Pharmacology from the University of Hong Kong (HKU) in 1978. He had postdoctoral training in Canada and started his first faculty position in the Yang Ming Medical College and Veterans General Hospital, Taiwan in 1981. He returned to Hong Kong and joined HKU in the Department of Pharmacology, Faculty of Medicine from 1984 and became Chair Professor of Pharmacology in 2000. He joined the Chinese University of Hong Kong (CUHK) in 2007 as a chairman of the Department of Pharmacology. Currently, he is the Professor of Pharmacology and Associate Director of the School of Biomedical Sciences, Faculty of Medicine in CUHK. Professionally he was the President (2006-2010) and is now the Chair of Presidential Council (2012-2014) of the Gastrointestinal Pharmacology Section of the International Union of Basic and Clinical Pharmacology and visiting and honorary professors of Peking University, Fudan University, Zhejiang University, Beijing Capital University of Medical Science, the Fourth Military Medical University, Virginia Tech, University of Maryland and University of California. His current research interests focus on drug development for inflammation and cancers in the gastrointestinal (GI) tract. His recent work in the discovery of novel peptides including small peptides and cathelicidin as shown in this review have promising potential for drug targeting against inflammatory and cancerous diseases in the stomach and colon. These findings received prominent recognition, had significant impact on biomedical and clinical sciences and attracted international pharmaceutical industry's interest in the development of drugs and agents for the treatment and diagnosis of GI disorders. Professor Cho trained more than 50 PhD and master students and 11 postdoctoral fellows so far in his academic career. He is also the editorial board member and editor in more than 30 journals in the fields of Gastroenterology and Pharmacology. He published more than 355 peer-reviewed articles and 48 reviews in scientific journals and is the editor of six books in GI ulcer and cancer. He also holds 2 patents related to therapeutic agents for GI disorders in CUHK.

**Core tip:** Cathelicidin is one of the most important host defense peptides known today. It carries multiple and yet unique biological functions against pathogens which contribute to the induction and also progression of infection, inflammation and cancer, the three major types of diseases in mankind. Deficiency of such peptide would cause multiple dysfunctions in the body. In this review we highlight the physiological role and therapeutic potential of cathelicidin in inflammation and cancer and also mucosal repair in the gut. All these information would shed new lights on the development of cathelicidin as therapeutic agent for different disorders in the gastrointestinal tract.

Chow JYC, Li ZJ, Kei WK, Cho CH. Cathelicidin a potential therapeutic peptide for gastrointestinal inflammation and cancer. *World J Gastroenterol* 2013; 19(18): 2731-2735 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2731.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2731>

## INTRODUCTION

Cathelicidins are innate immunity peptides. They are antimicrobial peptides (AMPs) that are produced by organisms as part of the defensive mechanism against various pathogenic microbes in humans and animals<sup>[1,2]</sup>. This class of pleiotropic peptides provides the first-line defense against infection by eliminating pathogens. Each AMP is encoded by a distinct gene. They show a great diversity in structures but have some common features, including: (1) relatively small molecular sizes (usually less than 50 amino acid residues); (2) cationic nature; (3) amphipathic helix structure; and (4) a substantial portion of hydrophobic amino acids<sup>[2,3]</sup>. Human cathelicidin (LL-37) consists of a long amphipathic helix spanning residues 2-31 with the C-terminal residues 32-37 unstructured. Another feature is that the structure is curved with a train of hydrophobic side chains. Such a cationic structure is perfect to associate with anionic micelles<sup>[4]</sup>. Indeed the cationic cathelicidin reacts electrostatically with anionic membrane components in particular cancer cells and microbes to disrupt cell membranes and induce cell death, while normal cells are neutral<sup>[5,6]</sup>. This specific property would enable cathelicidins directly and selectively attack membranes of microbes and cancer cells but spare the normal cells<sup>[7]</sup>. This uniqueness would make these peptides naturally exist and relatively non-toxic to normal mammalian system and have significant clinical implications as therapeutic agents for various diseases in particular those bacterial-related inflammation and cancer in the gastrointestinal (GI) tract<sup>[1,4,8]</sup>.

## CATHELICIDIN IN THE GI TRACT

Cathelicidins, a family of host defense peptides naturally expressed by cells of the GI tract. LL-37 is the mature

form of human cathelicidin. It is produced constitutively by differentiated surface and upper crypt epithelial cells in the colon and by the Brunner glands in the duodenum<sup>[9]</sup>. In normal stomach, the expression of the peptide is restricted to differentiated surface of various types of cells including epithelial cells, chief cells and parietal cells and is also present in the gastric secretion. They are upregulated during infection, inflammation and wound healing both in animals and humans<sup>[9-12]</sup>. These biological responses to external challenges could have significant implications as a host defense in protection against different disorders in the GI tract.

One good example is in the course of *Helicobacter pylori* (*H. pylori*) infection in which the expression of LL-37 is induced along the gastric glands. Induction of LL-37 may help to fight against bacterial infection at the early stage. However, the expression of LL-37 is dysregulated during *H. pylori*-associated gastric carcinogenesis. During the progression from atrophic gastritis to adenocarcinoma, the expression of LL-37 is reduced<sup>[12]</sup>. All these findings indicate that cathelicidin could play a significant role in preventing bacteria related inflammation and perhaps also carcinogenesis in the GI tract. It is envisaged that deficiency of this host defense peptide could facilitate the formation of inflammation and cancer.

## CATHELICIDIN AND GI REPAIR

Wound repair is a crucial adaptation to tissue damage. Based on the above information it comes to no surprise that soluble peptides like cathelicidins could evolve to orchestrate wound healing in response to mucosal damage in the GI tract. Along this line, LL-37 and mouse cathelicidin (mCRAMP) are strongly expressed in skin epithelium during wound healing in humans and mice<sup>[13]</sup>. In addition, the expression of LL-37 is low or absent in chronic ulcers, and antibodies to this peptide inhibit post-wounding re-epithelialization<sup>[14]</sup>.

Induction of angiogenesis by cathelicidin further highlights its potential role in wound repair<sup>[15]</sup>. In this context, it has been proposed that the healing-promoting effect of the peptide may be mediated through modification of growth factor and receptor interactions<sup>[16,17]</sup>. However the exact mechanisms by which cathelicidins promote wound healing have not yet been fully clarified. A recent study conducted by Yang *et al.*<sup>[11]</sup> in 2006 showed that rat cathelicidin can promote gastric ulcer healing in rats through induction of cell proliferation and angiogenesis. The same peptide stimulates cultured gastric epithelial cells through a transforming growth factor  $\alpha$ -dependent transactivation of epidermal growth factor and its related pathway to induce proliferation of gastric cells<sup>[11]</sup>.

## CATHELICIDIN AND INFLAMMATION

Experimental evidence shows that cathelicidin can modulate inflammation by altering cytokine response and chemoattraction of inflammatory cells in diseased tissues<sup>[1,18,19]</sup>. A recent study demonstrates that bacterial DNA

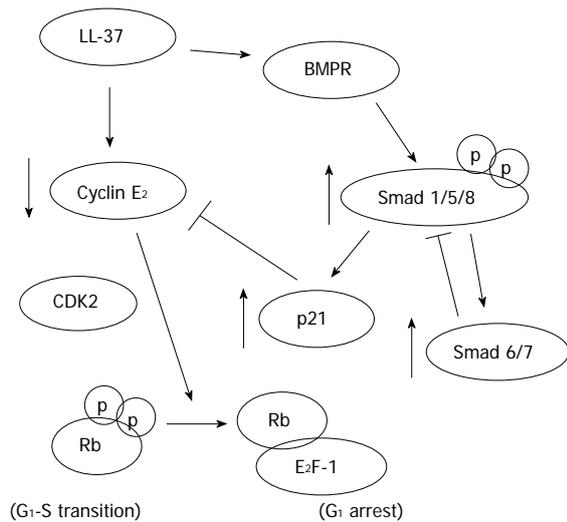


Figure 1 The possible signal pathway activated by cathelicidin (LL-37) to inhibit cell proliferation in gastric cancer cells. BMPR: Bone morphogenetic protein.

upregulates cathelicidin expression *via* a Toll-like receptor and mitogen-activated protein kinases/Erk pathway in colonic murine mucosa<sup>[20]</sup>. Clinical study also showed that cathelicidin expression was altered in inflammatory bowel diseases (IBD) patients. It was increased in both inflamed and non-inflamed mucosa in ulcerative colitis (UC) patients but not in Crohn's disease patients. The distribution of cathelicidin was also changed. Cathelicidin mainly expressed in the upper crypt of colons in healthy people in contrast to the basal part in IBD patients<sup>[21]</sup>. In another study, deficiency of cathelicidin in mCRAMP-knockout mice present more severe symptoms and mucosal disruption than the wild-type mice in response to dextran sulfate sodium challenge to induce UC. The inflammatory cytokines and the number of apoptotic cells are increased together with mucus secretion and gene expression are impaired. All these abnormalities are reversed by intrarectal administration of mCRAMP or mCRAMP-encoding plasmid<sup>[22]</sup>. On the other hand the increase of endogenous cathelicidin by agents such as butyrate and vitamin D has been suggested to modulate inflammatory responses either induced by chemical or bacteria in colonic cells<sup>[21,23-26]</sup>. Indeed butyrate treatment has been demonstrated to improve rectal histopathology in humans and eradicate *Shigella in vitro*<sup>[23,24]</sup> and vitamin D can prevent mucosal injury in chemical-induced acute colitis in mice<sup>[25]</sup>.

The peptide also significantly reduces the increased number of fecal microflora in UC animals<sup>[27]</sup>. Indeed exogenous cathelicidin modulates *Clostridium difficile* (*C. difficile*) colitis. In addition, *C. difficile*-induced colitic mice treated with cathelicidin inhibits toxin A-associated intestinal inflammation<sup>[28]</sup>. In view of the current UC therapies mainly focus on relieving the inflammatory responses or reducing the pathogenic microbes, cathelicidin would have both actions, and it further promotes the mucosal defensive mechanism through mucus secretion *via* a MAP kinase pathway<sup>[29]</sup>. All these actions would provide us a better therapeutic option in the treatment of inflamma-

tion in the colon. In this context, Cho and his group develop a new form of transporting system for this peptide by combining a probiotic *Lactococcus lactis* with cathelicidin gene into a single preparation. This preparation given orally instead of intrarectal administration<sup>[22]</sup> produces similar protection against UC in mice<sup>[30]</sup>. In a similar approach, we have applied the same mCRAMP-secreting strain of *Lactococcus lactis* to reduce *H. pylori* density in the stomach as well as the associated inflammatory cell infiltration and cytokine production<sup>[31]</sup>. These findings show the feasibility of using the transformed food-graded probiotic to deliver cathelicidin to the diseased organs and exert targeted therapy. This new biological preparation would have significant clinical applications in the future as potential therapeutic agent to alleviate inflammation induced by *H. pylori* infection in the stomach and bacteria overgrowth in the colon.

## CATHELICIDIN AND CANCER

Although studies have demonstrated that LL-37 could promote tumorigenesis in some cancers including lung and breast cancers as well as epithelial ovarian cancer<sup>[32-34]</sup>. Other reports have shown that LL-37 may induce cell death in many tissues. In human airway epithelial cells, LL-37 has been shown to result in apoptotic TUNEL positive cells in a caspases-dependent manner<sup>[35]</sup>. Analogue of LL-37 could induce the caspase-independent apoptosis in an oral squamous cell line SAS-H1 but not normal cells<sup>[36]</sup>. The anti-tumorigenic effect of LL-37 is dependent on its ability to induce DNA break and mitochondrial damage in Jurkat T leukemia and A549 cells which are independent of caspase activation<sup>[37]</sup>. It is likely that low tissue expression of LL-37 could promote tumor formation. Indeed downregulation of LL-37 in cancer tissues has also been reported in the GI tract. In normal gastric mucosa, LL-37 is expressed in surface epithelial cells and chief cells as well as parietal cells in the fundic glands. Immunochemical staining of LL-37 has revealed that the expression of LL-37 is down-regulated in gastric hyperplastic polyps, tubular adenomas, and adenocarcinomas<sup>[12]</sup>. After *H. pylori* infection, LL-37 is markedly up-regulated in the epithelium and gastric secretions. Such upregulation could not be detected in patients with *H. pylori*-independent gastric inflammation. Moreover, a higher level of LL-37 expression has been demonstrated in wild-type *H. pylori* infection of cultured gastric epithelial cells and this higher production of LL-37 requires an intact type IV secretion system<sup>[4,12]</sup>. Therefore, it is indicated that expression of LL-37 may be in a tissue- and disease-specific manner.

Our recent study shows that LL-37 may function as a putative tumor-suppressing gene in gastric carcinogenesis. We found that exogenous LL-37 inhibits proliferation and induces G<sub>0</sub>/G<sub>1</sub>-phase cell cycle arrest through a defined signal pathway in gastric cancer cells (Figure 1). Furthermore depletion of endogenous LL-37 stimulates gastric cancer cell DNA synthesis suggesting that the

**Table 1** Possible functional effects and mechanisms of action of cathelicidins in different gastrointestinal disorders

| Type of GI disorders | Functional effects   | Mechanisms   | Ref.       |
|----------------------|--|--|------------|
| Ulcer                | Increases of cell proliferation, re-epithelialization and angiogenesis                             | Activation of growth factors and their receptors   | [11,13-17] |
| Inflammation         | Decrease of pathogenic microbes, inflammatory cytokines and apoptosis; increase of mucus secretion | Activation of MAP kinase, formyl peptide receptor and mucin genes; electrostatic interaction on microbial membrane | [1,5,8,23] |
| Cancer               | Induction of apoptosis and cell cycle arrest   | Release of AIF/EndoG; activation of BMPR and Smads   | [29,30]    |

GI: Gastrointestinal; MAP: Mitogen-activated protein; AIF: Apoptosis-inducing factor; BMPR: Bone morphogenetic protein.

antiproliferative effect of LL-37 occurs at physiological concentrations. The direct anti-cancer action has also been confirmed in a gastric xenograft cancer model in nude mice<sup>[38]</sup>. In the lower GI tract, it has been shown that LL-37 is strongly expressed in the human normal colon mucosa but downregulated in colon cancer tissues. In both settings it is correlated with the number of apoptotic cells in colonic mucosa. To this end, the pro-apoptotic activity of LL-37 is confirmed in colon cancer cells in which the peptide activates a GPCR-p53-Bax/Bak/Bcl-2 signaling cascade that triggers off the AIF/EndoG-mediated apoptosis in colon cancer cells<sup>[39]</sup>. All these findings suggest that cathelicidin could be a tumor suppressor gene in the stomach and colon. Supplementation of which would have a great potential as a therapeutic agent for gastric and colon cancers.

## PERSPECTIVES AND CONCLUSION

The host defense peptide cathelicidin is highly expressed in the GI mucosa. This peptide and its recombinant protein in a deliverable preparation represent an appealing option for the treatment of inflammation and cancer and also promotion of mucosal repair in the GI tract (Table 1). This is especially true for those diseases associated with bacteria including gastritis and UC. Depletion of cathelicidin by unknown epigenetic mechanisms in the gastric and colonic tissues could be one of the causative factors in the promotion of inflammation and carcinogenesis in both organs. Supplementation with this host defense peptide orally through an effective delivery system seems to be a promising approach to treat different disorders in the GI tract.

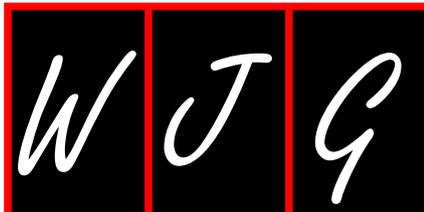
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P- Reviewer Koon HW S- Editor Wang JL L- Editor A  
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## Relationships of CDXs and apical sodium-dependent bile acid transporter in Barrett's esophagus

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Received: April 2, 2013 Revised: May 2, 2013

Accepted: May 7, 2013

Published online: May 14, 2013

### Abstract

Barrett's esophagus (BE) is characterized by intestinal metaplasia with the differentiated epithelium replaced by another type of epithelium morphologically similar to normal intestinal epithelium. The metaplasia is preceded by bile and acid reflux into the esophagus. BE is a premalignant condition associated with increased risk of esophageal cancer, especially esophageal adenocarcinoma. The Caudal-related homeodomain transcription factors Caudal-related homeodomain transcription factor CDX1 and CDX2 are expressed exclusively in the small and large intestine, playing important roles in proliferation and differentiation of intestinal epithelial cells. Ectopic expression of CDX1 and CDX2 occurs in BE. The apical sodium-dependent bile acid transporter (ASBT) is expressed primarily in terminal ileum where it is a key factor for intestinal reabsorption of bile salts. In addition to upregulation of CDX1 and CDX2, ASBT expression is up-regulated in BE. Furthermore, both CDX1/CDX2 and ASBT expressions are down-regulated in high-grade esophageal dysplasia. The alteration of the above-mentioned factors calls for attention: what is the relationship between CDXs and ASBT aberrant

expression in BE? In this commentary, we discuss this issue on basis of the recent study done by Ma *et al*.

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**Key words:** Esophagus; Intestinal metaplasia; Caudal-related homeodomain transcription factors; Apical sodium-dependent bile acid transporter; Aberrant expression

**Core tip:** Aberrant co-expression of Caudal-related homeodomain transcription factors (CDXs) and apical sodium-dependent bile acid transporter (ASBT) in the epithelium of Barrett's esophagus (BE) indicates association among these factors. Acid and bile reflux induce CDXs gene expression and can lead to formation of BE. CDX-mediated promoter activation can lead to aberrant expression of ASBT. The BE phenotype may be better than squamous epithelium to protect against refluxed acid and bile. On the other hand the BE phenotype is associated with increased risk of esophageal adenocarcinoma (EAC). Furthermore, the decreased expressions of CDXs and ASBT in high-grade esophageal dysplasia indicate that CDXs and ASBT are inhibitory factors to the progression of EAC.

Zhao J, Gregersen H. Relationships of CDXs and apical sodium-dependent bile acid transporter in Barrett's esophagus. *World J Gastroenterol* 2013; 19(18): 2736-2739 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2736.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2736>

### COMMENTARY ON HOT TOPICS

Recently, an interesting study by Ma *et al*<sup>[1]</sup> demonstrated that short interfering RNA-mediated knockdown of Caudal-related homeodomain transcription factors (CDXs) resulted in reduced apical sodium-dependent bile acid transporter (ASBT) mRNA expression in intestinal cells.

CDXs strongly induced activity of the ASBT promoter of esophageal and intestinal cells. Association with ASBT expression was found for CDX1, CDX2 and hepatocyte nuclear factor-1 $\alpha$  in Barrett's esophagus (BE) biopsies. Ma *et al*<sup>[1]</sup> concluded that CDX1 and CDX2 activate the human ASBT promoter by transcription. For the first time ASBT is added to the list of genes regulated by CDXs. We strongly recommend this paper to the readers.

BE is a clinically important disease. The human esophagus is lined by a multilayered squamous epithelium which withstands the potential damage from rapidly propelling food boluses through the esophagus and also from intermittent exposure to refluxed contents from the stomach. However, the esophageal epithelium, usually at the gastroesophageal junction, can be inflamed and injured if the esophageal epithelium chronically and repeatedly is in contact with refluxed bile and acid. This can result in intestinal metaplasia where the esophageal squamous epithelium is replaced by intestinal-type epithelium, which is the key feature of BE<sup>[2]</sup>. BE is characterized not only by the morphological intestinalization but also by changes in gene expression patterns. The intestinal specific transcription factors CDX1 and CDX2 and other intestinal proteins such as villin, sucrase isomaltase, and acidic mucins/MUC2 can be detected in human BE tissue<sup>[3]</sup>. BE is an important risk factor for esophageal adenocarcinoma (EAC)<sup>[4,5]</sup>. The molecular mechanisms related to BE are not yet fully understood. Currently, it is believed that the BE cell emerges from (1) the esophageal squamous epithelium; (2) the distal esophagus submucosal gland epithelium; (3) the proximally-migrating gastric cardia epithelium; or from (4) infiltrating bone marrow stem cells<sup>[6,7]</sup>. Hence, the mechanism of BE formation is not well understood.

ASBT is a 48-kDa transmembrane protein. At the apical membrane of ileal enterocytes, ASBT is the chief mediator of active sodium-dependent intestinal bile acid absorption<sup>[8]</sup>. The roles of ASBT on bile acid reabsorption, regulation of *ASBT* gene expression and its association with some diseases have been reviewed in detail<sup>[8-11]</sup>. ASBT is mainly expressed in the terminal ileum but is also expressed in renal tubule cells, cholangiocytes, and the gallbladder<sup>[10]</sup>. It was recently shown that the expression of ASBT is elevated in esophageal epithelial cells from BE patients whereas ASBT expression was decreased in esophageal adenocarcinoma at both mRNA and protein levels<sup>[12]</sup>. CDX1 and CDX2 are expressed exclusively in the small and large intestine, playing important roles in proliferation and differentiation of intestinal epithelial cells. The role of *CDX* genes in the gut has recently been reviewed<sup>[13]</sup>. In adults, CDX1 is expressed primarily in intestinal crypts<sup>[14,15]</sup> whereas CDX2 is expressed in the paracaecal region of the intestine. Furthermore, CDX2 is expressed relatively more in the villi than in the crypts<sup>[14]</sup>. CDX1 and CDX2 are not expressed in the normal human esophagus<sup>[16]</sup>. However, CDX1 and CDX2 levels are elevated in the epithelium of BE<sup>[16,17]</sup>. CDX2 expression has been demonstrated in all biopsy

specimens from BE without and with dysplasia, and from BE-associated adenocarcinomas<sup>[18,19]</sup>. Expression of CDX1 mRNA and protein was found in biopsy specimens from patients with BE but not from epithelium of normal esophagus<sup>[20]</sup>. Furthermore, similar to expression of ASBT, CDX2 expression decreased esophageal metaplasia progressed into adenocarcinoma<sup>[21,22]</sup>.

The aberrant co-expression of CDXs and ASBT in the BE epithelium makes us ask what is the relationship between CDXs and ASBT, how do these factors relate to BE, BE with dysplasia and BE-associated adenocarcinomas. In order to study the relationship between CDXs and ASBT, Ma *et al*<sup>[1]</sup> conducted a test series to (1) study whether endogenous human ASBT mRNA levels are regulated by CDX1 and CDX2; (2) study the possible direct role for CDX1 and CDX2 in the regulation of the ASBT promoter; (3) identify putative CDX response elements (CDXREs) within the ASBT; (4) study whether the proximal promoter region containing the predicted CDXREs mediate the CDX-dependent activation; (5) study the potential *in vitro* interaction between CDX1 and CDX2 with their predicted binding motifs within the ASBT promoter; and to (6) confirm the interaction between CDX1 and CDX2 with the ASBT promoter also within living cells. Ma *et al*<sup>[1]</sup> found in human esophageal and intestine-derived cell lines that the human ASBT promoter is a direct target for transcriptional activation by the transcription factors CDX1 and CDX2. In other words, ASBT expression is regulated by CDXs. Therefore, their study adds a new piece of knowledge to the already known complexity of transcriptional regulation of *ASBT* gene expression. Furthermore, Ma *et al*<sup>[1]</sup> discover close association of CDX and ASBT expression levels in human BE tissue. This indicate that CDX-mediated ASBT promoter activation can lead to aberrant esophageal expression of the bile acid uptake system ASBT and consequently to an increase in epithelial bile acid uptake activity by the BE mucosa.

It is well known that BE is closely associated with gastro-esophageal reflux disease (GERD). In animal models, GERD caused increased *Cdx2* expression in cells of the basal layer of esophageal squamous epithelium. The increased *Cdx2* expression preceded the development of intestinal metaplasia<sup>[23,24]</sup>. In esophageal biopsy specimens from patients with BE, Vallböhmer *et al*<sup>[17]</sup> found high levels of CDX2 mRNA in specialized intestinal metaplasia. A recent study done by Kazumori *et al*<sup>[25]</sup> shows that *Cdx1* is over-expressed in esophageal metaplastic tissue and that bile acids increase promoter activity in cultured esophageal epithelial cells. This in turn appears to induce production of *Cdx2* sufficient to cause intestinal metaplasia. It has been proposed that bile acids in refluxed contents cause tight junctions in squamous cells to break, allowing the bile acids to leak into the basal layer, resulting in cell transdifferentiation<sup>[26]</sup>.

From the above-mentioned studies it is evident that acid reflux and bile reflux contribute to increased CDX expression levels. The induction of *CDXs* genes precedes

the morphologic changes in BE. The BE phenotype may be better than squamous epithelium to protect against exposure to refluxed acid and bile. Furthermore, CDX-mediated promoter activation leads to aberrant expression of ASBT, resulting in increased epithelial bile acid uptake by the BE mucosa. However, the BE phenotype has 30-125 times increased risk of EAC compared to that of the general population<sup>[27]</sup>. Furthermore, although CDXs expression can be detected in well or moderately well differentiated EAC, expression of CDXs is decreased and may even be undetectable in poorly differentiated EAC<sup>[28,29]</sup>. Ma *et al*<sup>[11]</sup> found that ASBT like CDXs decrease its expression in high-grade esophageal dysplasia. All together this suggests that CDXs and ASBT expression have an inhibitory role for the progression of EAC. However, the exact mechanism about the effect of CDX1, CDX2 and ASBT on BE and BE-associated esophageal dysplasia is not well understood yet and need further study.

In summary, aberrant co-expression of CDXs and ASBT in BE epithelium stimulates further interest into learning more about the relationship between CDXs and ASBT and how they relate to BE. Based on reviewing the study by Ma *et al*<sup>[11]</sup> and other relevant literature, it is anticipated that ASBT gene expression is regulated by CDXs. Acid and bile reflux as well as inflammation induce CDXs gene expression preceding BE. CDX-mediated promoter activation can lead to aberrant expression of ASBT. The BE phenotype may be better than squamous epithelium to protect against refluxed acid and bile. On the other hand, BE phenotype is associated with increased risk of EAC. Furthermore, CDXs and ASBT expressions decrease in high-grade esophageal dysplasia. This indicates that CDXs and ASBT expression may be inhibitory factors for progression of EAC. Further research is needed for understanding the exact mechanism and effects of CDX1, CDX2 and ASBT on BE and BE-associated esophageal dysplasia.

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**P- Reviewers** Shi C, Chen XM **S- Editor** Gou SX **L- Editor** A  
**E- Editor** Li JY



## Persistent hypertransaminasemia in asymptomatic children: A stepwise approach

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Received: November 12, 2012 Revised: December 20, 2012

Accepted: January 17, 2013

Published online: May 14, 2013

### Abstract

We aimed to examine the major causes of isolated chronic hypertransaminasemia in asymptomatic children and develop a comprehensive diagnostic flow diagram. A MEDLINE search inclusive of publications throughout August 2012 was performed. We found only a small number of publications that had comprehensively investigated this topic. Consequently, it was difficult to construct a diagnostic flowchart similar to those already available for adults. In children, a "re-testing panel" prescription, including gamma-glutamyl transpeptidase and creatine kinase in addition to aminotransferases, is considered a reasonable approach for proficiently confirming the persistence of the abnormality, ruling out cholestatic hepatopathies and myopathies, and guiding the subsequent diagnostic steps. If re-evaluation of physical and historical findings suggests specific etiologies, then these should be evaluated in the initial enzyme retesting panel. A simple multi-step diagnostic algorithm incorporating a large number of possible pediatric scenarios, in addition to the few common to adults, is available. Accurately classifying a child with asymptomatic persistent hypertransaminas-

emia may be a difficult task, but the results are critical for preventing the progression of an underlying, possibly occult, condition later in childhood or during transition. Given the high benefit/cost ratio of preventing hepatic deterioration, no effort should be spared in diagnosing and properly treating each case of persistent hypertransaminasemia in pediatric patients.

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**Key words:** Transaminase; Aminotransferase; Hypertransaminasemia; Liver disease; Children

Vajro P, Maddaluno S, Veropalumbo C. Persistent hypertransaminasemia in asymptomatic children: A stepwise approach. *World J Gastroenterol* 2013; 19(18): 2740-2751 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2740.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2740>

### INTRODUCTION

The measurement of serum transaminase levels has become part of the routine biochemical evaluation that takes place before surgery or for the investigation of pathologies not necessarily related to liver injury in many countries. An investigation of unexpected hypertransaminasemia is important for differentiating muscular and hepatic disease; to institute timely and specific treatment for progressive, but still asymptomatic, treatable liver conditions [*e.g.*, Wilson's disease, autoimmune hepatitis (AIH), and non-alcoholic fatty liver disease (NAFLD)]; to furnish genetic counseling for hereditary disorders; and/or to setup appropriate preventive measures (*e.g.*, avoidance of viral hepatitis transmission). Moreover, prevention of possible hepatic deterioration has a high benefit/cost ratio by avoiding the need for eventual liver transplantation<sup>[1]</sup>.

Currently, the most frequent cause of hypertransaminasemia in both adults and children is obesity, although obesity-related liver disease is still sometimes erroneously

considered cryptogenic because of a poor perception of obesity among medical practitioners<sup>[2,4]</sup>. In adults, the causes of isolated hypertransaminasemia other than obesity-related liver disease are limited to viral hepatitis, toxic damage, autoimmune hepatobiliary diseases, celiac disease, Wilson's disease, and hereditary hemochromatosis<sup>[5,6]</sup>, whose diagnostic work-up is well established<sup>[4,6-11]</sup>. The problem is more complex in children, in whom individually rare genetic/metabolic conditions collectively constitute 20%-30% of liver diseases<sup>[12,13]</sup>. Therefore, persistent hypertransaminasemia in a child should alert the physician to the possibility of an underlying hepatic or multisystem metabolic disorder and prompt a referral to a specialized center for diagnostic evaluation. However, despite extensive investigation, the etiology of some cases may have to be defined as truly unknown (cryptogenic)<sup>[12,14]</sup>.

There are only a few reports examining the possible causes of isolated chronic hypertransaminasemia in asymptomatic pediatric populations<sup>[12,14-16]</sup>, and often these studies are biased by flawed inclusion and exclusion criteria, and/or an inadequate diagnostic work-up. Consequently, there is no diagnostic algorithm for the differential diagnosis of unexpected chronic hypertransaminasemia in pediatric patients.

The aim of this report is to develop a comprehensive diagnostic algorithm that includes many of the frequent causes of isolated asymptomatic hypertransaminasemia in children and adolescents. We examined current adult guidelines and expert opinions, and then performed a MEDLINE search inclusive of publications throughout August 2012 using the key words: transaminase, aminotransferase, hypertransaminasemia, liver disease, and children. We also evaluated reports from specialized tertiary pediatric hepatology centers. A secondary aim was to provide a basis for future studies on the cost/benefit ratio of diagnostic assessment procedures.

## TRANSAMINASES: BACKGROUND ON ORIGINS, LEVELS, AND THRESHOLD LEVELS

Aminotransferases are normally present in circulation at low levels. They are intracellular enzymes produced principally by hepatocytes, and their increase in serum is therefore indicative of liver cell injury. However, aspartate aminotransferase (AST) is also found in cardiac and skeletal muscles, the kidneys, brain, pancreas, and lungs, and in erythrocytes, in decreasing order of concentration. Additionally, alanine aminotransferase (ALT) is present in skeletal muscle and kidneys, but at low concentrations, and its increase in the circulation is more specific for liver damage than AST. Aminotransferase serum levels depend not only on the tissue of origin, but also on the enzyme half-life, which is longer for ALT than AST. Thus, in diseases such as muscular dystrophy, patients can have AST and ALT serum values that are elevated to the same degree, instead of the expected prevalent elevation of AST<sup>[17]</sup>.

In clinical practice, normal parameter values are within 2 standard deviations of the mean value obtained in healthy individuals. This implies that 5% of the results of healthy subjects fall outside this range. While transaminase reference intervals for adults have recently been revised<sup>[18,19]</sup>, this has not occurred for children. England *et al*<sup>[20]</sup> recently proposed ALT centiles stratified by sex and age in a healthy European population. They propose an ALT upper limit of normal of 60 IU/L in boys and 55 IU/L in girls during the first 18 mo of life. The range changes to 40 IU/L in boys and 35 IU/L in girls after the age of 18 mo. The Screening ALT for Elevation in Today's Youth (SAFETY) study conducted on a population of North American patients aged between 12 and 17 years shows that the upper limit of normal used by most laboratories for ALT is too high to detect chronic liver disease and that less than half of North American hospitals utilize gender-specific values. In that study, the ALT thresholds in use had a low sensitivity for the detection of chronic liver damage (30%-40%). Using the National Health and Nutrition Examination Survey (NHANES) ALT threshold of 25.8 IU/L for boys and 22.1 IU/L for girls, the sensitivity improved to 70%-80%, while the specificity was only reduced from approximately 90% to approximately 80%<sup>[21]</sup>.

### Clinical recommendation

In the pediatric population, there is no established reference range of ALT and AST. ALT thresholds currently in use have a low sensitivity for the detection of chronic liver damage. In teenagers, the biologically-determined and gender-specific ALT threshold of 25.8 U/L for boys and 22.1 U/L for girls established by NHANES increases this sensitivity, with only a modest specificity reduction.

## APPROACH TO ASYMPTOMATIC HYPERTRANSAMINASEMIA

Table 1 summarizes the most frequent causes of persistently-elevated transaminase levels in asymptomatic children, schematically classified under the categories of viral, autoimmune, metabolic, and other types of hepatobiliary diseases or extrahepatic causes of hypertransaminasemia.

A detailed evaluation of medical and family history and an accurate clinical examination are crucial in determining the likely etiology of hypertransaminasemia, as they may indicate towards possible muscle or liver conditions.

### Initial step: The retesting panel

A stepwise approach contemplates repeating the tests to confirm the results<sup>[5,9,10]</sup>. In adults, timing of retesting is not firmly established because it has usually been empirically guided by the degree of transaminase alterations found {mild [ $< 5$  times upper limit of normal ( $\times$  ULN)]; moderate ( $5-10 \times$  ULN); marked ( $> 10 \times$  ULN)}.

In pediatric patients, information about gamma-glutamyl transferase (GGT) rather than alkaline phosphatase values might help to determine whether the liver injury

**Table 1 Main causes of asymptomatic hypertransaminasemia in children**

| Hepatic origin  | Extrahepatic origin   |
|---|---|
| Obesity (non-alcoholic fatty liver disease)   | Duchenne/Becker muscular dystrophy (prevalence: 1:4700)                               |
| Viral infections (major and minor hepatotropic viruses)                                   | Other myopathies ( <i>e.g.</i> , caveolinopathies; prevalence: 1:14000 to 1:120000)   |
| Autoimmune liver disease (prevalence: 1:200000)   | Myocardiopathies  |
| Celiac disease and inflammatory bowel disease   | Nephropathies   |
| Wilson's disease (prevalence: 1:30000)  | Hemolytic disorders   |
| Cystic fibrosis (prevalence: 1:2500) and Shwachman-Diamond syndrome (prevalence: 1:50000) | Macro - AST (prevalence: 30% of children with isolated aspartate aminotransferasemia) |
| Alpha1 antitrypsin deficiency (prevalence: 1:7000)  |   |
| Other genetic and metabolic diseases <sup>1</sup>   |   |
| Toxic: Drugs and alcohol  |   |
| Cryptogenic hypertransaminasemia  |   |

<sup>1</sup>Refer to Table 3. AST: Aspartate aminotransferase.

is predominantly hepatocellular or cholestatic<sup>[5]</sup>. Creatine phosphokinase (CPK) should also be evaluated to rule out occult muscle disease<sup>[22]</sup>.

As in adults<sup>[5,10,23]</sup>, in many asymptomatic children, abnormal values can show normal values when retested<sup>[12,14-16,23]</sup>. The reported percentage of patients who normalize aminotransferase serum values within 6 mo from the first detection of abnormality ranges from 26% to 73.6%<sup>[12,14-16]</sup>. A fluctuating pattern (transient/self-limiting or intermittent) is frequently observed at all ages, and more than one retesting may be warranted even for a mild increase of transaminases. In areas with a high prevalence of hepatitis B (HBV) and C (HCV) virus infection and in high-risk populations, it is advisable to request viral markers at the time of repeat testing to accelerate the screening protocol<sup>[9,10]</sup>. Recently, Senadhi<sup>[24]</sup> recommended that HBV and HCV screening is warranted in all asymptomatic patients with mild transaminase elevations.

In selected patients who participate in strenuous sports, liver tests should be repeated after at least one week without exercise, especially if hypertransaminasemia was associated with high CPK or with elevation of other enzymes of muscle origin<sup>[25]</sup>. If high CPK and hypertransaminasemia are confirmed, it is mandatory to exclude muscular diseases, which are often clinically asymptomatic during the first 5-6 years of life and can be recognized only after a detailed and oriented neurologic examination<sup>[22]</sup>. In addition to the well-known muscular dystrophies, myocyte injury, necrosis induced by drugs or toxins, and increased exercise are possible causes of high CPK and hypertransaminasemia. Additionally, some mitochondrial, endocrine and metabolic myopathies, and gluten enteropathy are also causes of high CPK and hypertransaminasemia. A serum elevation of CPK ranging from 450 to 5000 U/L (normal upper limit: 150 U/L) accompanying isolated asymptomatic hypertransamina-

semia can also be a marker of a caveolinopathy, a group of newly described and still poorly understood muscle diseases that affect the limb-girdle, distal muscles, and heart. A diagnosis may be particularly challenging in pediatric patients that are only mildly symptomatic<sup>[26]</sup>.

### First, second, and third line investigations

The evaluation of patients with confirmed hypertransaminasemia should include first (and eventually second and third) line investigations (Table 2). If the patient's history or physical examination suggests a particular disease, selected first-line investigations should already be part of the retesting panel. However, some authors suggest that first-line exams for hepatocellular causes of liver disease should be performed without the need to confirm hypertransaminasemia in patients with increased serum levels of ALT > 3-5 × ULN<sup>[27]</sup>. Historically, a hypertransaminasemia duration of approximately 6 mo has been arbitrarily used to determine chronic liver disease. However, it is unwise to wait for 6 mo before investigating a possible cause of liver damage, as some hepatopathies, such as autoimmune liver disease or Wilson's disease, can become rapidly life-threatening without appropriate treatment<sup>[28]</sup>.

In the case of protracted isolated AST elevation, the possibility of macro-AST should be investigated by polyethylene glycol and/or electrophoresis testing. Macro-AST is a condition characterized by the presence in serum of a macromolecular complex formed by association with other plasma components [*e.g.*, immunoglobulin G (IgG)] or by self-polymerization. Due to their large size, macro-AST components cannot be filtered by renal glomeruli and are retained in the plasma. The condition is benign, but may cause diagnostic uncertainty and lead to useless, invasive, expensive, and time-consuming testing<sup>[29]</sup>.

### Clinical recommendation

Aminotransferase levels should be retested upon finding hypertransaminasemia in asymptomatic patients. At this time, the levels of CPK (for muscular disease) and GGT (for biliary involvement) should also be evaluated. An accurate clinical history and physical examination are of paramount importance for guiding an appropriate investigation and avoiding expensive and unnecessary tests.

## MOST COMMON LIVER DISEASES

### Non-alcoholic fatty liver disease

NAFLD is the most common cause of hypertransaminasemia in children and adolescents<sup>[12,14,16,21]</sup>. In clinical practice, NAFLD is usually suspected by the finding of hypertransaminasemia and ultrasonographic fatty liver in an obese child<sup>[30,31]</sup>. Although the ALT serum level is a useful diagnostic tool, it is not a sensitive marker of NAFLD. It is common to observe the entire histological spectrum of NAFLD in patients with normal ALT levels<sup>[22,32]</sup>. The evaluation of both AST and ALT values is important because an increased AST/ALT ratio has been reported to reflect a progressive and more serious condition [fibrotic

**Table 2 Retesting panel; first, second, and third line investigations in children with asymptomatic mild hypertransaminasemia**

| Retesting panel <sup>1</sup> | First line panel   |   | Second and third line panels  |
|------------------------------|--|---|---|
|                              | Liver function tests   | Etiology tests  |   |
| ALT                          | Conjugated and unconjugated bilirubin  | Viral markers (HAV, HBV, HCV)                           | Urinary copper, molecular ATP B7 analysis   |
| AST                          | Protein electrophoresis  | Minor hepatotropic viruses serology                     | HCV RNA, HBV DNA  |
| CPK                          | Serum albumin  | ( <i>e.g.</i> , EBV, CMV)                               | Genetic and metabolic enlarged screening <sup>2</sup> (“non-alcoholic fatty liver disease bin”) |
| GGT                          | Prothrombin time and partial thromboplastin time                               | Ceruloplasmin, serum copper                             | Sweat test  |
|                              | Blood cell count   | ANA, SMA, LKM, LCI, anti-SLA, total IgG                 | Fecal elastase, steatocrit  |
|                              | Hepatic ultrasonography  | Serum $\alpha$ 1 antitrypsin                            | Other hepatic imaging techniques (MRI, ERCP, CT, <i>etc.</i> )                                  |
|                              | If only AST elevation is confirmed: PEG test and electrophoresis for macro-AST | EMA, tTgasi IgA, deamidated AGA IgA (< 2 yr), total IgA | Liver biopsy <sup>3</sup><br>Jejunal biopsy (after celiac disease serology)                     |

<sup>1</sup>After at least one week off from exercise; <sup>2</sup>Genetic and metabolic enlarged screening: blood gases, lactic acid, serum ammonium concentrations, blood and urinary amino acids, urinary reducing substances, urinary organic acids, transferrin isoforms, screening test for congenital disorders of glycosylation,  $\alpha$ 1 antitrypsin phenotype; <sup>3</sup>Modified from the reference of 28. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; PEG: Polyethylene glycol; CPK: Creatine kinase; GGT: Gamma-glutamyl transferase; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; EBV: Epstein-Barr virus; CMV: Cytomegalovirus; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; LKM: Anti-microsomal antibodies; LCI: Anti-liver cytosol antibodies type 1; SLA: Soluble liver antigen; IgG: Immunoglobulin G; EMA: Anti-endomysial antibodies; tTgasi: Anti-tissue transglutaminase antibodies; AGA: Anti-gliadin antibodies; IgA: Immunoglobulin A; ATP B7: ATP binding protein 7; MRI: Magnetic resonance imaging; ERCP: Endoscopic retrograde cholangiopancreatography; CT: Computed tomography.

**Table 3 Fatty liver disease; possible causes in likely asymptomatic children and adolescents**

| General or systemic                   | Genetic-metabolic causes                     | Drugs/chemicals               |
|---------------------------------------|--|-------------------------------|
| Obesity                               | Cystic fibrosis                              | Ethanol                       |
| Metabolic syndrome                    | Shwachman syndrome                           | Ecstasy, cocaine, solvents    |
| Obstructive sleep apnea               | Wilson’s disease                             | Nifedipine                    |
| Polycystic ovary syndrome             | Alpha 1-antitrypsin deficiency               | Diltiazem                     |
| Diabetes mellitus type 1              | Fructosemia                                  | Estrogens                     |
| Thyroid disorders                     | Cholesterol ester storage disease            | Corticosteroids               |
| Hypothalamic-pituitary disorders      | Glycogen storage disease (type I, VI and IX) | Methotrexate                  |
| Inflammatory bowel disease            | Mitochondrial and peroxisomal defects        | Prednisolone                  |
| Celiac disease                        | $\alpha$ - and $\beta$ -oxidation defects    | Valproate                     |
| Protein calorie malnutrition          | Organic acidosis                             | Vitamin                       |
| Rapid weight loss                     | Abeta or hypobetalipoproteinemia             | Zidovudine and HIV treatments |
| Anorexia nervosa                      | Porphyria cutanea tarda                      | Solvents                      |
| Small intestinal bacterial overgrowth | Homocystinuria                               | Pesticides                    |
| Hepatitis C                           | Familial hyperlipoproteinemias               |                               |
|                                       | Bile acids synthesis defects                 |                               |
|                                       | Congenital disorders of glycosylation        |                               |
|                                       | Citrin deficiency                            |                               |
|                                       | Turner syndrome                              |                               |

Modified from the references of 30 and 38. HIV: Human immunodeficiency virus.

non-alcoholic steatohepatitis (NASH)]<sup>[33]</sup>.

Due to the worldwide obesity epidemic, obesity may be erroneously accepted as a normal condition even in the presence of abnormal liver function tests<sup>[2]</sup>. Conversely, in the presence of obesity, all other conditions associated with abnormal transaminase levels, such as celiac disease<sup>[34,35]</sup>, Wilson’s disease<sup>[36]</sup>, autoimmune hepatitis<sup>[37]</sup>, or muscular diseases<sup>[22]</sup>, must be investigated and excluded. In many of these diseases, prompt treatment will avoid irreversible disease progression<sup>[38]</sup> (Table 3).

Abdominal ultrasound and liver function tests should be performed as a first step in the diagnosis of possible NAFLD in children<sup>[39]</sup>. Ultrasonography, however, detects fat levels above 30% only. Waist circumference is a valid surrogate marker of central obesity, which is closely correlated with liver involvement<sup>[40]</sup>. When pediatric NAFLD is suspected, other liver diseases should be excluded

based on an age-driven algorithm<sup>[39]</sup>. In obese children and adolescents with disordered breathing during sleep, sleep apnea syndrome should be viewed as a risk factor for NAFLD. Interestingly, weight loss, avoidance of a sedentary life, and effective treatment of sleep apnea syndromes resulted in a significant improvement of liver enzyme levels<sup>[41,42]</sup>.

**Clinical recommendation:** NAFLD and NASH are diagnoses of exclusion even in obese children with hypertransaminasemia. Other fatty liver causing diseases should be investigated based on the patient’s age and clinical features.

**Viral infections**

Serological markers of hepatitis viruses are part of the first-line etiology investigation panel (Table 2). A positive

history of blood or blood-derivative transfusion, tattooing, ingestion of potentially-contaminated food, and the ethno-geographic origin of the patients give a clue as to a possible infectious etiology. Although immigration and international adoption from endemic areas are well-recognized risk factors, hepatitis B shows an emerging presentation of subclinical chronic hepatitis B in children<sup>[43]</sup>.

In countries where universal vaccination against HBV has been introduced, a significantly-reduced rate of HBV infection in children and mother-to-infant transmission has been observed. However, international adoption and immigration maintain an ample reservoir of HBV infection. Although significantly less infectious than HBV, HCV infection remains a widespread problem in the absence of an effective vaccine. In Western countries, perinatal transmission is currently the primary mode of HCV spread in children, which accounts for approximately 65% of cases<sup>[44]</sup>. Although hypertransaminasemia may suggest HCV infection, adult<sup>[45]</sup> and pediatric HCV carriers<sup>[46]</sup> may have normal or fluctuating aminotransferase levels even in the presence of serious liver damage.

Among the conditions falling within the category of adult cryptogenic hypertransaminasemia is a new entity called “occult” HCV infection. Said entity is characterized by fluctuating serum levels of HCV RNA, while the virus genome and its replicative intermediate RNA-negative strand are detectable in the liver and in peripheral blood mononuclear cells with or without antibodies to HCV<sup>[47]</sup>. Similarly, “occult” HBV infection has been recognized in patients with cryptogenic hepatitis B surface antigen (HBsAg)-negative chronic liver disease<sup>[48]</sup>. Intrahepatic replicative activity (HBV covalently closed circular DNA and pregenomic RNA) has recently been described in adults with occult hepatitis B infection. HBV occult infection seems to be relatively frequent in immunized children born to HBsAg-positive mothers. HBsAg negativity is not sufficient for excluding the presence of HBV DNA. These findings emphasize the importance of considering occult HBV infection in hypo-endemic areas<sup>[49]</sup>.

In pediatric patients, hepatitis A virus infection may be followed by up to 1 year intermittent hypertransaminasemia due to relapsing viremia, which does not evolve to chronic liver damage<sup>[50]</sup>. Other hepatotropic viruses are a frequent cause of liver disease. Epstein-Barr virus and/or cytomegalovirus should be excluded in patients with a history of fever, lymphadenopathy (mainly latero-cervical), pharyngitis, asthenia, and/or hepatosplenomegaly. In patients with paucisymptomatic immunodeficiency, coxsackievirus and adenovirus may be the cause of liver enzyme alteration. Gastrointestinal infection by rotavirus can be accompanied by self-limiting hepatic and non-hepatic transaminase elevation in 20% of infected patients<sup>[51]</sup>.

Currently minor, rather than major, hepatotropic viruses are a common cause of hypertransaminasemia in children in developed countries<sup>[12,15,16]</sup>.

**Clinical recommendation:** Viral serological markers are part of the first-line investigation panel.

### Toxic causes

An accurate medical history is crucial in determining the possible role of toxin-induced hepatotoxicity in children with hypertransaminasemia<sup>[52]</sup>. In adults, diagnostic scores have been proposed and evaluated to define the association between drugs and liver disease<sup>[53,54]</sup>. Alcohol abuse can induce liver disease and hypertransaminasemia even in childhood, especially in adolescents<sup>[6]</sup>. It has been suggested that elevated serum GGT levels, and/or an AST/ALT ratio > 1, and/or an increase in mean corpuscular volume may help in identifying excessive drinking<sup>[55]</sup>. Carbohydrate deficient transferrin is another tool for identifying unhealthy occult drinking<sup>[56]</sup>. If medication or alcohol are suspected causes of hypertransaminasemia in an adolescent, aminotransferases should be retested after 6-8 wk of controlled or monitored abstinence<sup>[57]</sup>. In addition to prescribed or over-the-counter medications and herbal remedies, illegal drugs or substances of abuse should be considered in differential diagnoses and be carefully investigated<sup>[58,59]</sup>.

### Autoimmune liver disease

AIH is a progressive inflammatory liver disease without a known etiology, and is characterized histologically by interface hepatitis and serologically by high levels of transaminases, IgG, and positive autoantibodies<sup>[60]</sup>. The exact prevalence of autoimmune hepatitis is unknown, but it is approximately one in 200000 in the general population of the United States<sup>[61]</sup>. Sometimes, the histology of autoimmune hepatitis is associated with bile duct injury-determining overlap syndrome or autoimmune sclerosing cholangitis (ASC); this condition is different from primary sclerosing cholangitis, which is characterized by inflammation and fibrosis in the intrahepatic and/or extrahepatic bile load in the absence of interface hepatitis<sup>[62,63]</sup>. It is important to perform a cholangiography in all children with the histological features of autoimmune hepatitis, as ASC is as prevalent as AIH in childhood, and thus only cholangiography can differentiate between these conditions<sup>[64]</sup>. There are two types of AIH classified according to antibody profile: type 1 [anti-nuclear antibodies and anti-smooth muscle antibodies (SMA)] and type 2 [anti-liver kidney microsomal antibody (LKM1); and/or antibodies to liver cytosol type 1 (anti-LC1)]<sup>[65]</sup>. Anti-soluble liver antigen (anti-SLA) antibodies can be positive in otherwise autoAb-negative patients. These antibodies cannot be detected by immunofluorescence, and require enzyme-linked immunosorbent assay and immunoassays for identification. Type 2 may tend to be more severe and prevalent in children, adolescents, and young adults than in the older population<sup>[60]</sup>. The absence of autoantibodies in a child with hypertransaminasemia does not exclude the diagnosis of autoimmune hepatitis. In fact, seronegative but empirically steroid-responsive autoimmune hepatitis has been reported<sup>[66-68]</sup>. Conversely, the increase in serum gamma-globulins is not universal in AIH. As for Wilson's disease, the diagnosis of autoimmune hepatitis may be difficult because it is not based on specific markers. Consequently, a scoring system

has been devised<sup>[69,70]</sup> that gives positive predictive values for females, the presence of other autoimmune diseases, hypergammaglobulinemia, and positivity for ANA, SMA, LKM1, LC1 and ASLA. It also gives negative scores for viral markers and a positive history for drugs and alcohol use. A simpler score based on only four items<sup>[71]</sup> has not yet been fully validated in children. Primary biliary cirrhosis is not observed during childhood, although very rare cases of anti-mitochondrial autoantibody positivity have been reported<sup>[72]</sup>.

AIH (and ASC) may present with only hypertransaminasemia, which can occur in children with apparent good health. It should therefore be investigated with appropriate examinations; if left untreated, cirrhosis may develop.

**Clinical recommendation:** Autoimmune hepatitis should be rapidly identified in order to administer prompt therapy and avoid cirrhotic evolution. Hypertransaminasemia and hypergammaglobulinemia may be the only findings of seronegative autoimmune hepatitis. It is important to perform a cholangiography in all children with the histological features of autoimmune hepatitis because, in childhood, autoimmune sclerosing cholangitis is as prevalent as autoimmune hepatitis.

### *Celiac disease and hypertransaminasemia*

Celiac disease may be associated with liver involvement in both adults and children. Isolated hypertransaminasemia may be the first manifestation of clinically silent celiac disease<sup>[73,74]</sup>. It is currently controversial<sup>[75]</sup> as to whether, in children less than 2 years old, AGA-IgA and IgG should be tested, because of a diagnostic sensitivity higher than that of anti-endomysium antibodies and anti-transglutaminase antibodies at that age. Selective non-responsiveness to HBV immunization in a hypertransaminasemic child may be a clue to undiagnosed celiac disease<sup>[76]</sup>.

So-called “celiac hepatitis” is the most common hepatopathy in celiac patients and is characterized by a moderate increase of transaminase levels usually associated with minimal and non-specific liver lesions of the lobule and portal tracts. A gluten-free diet generally results in normalization of the liver enzymes and repair of histological damage. Due to high disease prevalence, patients with a known diagnosis of celiac disease and persistent hypertransaminasemia should be tested for other possible causes of liver damage before ascribing liver function test abnormalities to celiac disease. Co-existing causes of hepatopathy have been reported<sup>[77]</sup>. Celiac disease may present as hepatic steatosis in obese children with hypertransaminasemia resistant to weight loss. In such cases, the addition of a gluten-free diet is necessary to resolve their liver abnormalities<sup>[34]</sup>.

Celiac disease can be associated with a variety of autoimmune liver diseases, including AIH, autoimmune cholangitis, and overlap syndromes, with a frequency of AIH peaking at 2.9% in celiac disease children less than 10 years old<sup>[74]</sup>. Autoimmune hepatic involvement can ei-

ther precede or follow the diagnosis of celiac disease. It is necessary to diagnose AIH associated with celiac disease promptly, because it is not responsive to a gluten-free diet alone and requires long-term associated immunosuppressive therapy<sup>[78]</sup>.

### *Wilson's disease and hypertransaminasemia*

The prevalence of Wilson's disease is estimated at one in 30000 in most populations (Table 4). The prevalence is as high as one in 10000 in China, Japan, and Sardinia<sup>[79]</sup>. It may present at any age. Usually, the onset of symptomatic liver disease is at approximately 12 years of age<sup>[80]</sup>. It is only during adolescence or early adult life that patients usually present with complicating neurological and psychiatric manifestations. The most important laboratory diagnostic clues are hypoceruloplasminemia (< 20 mg/dL in 85%-95% cases), increased free serum copper, increased intrahepatic copper (> 250 µg/g dry weight), and increased basal and post-penicillamine challenge urinary copper excretion<sup>[81,82]</sup>. These findings are not specific if considered individually, and several conditions may be responsible for false negative and false positive results. Therefore, a score (the “Ferenci score”), which takes into account the Kayser-Fleischer ring, neuropsychiatric symptoms, the occurrence of Coombs negative hemolytic anemia, increased urinary copper, decreased serum ceruloplasmin, increased copper content of hepatocytes, and the presence of causative mutations, has been devised to distinguish between an unlikely/probable/highly-likely diagnosis of Wilson's disease<sup>[81]</sup>. More recently, a new cut-off value for urinary copper excretion in asymptomatic children with Wilson's disease has been suggested. The new value of 40 µg/24 h replaces the previously-used 100 µg/24 h<sup>[83,84]</sup>. The post-penicillamine challenge urinary copper estimation has been reported to be poorly sensitive in asymptomatic children<sup>[84]</sup>. A molecular diagnosis and/or haplotype analysis of the region surrounding ATP7B on chromosome 13 should be considered in children with enigmatic liver disease, especially those with features of NAFLD<sup>[84,85]</sup>. Wilson's disease-like hypoceruloplasminemic liver disease has been recently described in congenital disorders of glycosylation (CDG) type X<sup>[84,86,87]</sup>. Non-Wilsonian high urinary copper excretion has been reported in pediatric nodular regenerative hyperplasia<sup>[84]</sup>.

**Clinical recommendation:** The criteria adopted for the diagnosis of Wilson's disease are non-specific if considered individually. The Ferenci score will distinguish between unlikely, probable, and highly-likely diagnoses of Wilson's disease. New pediatric cut-off values and the real value of post-penicillamine urinary test in asymptomatic cases need careful consideration.

## **OTHER DISEASES**

Disorders, such as inborn errors of metabolism and/or congenital conditions affecting the liver, are much more

**Table 4 Clinical and laboratory findings for orienting diagnosis of some genetic metabolic liver diseases**

| Clinical/laboratory findings                                 | Possible genetic-metabolic cause             | Prevalence                 | Liver involvement |
|--|--|----------------------------|-------------------|
| Pancreatic failure, hematological disorders                  | Shwachman syndrome                           | 1:50000                    | +++ <sup>1</sup>  |
| Asymptomatic, hemolysis                                      | Wilson's disease                             | 1:30000                    | +++               |
| Previous neonatal cholestasis, hepatomegaly                  | Alpha 1 antitrypsin deficiency               | 1:7000                     | +++               |
| Hypoglycemia, hepatomegaly                                   | Glycogen storage disease (type I, VI and IX) | From 1:100000 to 1:1000000 | +++               |
| Fructose refusal, hepatomegaly                               | Hereditary fructose intolerance              | 1:20000                    | +++               |
| Lethargy, increased serum ammonia levels                     | Urea cycle defects                           | 1:30000 (all disorders)    | ++                |
| Lethargy, increased serum ammonia levels                     | Urea cycle defects                           | 1:30000 (all disorders)    | ++                |
| Chubby face, fatty liver, specific serum amino acids pattern | Citrin deficiency                            | 1:20000 in East Asia       | ++                |
| Failure to thrive, lactic acidosis                           | Mitochondrial diseases                       | 1:8500 (all disorders)     | +                 |
| Failure to thrive, ketoacidosis, hypoglycemia                | Organic acidosis                             | 1:1000 (all disorders)     | +                 |
| Mild coagulopathy, clinical phenotype                        | Congenital disorders of glycosylation        | From 1:10000 to 1:100000   | +                 |
| Short stature, female gender, karyotype                      | Turner syndrome                              | 1:2000                     | +                 |
| Failure to thrive, positive sweat test                       | Cystic fibrosis                              | 1:2500                     | +                 |

<sup>1</sup>First 1-2 yr of life; +: Possible; ++: Frequent; +++: Almost always.

common in pediatric patients than in adults (Table 3). These diseases are rare when considered individually, but represent a large group if considered collectively. It is difficult to establish their incidences; in most cases, they are relatively asymptomatic and may therefore remain undiagnosed<sup>[88]</sup>. Although many etiologies present in the neonatal period with cholestasis or acute illness, several may become manifest only later in infancy or childhood. Extreme care is required to avoid misdiagnosing these cases. This is particularly true if the patients have NAFLD-like symptoms that risk being considered part of the NASH syndrome<sup>[58]</sup>. Laboratory investigations for genetic metabolic diseases that may often be responsible for a NAFLD-like fatty liver picture should be guided by age and clinical/family history (Table 4). We will comment only on the most relevant metabolic liver diseases and refer the reader to a series of comprehensive reviews for specific information regarding the less common ones<sup>[89,90]</sup>.

Hepatic derangement with severe, but self-limiting, hypertransaminasemia and variable histological patterns may be the sole initial evident manifestation of Shwachman-Diamond syndrome (incidence: 1:50000 worldwide). The mechanisms that can contribute to liver damage in these patients are not known<sup>[91]</sup>. Citrin deficiency (incidence: 1:20000 in East Asia), a condition now also recognized in Western countries, may present with a pattern of neonatal cholestasis and increased levels of blood citrulline, or NAFLD in children and adolescents<sup>[92]</sup>. Hereditary fructose intolerance (incidence: 1:20000 worldwide) typically occurs with a pattern of early-onset cholestasis during weaning. However, it may present later in patients who spontaneously follow a low fructose diet because of instinctive fructose refusal/dislike or avoidance. In these cases, medical observation may be dictated by the incidental finding of hypertransaminasemia, hepatomegaly, and/or bright liver by ultrasound observation. Feeding history is crucial for the diagnosis, which is confirmed by molecular analysis of gene mutations<sup>[93]</sup>. Mitochondrial diseases are often multisystem, but some cases may present with exclusive or prevalent mild liver involvement [*e.g.*, mitochondrial DNA depletion syndrome due

to deoxyguanosine kinase (DGUOK, OMIM 251880)]. Some congenital disorders of glycosylation (incidence: 1:50000-1:100000) may present as chronic isolated hypertransaminasemia. Children with clinically asymptomatic, cryptogenic hypertransaminasemia with liver steatosis-fibrosis and mild coagulopathy should be screened for CDG<sup>[86,87]</sup>. Congenital hepatic fibrosis (CHF, incidence: unknown) is a developmental disorder of the portobiliary system. It is one of the fibropolycystic diseases, which include Caroli disease, autosomal dominant polycystic kidney disease, and autosomal recessive polycystic kidney disease. Clinically, it is characterized by non-cirrhotic portal hypertension, and rarely complicated by (porto-) pulmonary hypertension and hepatopulmonary syndrome. Hepatocellular function is relatively well-preserved, unless cholangitic episodes are present. Hereditary familial hemochromatosis (incidence: 1:20000 worldwide) most often presents after the transition to maturity, and is well-discussed in studies on adult patients and outside the scope of this article. Alpha 1-antitrypsin deficiency (incidence: 1:7000) presents clinical symptoms only in a minority of affected people. In infancy, the most common presentation is cholestasis. During childhood, it may present with minimally symptomatic disease that becomes significant liver disease only in 10%-15% of patients, often after several years of a near-normal quality of life, and may progress to decompensated liver disease<sup>[94]</sup>. Glycogenosis types VI and IX may be associated with elevated ALT and a soft hepatomegaly that is often not discernible at clinical examination, but without the gross metabolic abnormalities that are typically observed in the other types of glycogenosis. Cystic fibrosis rarely has a prevalent or exclusive hepatic presentation<sup>[95]</sup>. Nodular regenerative hyperplasia is an infrequently-identified liver disease characterized by non-fibrotic nodular hepatocyte regeneration, secondary portal hypertension, and mild stable abnormalities of liver function tests. In adults, it is usually associated with malignant prothrombotic or rheumatological conditions. These associations are rarely encountered in pediatric practice. The diagnosis is sometimes suggested by minimal histological changes

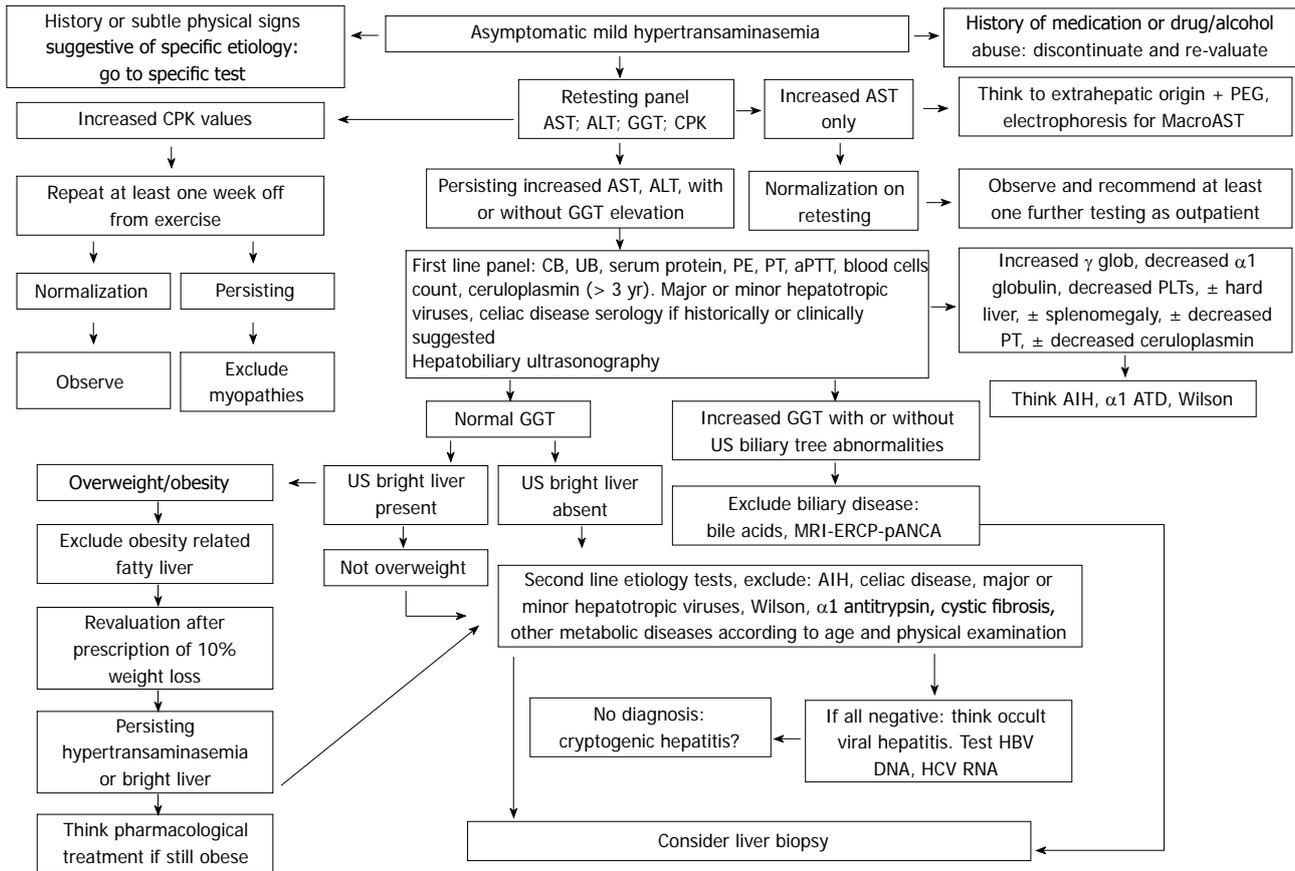


Figure 1 Diagnostic algorithm for the diagnosis of pediatric mild chronic asymptomatic hypertransaminasemia. Modified from the reference of 28. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CB: Conjugated bilirubin; UB: Unconjugated bilirubin; CPK: Creatine kinase; GGT: Gamma-glutamyl transferase; PE: Pulmonary embolism; PEG: Polyethylene glycol; PT: Prothrombin time; PTT: Partial thromboplastin time; US: Ultrasound; MRI: Magnetic resonance imaging; ERCP: Endoscopic retrograde cholangiopancreatography; pANCA: Perinuclear anti-neutrophil cytoplasmic antibodies; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AIH: Autoimmune hepatitis;  $\alpha$ 1 ATD:  $\alpha$ 1-antitrypsin deficiency.

(*e.g.*, sinusoidal dilation)<sup>[96]</sup>.

## TRANSAMINITIS AND CRYPTOGENIC HEPATITIS

The term “transaminitis” was coined to describe overall liver enzyme leakage without hepatotoxic consequences in adult patients receiving drug therapy of any type. Hypertransaminasemia is due to an unknown effect of the drug (*e.g.*, changes in hepatocellular membranes due to lipid lowering) or underlying conditions (*i.e.*, fatty liver)<sup>[97]</sup>. Childhood “cryptogenic” hepatitis appears to be a symptomless disease characterized by isolated hypertransaminasemia that onsets during the first 4 years of life, and mild to moderate histologic liver lesions. Although the frequency of spontaneous remissions is low, childhood chronic cryptogenic hepatitis appears, in the short-term, to be a non-progressive disease. This diagnosis can only be attempted once all other known causes of liver disease are excluded<sup>[14,15]</sup>.

## DISCUSSION AND CONCLUSION

Given the large number of pediatric conditions that may

be responsible for isolated persistent hypertransaminasemia, one can conclude that even asymptomatic children should undergo an intensive liver work-up. Based on the possibility of a fluctuating pattern, it seems reasonable to repeat ALT and AST testing to confirm hypertransaminasemia, rather than embarking on expensive investigations that may prove to be useless. An enzyme panel costs approximately \$30, whereas tests to identify only some of the most common causes of elevated liver enzymes (such as serology for hepatitis B and C infection and ultrasonography for NAFLD) cost approximately \$400<sup>[10]</sup>. However, the cost-benefit considerations of a stepwise diagnostic approach *vs* a simultaneous (and timesaving) testing approach in children should not deter from the need to avoid repeated vein punctures, which is often a traumatic experience. As seen in patients with a fever of unknown origin, in asymptomatic children with cryptogenic hypertransaminasemia, ordering investigations as screening procedures in the hope that something abnormal will be identified might have a number of disadvantages. These disadvantages include: possible adverse reactions or complications, loss of the patient’s faith in the medical staff, high testing costs, and a soporific effect on the doctor’s diagnostic mental activities<sup>[98]</sup>.

The prescription of a “retesting panel”, which in-

cludes the determination of GGT and CPK in addition to aminotransferase levels, has the advantage of confirming the persistence of the abnormality, helping to rule out, at least in part, cholestatic hepatopathies and myopathies, and guiding the subsequent diagnostic steps that are shown in Figure 1. Testing serum bile acids and cholangiography are other means to better assess cholestasis. If reassessment of physical and anamnestic findings suggests specific etiologies, these should be checked in the initial enzyme retesting panel (*e.g.*, viral serologies or hepatorenal ultrasonography for viral hepatitis and NAFLD, respectively). In the presence of even subtle symptoms or signs (*e.g.*, jaundice, ascites, pruritus, hepatomegaly, and/or splenomegaly), complete testing to identify the possible cause of liver disease should be included in the initial retesting.

The first line panel in asymptomatic hypertransaminasemic patients should consist of liver ultrasonography, liver function tests, and a number of investigations for the most frequent etiologies. Second and third line investigations are justified either by the inconclusive first line panel or to explore specific plausible conditions. Liver biopsy is part of these panels, but its exact timing and role remains a controversial issue<sup>[28,39,99-101]</sup>. It has been shown that in those patients with negative etiological investigations, a liver biopsy will most likely not add further useful information<sup>[10,15]</sup>, and considering that a percutaneous liver biopsy samples only 1:50000 of the liver, sampling error is an obvious limitation which can lead to misdiagnosis and staging inaccuracies<sup>[102]</sup>. The competence of the pediatric liver disease pathologist is paramount. Steatosis of the liver in a non-obese individual may suggest a metabolic/genetic hepatopathy<sup>[14,38]</sup>.

In conclusion, here we provide an overview of pediatric persistent hypertransaminasemia and list a series of metabolic, genetic, gastrointestinal, and extrahepatic causes that should be taken into account in clinical practice. The number of these etiologies constitutes a wider field of what one usually considers in adulthood. Importantly, information derived from the combination of the patient's history, physical examination, and basic laboratory data are necessary to reach a timely and correct diagnosis. We also provide a stepwise approach that should always be guided by clinical scenarios.

## ACKNOWLEDGMENTS

We are grateful to Professor Giorgina Mieli Vergani (London) for thoughtful discussion and suggestions.

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**P- Reviewer** Yu DZ **S- Editor** Song XX **L- Editor** Rutherford A  
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## Efficacy and safety of over-the-scope clip: Including complications after endoscopic submucosal dissection

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Received: December 26, 2012 Revised: February 22, 2013

Accepted: March 6, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To retrospectively review the results of over-the-scope clip (OTSC) use in our hospital and to examine the feasibility of using the OTSC to treat perforations after endoscopic submucosal dissection (ESD).

**METHODS:** We enrolled 23 patients who presented with gastrointestinal (GI) bleeding, fistulae and perforations and were treated with OTSCs (Ovesco Endoscopy GmbH, Tuebingen, Germany) between November 2011 and September 2012. Maximum lesion size was defined as lesion diameter. The number of OTSCs to be used per patient was not decided until the lesion was completely closed. We used a twin grasper (Ovesco Endoscopy GmbH, Tuebingen, Germany) as a grasping device for all the patients. A 9 mm OTSC was chosen for use in the esophagus and colon, and a 10 mm device was used for the stomach, duodenum and rectum. The overall success rate and complications were evaluated, with a particular emphasis on patients who had

undergone ESD due to adenocarcinoma. In technical successful cases we included not only complete closing by using OTSCs, but also partial closing where complete closure with OTSCs is almost difficult. In overall clinical successful cases we included only complete closing by using only OTSCs perfectly. All the OTSCs were placed by 2 experienced endoscopists. The sites closed after ESD included not only the perforation site but also all defective ulcers sites.

**RESULTS:** A total of 23 patients [mean age 77 years (range 64-98 years)] underwent OTSC placement during the study period. The indications for OTSC placement were GI bleeding ( $n = 9$ ), perforation ( $n = 10$ ), fistula ( $n = 4$ ) and the prevention of post-ESD duodenal artificial ulcer perforation ( $n = 1$ ). One patient had a perforation caused by a glycerin enema, after which a fistula formed. Lesion closure using the OTSC alone was successful in 19 out of 23 patients, and overall success rate was 82.6%. A large lesion size (greater than 20 mm) and a delayed diagnosis (more than 1 wk) were the major contributing factors for the overall unsuccessful clinical cases. The location of the unsuccessful lesion was in the stomach. The median operation time in the successful cases was 18 min, and the average observation time was 67 d. During the observation period, none of the patients experienced any complications associated with OTSC placement. In addition, we successfully used the OTSC to close the perforation site after ESD in 6 patients. This was a single-center, retrospective study with a small sample size.

**CONCLUSION:** The OTSC is effective for treating GI bleeding, fistulae as well as perforations, and the OTSC technique proved effective treatment for perforation after ESD.

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**Key words:** Over-the-scope clip; Gastrointestinal bleed-

ing; Endoscopic submucosal dissection complications; Gastrointestinal fistulae; Gastrointestinal perforation

**Core tip:** We have reported our experiences with the over-the-scope clip (OTSC) and the outcomes of several cases in Japan. The aims of the present study were to retrospectively review the results of OTSC use in our hospital and to examine the feasibility of using the OTSC to completely close perforations after endoscopic submucosal dissection for early gastrointestinal cancers.

Nishiyama N, Mori H, Kobara H, Rafiq K, Fujihara S, Kobayashi M, Oryu M, Masaki T. Efficacy and safety of over-the-scope clip: Including complications after endoscopic submucosal dissection. *World J Gastroenterol* 2013; 19(18): 2752-2760 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2752.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2752>

## INTRODUCTION

Historically, the standard treatment for gastrointestinal (GI) perforations has been surgery. Recent, invasive endoscopic treatments, such as endoscopic submucosal dissection (ESD) and natural orifice transluminal endoscopic surgery (NOTES), have provided alternative approaches to surgery. Our recent attempts to develop a new device, such as a full-thickness suturing device, constitute progress in the development of NOTES<sup>[1-4]</sup>. In addition, several devices are currently available for endoscopic management<sup>[5,6]</sup>. However, there is little convenient, safety and perfect device for complication of endoscopic treatment<sup>[7,8]</sup>. Since its introduction, the over-the-scope clip (OTSC) (Ovesco Endoscopy GmbH, Tuebingen, Germany) has been used to treat GI bleeding, fistulae and perforations in the United States and several European countries. Reports describing the use and value of OTSCs have primarily consisted of animal studies and clinical cases<sup>[9-14]</sup>. Retrospective studies have demonstrated the feasibility and the preliminary safety of the OTSC for the treatment of GI bleeding and fistulae, as well as for the closure of acute GI perforations<sup>[15,16]</sup>. The OTSC was approved by the Japanese Drug Administration and was made commercially available in November 2011. We have reported on our experiences with the OTSC and the outcomes of several cases involving its use since this device became available in Japan. Here, we retrospectively report the results of using the OTSC in our hospital. We also describe the potential use of the OTSC to completely close perforations after ESD for early GI cancers.

## MATERIALS AND METHODS

We retrospectively analyzed our database of all 23 patients who underwent OTSC placement (Ovesco Endoscopy GmbH, Tuebingen, Germany) in our hospital from November 2011 to September 2012, as summarized in Table 1. The indications for OTSC placement were GI

bleeding, perforations, fistulae and the prevention of post-ESD duodenal artificial ulcer perforation. ESD were performed because of dissection of adenocarcinoma. Maximum lesion size was defined as lesion diameter, not lesion surface area. The number of OTSCs to be used per patient was not decided until the lesion was completely closed. We used a twin grasper (Ovesco Endoscopy GmbH, Tuebingen, Germany) as a grasping device for all the patients. A 9-mm OTSC was chosen for use in the esophagus and colon, and a 10-mm device was used for the stomach, duodenum and rectum. Clinical success was defined by the results of a computed tomography scan and a blood analysis. Cases considered to be failures were those requiring hemostasis to control GI bleeding. In technical successful cases we included not only complete closing by using OTSCs, but also partial closing where complete closure with OTSCs is almost difficult. In overall clinical successful cases we included only complete closing by using only OTSCs perfectly.

All the OTSCs were placed by 2 experienced endoscopists. The sites closed after ESD included not only the perforation site but also all defect ulcers.

We obtained written, informed consent related to the use of OTSCs from all the patients.

### *Institution participating in the study*

Kagawa University Hospital, Kagawa, Japan, participated in the study.

## RESULTS

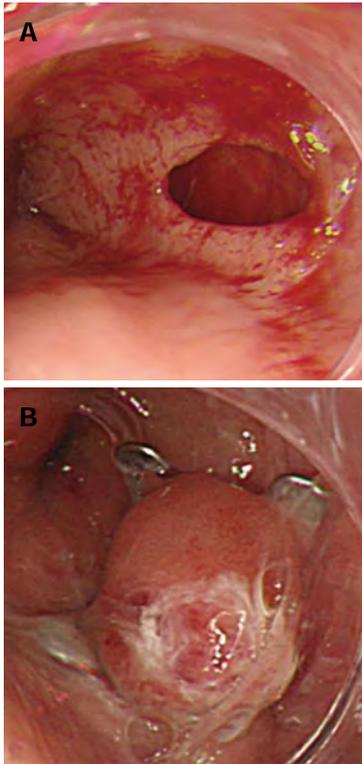
A total of 23 patients [mean age 77 years (range 64-98 years)] underwent OTSC placement during the study period. Of the 23 patients, 14 were male (60%) and 9 were female (40%). The indications for OTSC placement were GI bleeding ( $n = 9$ ), perforation ( $n = 10$ ), fistula ( $n = 4$ ) and the prevention of post-ESD duodenal artificial ulcer perforation ( $n = 1$ ). One patient had a perforation caused by a glycerin enema, after which a fistula formed. The perforations that were observed included iatrogenic perforations ( $n = 8$ ) and hemorrhagic peptic ulcers ( $n = 2$ ). The iatrogenic perforations included post-ESD artificial ulcer perforations ( $n = 6$ ), a perforation by a local steroid injection into an ulcer following ESD to prevent gastric stenosis ( $n = 1$ ), an esophageal perforation by a nasogastric tube ( $n = 1$ ) (Figure 1) and a rectal perforation by a glycerin enema ( $n = 1$ ). The fistulae included rectal fistulae ( $n = 2$ ), a stomach-to-skin fistulae following percutaneous endoscopic gastronomy (PEG) tube removal ( $n = 1$ ) (Figure 2) and a stomach-to-brachial tube fistula ( $n = 1$ ) (Figure 3).

The mean maximum size of the lesions was 23.1 mm (range 5 to 50 mm). The lesions were located in the esophagus ( $n = 1$ ), the stomach ( $n = 10$ ), the duodenum ( $n = 5$ ), the sigmoid colon ( $n = 3$ ) and the rectum ( $n = 4$ ). The time required for the procedure was measured from the time that the OTSC was applied to the time that it was released in the lesion. The median operation time for the successful cases was 18 min (range 5 to 51 min). The

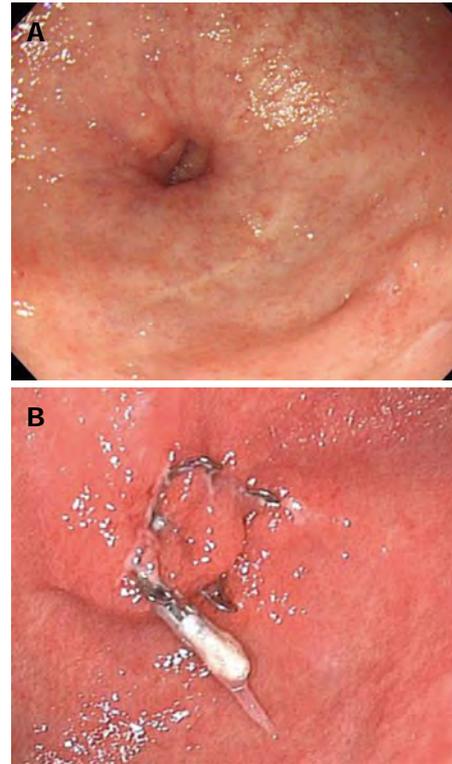
**Table 1 Database of patients who underwent over-the-scope clip device placement**

| No. | Sex | Age | Location                            | Primary disease                                | Maximum lesion size (mm) | Prior treatment history      | No. of OTSCs | Operation time (min) | Time from diagnosis (wk) | Technical/overall clinical successful | Additional treatment   | Complication | Stay in hospital after OTSC placement (d) | Duration of follow-up (d) |
|-----|-----|-----|-------------------------------------|--|--------------------------|------------------------------|--------------|----------------------|--------------------------|---------------------------------------|------------------------|--------------|---|---------------------------|
| 1   | M   | 86  | Lower esophagus                     | Iatrogenic perforation caused by stomach tube  | 20                       | None                         | 1            | 5                    | <1                       | Yes/yes                               | None                   | None         | 6   | 56                        |
| 2   | M   | 74  | Stomach                             | Delayed perforation after ESD                  | 40                       | None                         | 2            | 24                   | <1                       | Yes/yes                               | None                   | None         | 10  | 90                        |
| 3   | F   | 82  | Stomach                             | Perforation after ESD                          | 25                       | None                         | 2            | 20                   | <1                       | Yes/yes                               | None                   | None         | 7   | 8                         |
| 4   | M   | 80  | Stomach                             | Peptic ulcer with bleeding                     | 15                       | Hemostatic forceps           | 1            | 8                    | <1                       | Yes/yes                               | None                   | None         | 21  | 30                        |
| 5   | F   | 71  | Stomach                             | Peptic ulcer with perforation                  | 40                       | None                         | 2            | 23                   | <1                       | Yes/yes                               | None                   | None         | 7   | 58                        |
| 6   | F   | 88  | Stomach                             | Gastrocutaneous fistula                        | 10                       | None                         | 1            | 18                   | >4                       | Yes/yes                               | None                   | None         | 8   | 84                        |
| 7   | M   | 98  | Stomach                             | Bleeding due to Mallory-Weiss syndrome         | 12                       | None                         | 1            | 12                   | <1                       | Yes/yes                               | None                   | None         | 6   | 15                        |
| 8   | M   | 73  | Duodenum                            | Para-anastomotic ulcer bleeding                | 15                       | Clips                        | 2            | 21                   | <1                       | Yes/yes                               | None                   | None         | 8   | 18                        |
| 9   | M   | 80  | Duodenal bulb                       | Peptic ulcer with bleeding                     | 23                       | Clips and hemostatic forceps | 2            | 30                   | <1                       | Yes/yes                               | None                   | None         | 10  | 52                        |
| 10  | M   | 74  | Duodenal bulb                       | Peptic ulcer with perforation                  | 5                        | None                         | 1            | 8                    | <1                       | Yes/yes                               | None                   | None         | 13  | 194                       |
| 11  | F   | 73  | Duodenal bulb                       | Prevention of post-ESD perforation             | 25                       | None                         | 1            | 10                   | <1                       | Yes/yes                               | None                   | None         | 7   | 95                        |
| 12  | M   | 75  | 3 <sup>rd</sup> portion of duodenum | Delayed perforation after ESD                  | 28                       | None                         | 2            | 36                   | <1                       | Yes/yes                               | None                   | None         | 15  | 210                       |
| 13  | F   | 85  | Rectum                              | Rectovesical fistula                           | 15                       | None                         | 2            | 30                   | <1                       | Yes/yes                               | None                   | None         | 9   | 28                        |
| 14  | F   | 88  | Rectum                              | Iatrogenic rectal perforation/fistula          | 25                       | None                         | 1            | 8                    | <1                       | Yes/yes                               | None                   | None         | 10  | 30                        |
| 15  | M   | 73  | Stomach                             | Peptic ulcer with bleeding                     | 20                       | Clips and HSE                | N/A          | N/A                  | 1-4                      | No/no                                 | Hemostatic forceps     | N/A          | N/A                                       | N/A                       |
| 16  | M   | 64  | Stomach                             | Peptic ulcer with bleeding                     | 50                       | Hemostatic forceps           | N/A          | N/A                  | 1-4                      | No/no                                 | Hemostatic forceps     | N/A          | N/A                                       | N/A                       |
| 17  | F   | 65  | Sigmoid colon                       | Perforation after ESD                          | 35                       | None                         | 1            | 7                    | <1                       | Yes/yes                               | None                   | None         | 8   | 160                       |
| 18  | F   | 83  | Sigmoid colon                       | Perforation after ESD                          | 40                       | None                         | 3            | 16                   | <1                       | Yes/yes                               | None                   | None         | 8   | 90                        |
| 19  | F   | 88  | Stomach                             | Perforation caused by a local injection needle | 50                       | None                         | 3            | 51                   | 1-4                      | Yes/no                                | Surgery                | N/A          | N/A                                       | N/A                       |
| 20  | M   | 65  | Rectum                              | Postoperative anastomotic ulcer bleeding       | 5                        | Hemostatic forceps           | 1            | 6                    | <1                       | Yes/yes                               | None                   | None         | 8   | 14                        |
| 21  | M   | 65  | Rectum                              | Postoperative anastomotic ulcer bleeding       | 5                        | Hemostatic forceps           | 2            | 19                   | <1                       | Yes/yes                               | None                   | None         | 7   | 30                        |
| 22  | M   | 73  | Sigmoid colon                       | Refractory diverticular bleeding               | 5                        | Clips                        | 1            | 7                    | <1                       | Yes/yes                               | None                   | None         | 5   | 10                        |
| 23  | M   | 73  | Stomach                             | Gastrobronchial fistula                        | 28                       | Bronchial embolization       | 1            | 40                   | >4                       | Yes/no                                | May be given in future | N/A          | N/A                                       | N/A                       |

OTSC: Over-the-scope clip; ESD: Endoscopic submucosal dissection; M: Male; F: Female; N/A: Not available; HSE: Hypertonic saline-epinephrine injection therapy.



**Figure 1** Esophageal perforation caused by a nasogastric tube. A: During the insertion of a nasogastric tube, the tip of the tube perforated the lower esophagus; B: The wound was successfully closed with a single over-the-scope clip, and there was no leakage.



**Figure 2** Gastrocutaneous fistula that occurred after percutaneous endoscopic gastrostomy removal. A: After the removal of a gastrostomy tube, the patient was able to eat orally, but a gastrocutaneous fistula was diagnosed; B: The wound was slightly hardened, but was successfully closed with a single over-the-scope clip.

numbers of OTSC devices used per session were one ( $n = 11$  patients), two ( $n = 8$  patients) and three ( $n = 2$  patients). The post-OTSC placement mean observation period was 67 d (range 8-210 d).

The technical success rate for OTSC placement was 91.3% (21/23). The OTSC was successfully and safely released in all the cases except for 2, in which we did not release the OTSC because the ulcer was extremely stiff due to being a chronic hemorrhagic gastric ulcer. We could not include the entire thickness of the mucosa of these ulcers within the applicator cap. Ultimately, we stopped the bleeding by reapplying the hemostatic forceps (Coagrasper, FD-410LR, Olympus, Tokyo, Japan). The overall clinical success rate using the OTSC alone was 82.6% (19/23). A large lesion size (greater than 20 mm) and a delayed diagnosis (more than 1 wk) were the major contributing factors in the overall unsuccessful clinical cases. The location of the unsuccessful lesion was in the stomach (Table 2). In 2 patients, we were unable to place the OTSC on the refractory peptic ulcer. In 1 patient, a perforation occurred because of a local steroid injection into the artificial ulcer after ESD to prevent stenosis. We could not place the OTSC correctly because the wound was large and the ulcer was exceptionally stiff. In another patient, who was suffering from a gastrobrachial fistula (Figure 3), we were able to place only one OTSC, but not the additional OTSCs necessary for the complete closure of the fistula.

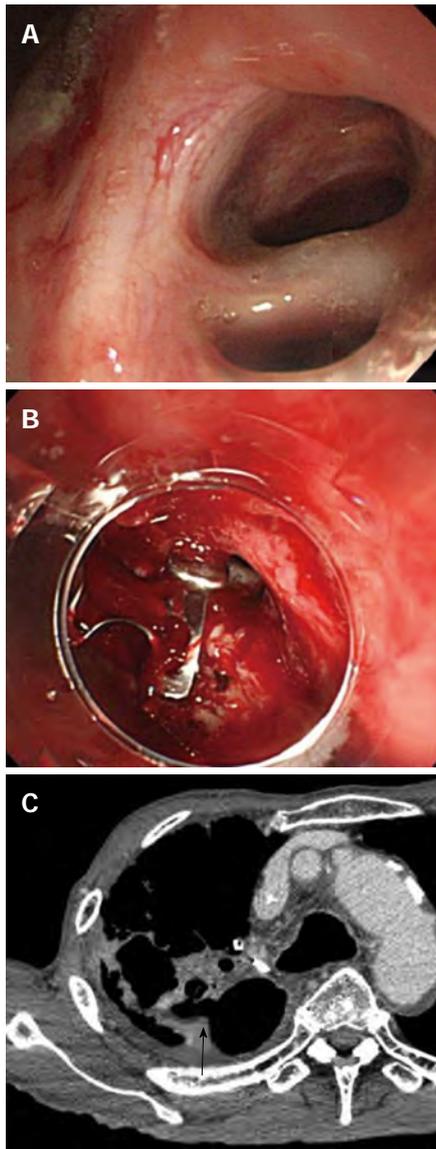
There was no adjunct therapy used for OTSC placement in the 19 overall clinical success cases. In the two technical failure cases, we used coagulation forceps for additional hemostasis. All the patients were hospitalized for observation after the OTSC placement. The median hospital stay was 9 d (range 6-21 d).

### Complications

During the observation period, none of the patients experienced any complications associated with OTSC placement.

## DISCUSSION

The development of new NOTES devices has introduced advanced therapeutic techniques that can be used in minimally invasive treatments<sup>[1-3]</sup>, including various full-thickness suturing devices that are applied in clinical practice. One of these devices is the OTSC system, the clinical utility of which has been reported in Europe and the United States. The OTSC system shows great potential for use in endoscopic treatments that require speed and simplicity<sup>[2,3,17,18]</sup>. This system received a pharmaceutical license in Japan in November 2011. Although animal experiments and clinical studies have been performed in Europe and the United States, few clinical cases have been reported in Japan<sup>[13,14]</sup>. We used the OTSC in NOTES animal experiments prior to its approval for



**Figure 3** A gastric tube bronchial fistula following a subtotal esophagectomy for esophageal cancer. A: A gastric tube bronchial fistula occurred after a subtotal esophagectomy for esophageal cancer. Bronchial embolization was performed, but it failed to close the fistula; B: The authors attempted to close the fistula using over-the-scope clip (OTSC) but were unsuccessful. Although 1 OTSC was placed, mucosal hardening (resulting from the prolonged duration of the untreated ulcer) prevented the placement of the additional OTSCs required for closure; C: A chest-abdominal computed tomography scan revealed a gastrobronchial fistula (arrow).

humans. Recognizing its potential for use in Japan, we began using the OTSC clinically immediately after the pharmaceutical license was granted. We have employed this device in 23 patients within a short period in our hospital, with an overall success rate of 82%. Animal and clinical studies of the OTSC have demonstrated that gastric, duodenal or colonic perforations up to 15 mm in diameter can be completely closed with a single OTSC. For perforations up to 20 mm in diameter, closure can also be achieved using some OTSCs, which indicates that there is sufficient working space for the unobstructed use of the endoscope during NOTES<sup>[17]</sup>. There are reports that

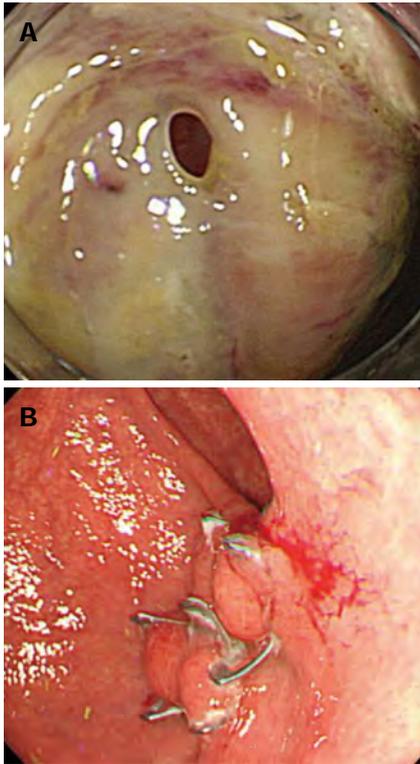
**Table 2** Relationship between each characters and overall clinical success rate *n* (%)

|                          | Patients | Overall clinical success |
|--------------------------|----------|--------------------------|
| Location                 |          |                          |
| Esophagus                | 1        | 1 (100)                  |
| Stomach                  | 10       | 6 (60)                   |
| Duodenum                 | 5        | 5 (100)                  |
| Colon                    | 3        | 3 (100)                  |
| Rectum                   | 4        | 4 (100)                  |
| Primary disease          |          |                          |
| GI bleeding              | 9        | 7 (77)                   |
| Chronic fistulae         | 4        | 3 (75)                   |
| Perforation              | 11       | 10 (90)                  |
| Maximum lesion size (mm) |          |                          |
| < 20                     | 9        | 9 (100)                  |
| 20-30                    | 8        | 6 (75)                   |
| > 30                     | 6        | 4 (66)                   |
| Time from diagnosis (wk) |          |                          |
| < 1                      | 18       | 18 (100)                 |
| 1-4                      | 3        | 0 (0)                    |
| > 4                      | 2        | 1 (50)                   |

full-thickness closures of defects 18-27 mm in diameter can be performed using OTSC; however, it is difficult to completely close defects > 30 mm in diameter<sup>[18]</sup>.

In the present series, OTSC closure was successful for wounds with a maximum diameter of ≤ 30 mm but unsuccessful in 1 case of refractory GI bleeding and 1 case of gastrobronchial fistula (Figure 3). Even in cases with a maximum wound diameter of > 30 mm, we placed the OTSC successfully because the tissue was well extensible and not hardened. In our clinical experience, unsuccessful OTSC closure had a chronic course and OTSC failures were due to chronic fibrotic changes and scarring at the perforation site. Specifically, we used OTSCs in 4 chronic patients and failed to close the perforation site in 3 of them (75%). It appears that wound closure with the OTSC is suitable for wounds with easy extensibility of the surrounding tissues; such lesions have little fibrosis and can be easily grasped by the twin grasper. Considering that successful closure was also achieved in cases with a rectal fistula < 20 mm in diameter or a gastrocutaneous fistula after PEG removal (Figure 2), we believe that OTSC use should be considered when surgery is the only remaining option, provided that the lesion (even if presumably hardened) is ≤ 30 mm in diameter and can be sucked into the cap to lift the mucosa. In addition, the success of the OTSC closures in 4 cases with lesions > 30 mm suggests that this device can be used in acute cases with good extensibility of surrounding mucosa.

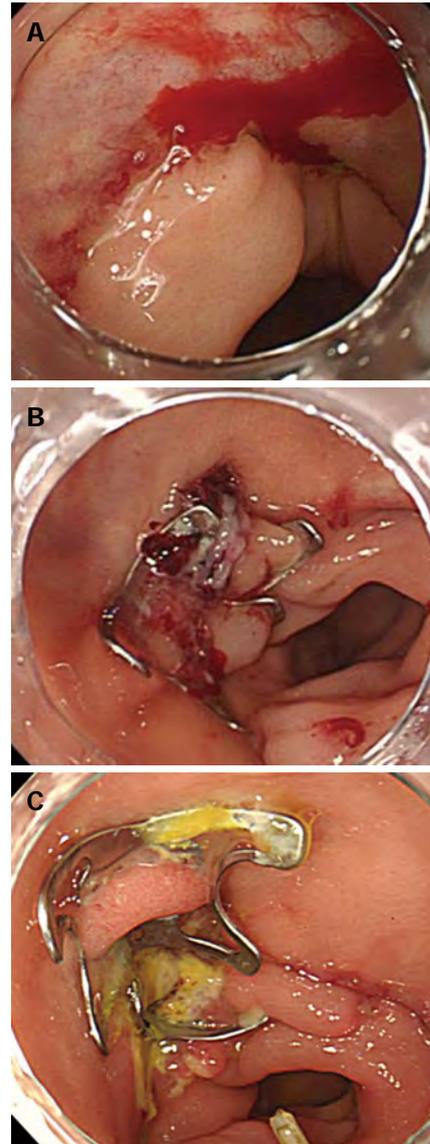
ESD was developed for *en bloc* removal of large gastric cancer, which decrease the risk of local recurrence, and specimens can be accurately evaluated by histological examination<sup>[19]</sup>. However, the procedure is associated with a high incident of complication with bleeding and perforation<sup>[20-22]</sup>. Now, bleeding has been good controlled with new hemostatic forceps and clips<sup>[23]</sup>. We also used the OTSC for the complete wound closure of post-ESD perforations in the stomachs of 2 patients who had undergone ESD for early gastric cancer. In both cases,



**Figure 4** Iatrogenic perforation after endoscopic submucosal dissection for early gastric cancer in the greater curvature of the stomach. A: A post-endoscopic submucosal dissection ulcer was found in the greater curvature of the stomach. An examination by retroflex view revealed that the muscle layer was separated and perforated; B: The wound was successfully closed using an over-the-scope clip (OTSC). An upper endoscopy performed 2 mo after the closure revealed no displacement of the OTSC or complications, such as ulceration or deformation.

the lesions were located in the greater curvature of the stomach (Figure 4). Because it is anatomically thinner than other parts of the stomach, the greater curvature of the stomach is considered to be easy to perforate<sup>[24-29]</sup>. In addition, because the knife is applied vertically to the mucosa during ESD, it is difficult to perform the procedure while maintaining an appropriate dissection depth into the submucosal layer. The endoscope must also be retroflexed<sup>[24]</sup>. These limitations have led to several cases in which our attempts to create a partial closure of an ulcer base using conventional clips caused the further extensive separation of the muscle layer and the subsequent need for surgery. In such cases, complete closure using the OTSC is thus preferred for a post-ESD ulcer in the greater curvature of the stomach, even if perforation is suspected.

The use of duodenal ESD is controversial among Japanese endoscopists because the narrow lumen of the duodenum makes it difficult to perform the procedure, and the base of a post-ESD duodenal ulcer is continuously exposed to bile, causing an increased incidence of delayed perforation compared with other ESD sites<sup>[30-35]</sup>. Nevertheless, duodenal surgery is highly invasive because of the anatomical position. ESD should be preferentially performed instead of surgery if clinically indicated. The



**Figure 5** Bleeding from an anastomotic ulcer caused by a sigmoidectomy for sigmoid cancer. A: Bleeding was observed from the anastomotic site after surgery for sigmoid colon cancer; B: The wound was successfully closed using an over-the-scope clip; C: The postoperative course has been uneventful, with no rebleeding.

Japan Gastroenterological Endoscopy Society reported in April 2009 that the complete closure of a post-ESD duodenal ulcer using conventional clips helps prevent delayed perforation. However, conventional clips are too small and do not provide sufficient grip strength to achieve the complete closure of an ulcer base. Therefore, the OTSC, which is larger and provides greater grip strength, is recommended for the complete closure of post-ESD duodenal ulcers<sup>[36]</sup>.

At our hospital, patient 11 (Table 1) experienced a small perforation of a post-ESD ulcer that formed in the duodenal bulb, and the OTSC was used for its closure. In patient 12 (Table 1), the lesion occurred in the descending portion of the duodenum and was exposed to bile, indicating an increased risk of delayed perforation. Thus,

the ulcer was closed using the OTSC, which helped prevent perforation and bleeding.

We experienced 2 cases of OTSC closure for post-ESD ulcers in the colon. In both cases, the lesions were located in the sigmoid colon, with post-ESD perforations requiring complete wound closure. The wound was > 30 mm in diameter in both cases and was successfully closed using the OTSC.

We experienced 9 cases of GI bleeding that were treated with OTSCs. Widely used hemostatic procedures, such as hemostatic clips and local injections, are economically advantageous but sometimes fail to achieve complete primary hemostasis. The use of coagulation hemostasis with hemostatic forceps is also increasing because the reliable coagulation of exposed vessels under direct visualization can minimize the risk of rebleeding. However, the application of coagulation hemostasis to a deep ulcer or a thin wall of the duodenum is associated with a risk of perforation. For patients who do not tolerate surgery well and are in shock due to rebleeding or who do not respond to conventional treatment, the use of the OTSC should be considered. Based on these criteria, we applied OTSCs to the 9 patients with GI bleeding and achieved complete hemostasis in 7 of them. The remaining 2 patients with failed hemostasis using the OTSC system had a personal status  $\geq 3$  and could not tolerate open abdominal surgery. One of the patients had a large ulcer (50 mm in diameter) to which hemostasis with hemostatic forceps was applied. However, the patient experienced 2 episodes of shock due to bleeding from other sites of neovascularization. During the third episode of bleeding, ulcer closure with the OTSC was attempted but was unsuccessful due to a hardened ulcer base. Hemostasis with hemostatic forceps was again performed, after which no rebleeding was observed.

Hemostatic treatment of an anastomotic ulcer using the OTSC for was successful in 1 patient with a duodenal lesion and for 2 patients with lesions in the sigmoid colon (Figure 5). The aggressive treatment of bleeding with hemostatic forceps may cause perforations because anastomotic sites are usually thin and fragile. We consider the OTSC to be an effective tool for the treatment of lesions at anastomotic sites.

Regarding safety and complications, none of our patients treated with the OTSC reported any complications. Assessments performed 7 d after closure using the OTSC also revealed no displacements of the OTSC or tissue necrosis at the wound sites. Endoscopic examinations revealed OTSC losses in 2 cases: 1 occurred 1 mo after OTSC placement for duodenal lesions, and the other occurred 2 mo after OTSC placement for colonic lesions. No associated complications were observed in either case. Other possible complications include mucosal damage caused by the teeth of the OTSC protruding out of the hood top during insertion. Therefore, special care should be taken when the OTSC is inserted into physiologically narrow sites, such as the esophageal entrance, pyloric ring or anal ring. There have been no reports of

OTSCs causing mucosal deformation or stenosis of the gastrointestinal<sup>[9]</sup>. However, we must consider the possibility that a failure to extract the fibrotic mucosa may result in the tissue being crushed by the twin grasper during the extraction of hardened tissue.

Based on our 23 cases and those reported in the literature, we consider OTSC to be a highly useful device that can be safely utilized in the treatment of GI perforations, fistulae and refractory bleeding. However, OTSC is not suitable for the closure of chronic lesions with hard, severely fibrotic wounds because it is difficult to draw such a lesion into the top of the device. However, we have used the OTSC to close post-ESD perforations with a 100% success rate. Although our sample size was small, we believe that the OTSC is a viable treatment option for post-ESD perforation.

## COMMENTS

### Background

Recently, over-the-scope clip (OTSC) devices have been used for gastrointestinal (GI) bleeding, fistulae and perforations in the United States and several European countries. OTSC devices became pharmaceutically licensed in Japan in August 2011. The authors have reported their experiences with the OTSC and the outcomes of several cases in Japan. The aims of the present study were to retrospectively review the results of OTSC use in the authors' hospital and to examine the feasibility of using the OTSC to completely close perforations after endoscopic submucosal dissection (ESD) for early GI cancers.

### Research frontiers

Historically, the standard treatment for GI perforations has been surgery. Recently, invasive endoscopic treatments, such as ESD and natural orifice transluminal endoscopic surgery (NOTES), have provided alternative approaches to surgery. However, the devices used to treat complications following endoscopic treatments are less convenient and not as safe. OTSCs have been used to treat GI bleeding, fistulae and perforations in several countries. In this study, the authors retrospectively report the results of using the OTSC in their hospital. They also discuss the potential use of the OTSC to completely close perforations after ESD for early GI cancers.

### Innovations and breakthroughs

This is the first retrospective study of the OTSC in Japan, and it includes more cases of post-ESD perforation closure compared with other published reports on OTSC. All the post-ESD perforation closure cases in the present study were successes. Thus, the authors consider OTSC to be a possible tool for the treatment of perforations after ESD.

### Applications

Based on the present 23 cases and those reported in the literature, the authors assert that the OTSC is useful and safe for the treatment of GI perforations, fistulae and refractory bleeding.

### Terminology

ESD is the only nonsurgical, endoscopic method of treating early GI cancers; NOTES, a fusion of flexible endoscopy and operative techniques, is a less invasive form of treatment than surgery.

### Peer review

The OTSC is an interesting and novel device that enhances the armamentarium of therapeutic gastroenterologists. This report illustrates the use of this novel device, which facilitates interventions that were previously impossible to perform endoscopically.

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**P- Reviewers** Van Rensburg C, Kumar A **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Li JY



## Lipoic acid suppresses portal endotoxemia-induced steatohepatitis and pancreatic inflammation in rats

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**Author contributions:** All authors participated in designing the study, analyzing and interpreting the data, and approved the final version of the manuscript; Tian YF, He CT and Chen YT conducted the experiments; Hsieh PS wrote the manuscript.

**Supported by Grants from the National Science Council of the ROC, No. NSC98-2320-B-016-011 MY3, CMNDMC 100-05 and TSGH-C100-011-015-S03**

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Received: December 11, 2012 Revised: February 11, 2013

Accepted: February 28, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To examine the effect of  $\alpha$ -lipoic acid (LA) on mild portal endotoxemia-induced steatohepatitis and associated pancreatic abnormalities in fructose-fed rats.

**METHODS:** Rats were randomly assigned into two groups with a regular or 60% fructose-enriched diet for 8 wk. After fructose feeding for 4 wk, rats were further divided into four subgroups: with intraportal saline (F<sub>PV</sub>), with intraportal saline plus administration of LA (F<sub>PV</sub> + LA), with lipopolysaccharide (LPS) infusion (F<sub>PLPS</sub>), and with LPS infusion plus administration of LA

(F<sub>PLPS</sub> + LA). Rats were treated with LPS using intraportal infusion while LA was administered orally. Metabolite levels, superoxide levels, inflammatory markers, malondialdehyde content, glutathione content and toll-like receptor 4 (*TLR4*) gene expression were all measured using standard biochemical techniques. Pancreatic insulin secretion was evaluated by a hyperglycemic clamp technique. Histology of liver and pancreas tissues were evaluated using hematoxylin and eosin staining and immunohistochemistry.

**RESULTS:** Fructose-induced elevation in plasma C-reactive protein, amylase, superoxide, white blood cell count as well as in hepatic and pancreatic contents of malondialdehyde, tumor necrosis factor alpha and interleukin-6 were increased in animals treated with LPS and reversed with LA administration. The augmented hepatic gene expression of *TLR4* in fructose-fed rats was further increased in those with intraportal LPS infusion, which was partially reversed by LA administration. Pathological examination showed inflammatory changes and leukocyte infiltration in hepatic and pancreatic islets of animals treated with LPS but were rarely observed in those with LA treatment. In addition to effects on the liver, impaired pancreatic insulin secretion seen in fructose-fed rats was deteriorated in with LPS treatment and partially reversed with LA administration.

**CONCLUSION:** These data suggest LA could significantly suppress mild portal-endotoxemia but not fructose-induced liver and pancreatic abnormalities in a rodent model for metabolic syndrome.

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**Key words:** Lipoic acid; Oxidative stress; Steatohepatitis; Portal endotoxemia; Insulin secretion; Fructose

**Core tip:**  $\alpha$ -lipoic acid (LA), a potent antioxidant and also an inducer of endogenous antioxidants has been

reported to protect the liver and pancreas from injury. Our observations suggest that LA could significantly suppress inflammatory change of steatosis induced by low-dose intraportal lipopolysaccharide infusion and associated deterioration of insulin secretion in this metabolic syndrome rodent model. In addition, our data suggest that hepatic toll-like receptor 4 signaling might not only play a significant role in chronic fructose-feeding-induced hepatic steatosis but also in its subacute inflammatory change induced by mild portal endotoxemia and associated extrahepatic disorders.

Tian YF, He CT, Chen YT, Hsieh PS. Lipoic acid suppresses portal endotoxemia-induced steatohepatitis and pancreatic inflammation in rats. *World J Gastroenterol* 2013; 19(18): 2761-2771 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2761.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2761>

## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is currently the most common liver disease in the world<sup>[1]</sup>. NAFLD is mainly associated with obesity, diabetes, hyperlipidemia and insulin resistance, which are the main characters of metabolic syndrome<sup>[1,2]</sup>. The development of liver inflammation in NAFLD, defined as non-alcoholic steatohepatitis (NASH), is one of the crucial steps in causing liver-related morbidity and mortality<sup>[3]</sup>. Moreover, a pathological link between portal endotoxemia and NASH and also alcoholic liver disease has been speculated in several animal and human studies<sup>[4-8]</sup>. For instance, genetically obese fatty/fatty rats and *ob/ob* mice showed increased sensitivity to endotoxin hepatotoxicity, quickly developing steatohepatitis after exposure to low doses of lipopolysaccharide (LPS)<sup>[5]</sup>. These studies implicate that portal endotoxemia might significantly contribute to the pathogenesis of chronic hepatic inflammation, especially in NAFLD.

The release of liver-derived acute-phase proteins and inflammatory cytokines under chronic liver injury significantly contributes to the extrahepatic effect of inflamed liver and the pathogenesis of metabolic syndrome<sup>[9,10]</sup>. We have shown that mild portal endotoxemia induced by low-dose intraportal LPS infusion could significantly cause chronic hepatic and pancreatic inflammation, and impair pancreatic insulin secretion in rats<sup>[11]</sup>. Furthermore, low-dose intraportal LPS infusion could also accelerate the process of NAFLD to NASH in fructose-fed rats, an animal model of metabolic syndrome with NAFLD<sup>[4]</sup>. However, the possible underlying mechanisms behind the detrimental effects of mild portal endotoxemia on liver and pancreas remain unclear.

Chronic stress such as portal endotoxemia has been documented to activate hepatic Kupffer cells and cause the release of reactive oxygen species, potentially inducing inflammatory changes in the liver and impairing

pancreatic functions<sup>[5,12]</sup>.  $\alpha$ -lipoic acid (LA) is a potent antioxidant and an inducer of endogenous antioxidants<sup>[13,14]</sup>. It also has a protective effect on hepatic and pancreatic injury<sup>[15-18]</sup>. In this study, we sought to test the potential therapeutic effect of LA on mild portal endotoxemia and fructose-induced inflammatory changes of fatty liver and impaired pancreatic insulin secretion in rats.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats aged five to six weeks were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The rats were housed in an animal center certified by Association of Assessment and Accreditation of Laboratory Animal Care. All animals were handled according to the guidelines and manual of the institutional animal care and use committee of this institute. Rats weighing 250-300 g were randomly assigned into six groups: (1) two control groups: on regular chow diets for 4 wk and then separated into those combined with or without intraportal vehicle (saline) infusion (C<sub>PV</sub> vs C) for another 4 wk; (2) four experimental groups: on high-fructose enriched diet (60% of the calories from fructose, TD89247, Teklad Primer Labs, Madison, WI) for 4 wk and thereafter (F<sub>PV</sub>), those combined with intraportal vehicle or low-dose LPS infusion (F<sub>LPS</sub>), cotreated with vehicle or LA ( $\alpha$ -LA in saline, pH 7.8, Sigma Co. Germany, 60 mg/kg per day, oral gavage) for additional 4 wk (F<sub>PV</sub> + LA and F<sub>LPS</sub> + LA respectively)<sup>[19]</sup>. In the time-course study, blood samples were taken by tail bleeding method after overnight fasting. At the end of week 8, one set of rats from the above grouping ( $n = 6$  per group) was used for an *in vivo* hyperglycemic clamp study. The second set ( $n = 6$  per group) was euthanized by overdose pentobarbital injection (100 mg/kg, intraperitoneal) immediately after overnight fasting and blood sampling by cardiac puncture was carried out for the measurement of plasma C-reactive protein (CRP), superoxide, white blood cell (WBC) count and endotoxin levels. Tissue samples were dissected for further analysis. The liver and pancreas dissected from the third set of rats ( $n = 6$  per group) were fixed by perfusion with 4% paraformaldehyde. The tissues were embedded in paraffin for further staining. Notably, the metabolic and hemodynamic parameters and histopathological examination of group C<sub>PV</sub> were similar to those without intraportal vehicle infusion (data not shown). The group C<sub>PV</sub> was used as the only control group on regular chow diet in the following result section.

### Intraportal infusion

A laparotomy was performed under anesthesia (sodium pentobarbital 25 mg/kg, intraperitoneal injection) in rats with intraportal infusion. A catheter (PE-5 tubing, 0.008 inch inner diameter  $\times$  0.020 inch outer diameter, SCI micro medical grade polyethylene; Scientific Commodities Inc., Lake Havasu City, AZ, United States) was inserted

into the distal end of a colic vein and the tip of the catheter was placed about 3 mm distal to the point at which the catheterized vein enters portal vein. After insertion, the catheters were filled with LPS-saline solution or saline alone and were connected to osmotic mini-pumps (model 2004, Alzet corporation, Cupertino, CA, United States) filled with LPS, 0.42 ng/kg per minute or saline as shown in our previous study<sup>[20]</sup>. Three days before the surgery, a safe dosage of LA was orally administered once per day for 4 wk<sup>[21,22]</sup>.

### **Hyperglycemic clamp**

In one set of rats, vascular catheters were placed in the left femoral artery and right femoral vein under anesthesia and their proximal ends were placed in subcutaneous pockets under scapular area at the end of week 8. The hyperglycemic clamp experiment was performed after recovery for 3 to 4 d, as described elsewhere<sup>[23,24]</sup>. The insulin secretions of the first and second phases were indicated by the incremental plasma insulin values during time 0-10 and 10-240 min in the glucose clamp period, respectively.

### **Plasma metabolic parameters**

The WBC count was determined by using a Bright-Line-hemocytometer (Hausser Scientific, Horsham, PA, United States). Whole blood glucose levels were measured by the glucose oxidase method. Plasma and tissue triglyceride levels were determined by using appropriate enzymatic colorimetric method (Randox Laboratories, Antrim, United Kingdom). Plasma insulin levels were determined by commercial rat enzyme-linked immuno sorbent assay (ELISA) kits (MercoDIA AB, Uppsala, Sweden). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT), albumin and amylase levels were measured by Randox reagent kits (Randox Laboratories Antrim, United Kingdom). CRP levels were measured by a commercial rat ELISA kit (Alpha Diagnostic, San Antonio, TX, United States). Arterial plasma endotoxin level was assayed by using the limulus amoebocyte lysate test (Kinetic-QLC; Whittaker Bioproducts, Walkersville, MD, United States).

### **Plasma superoxide levels**

A lucigenin-dependent chemiluminescence assay (Sigma Chemical Co. St. Louis, MO, United States) was used to quantify plasma superoxide levels with the MLA-GOLDS chemiluminescence analyzing system (Tohoku Electronic Industrial Co., Sendai, Japan) as described previously<sup>[4]</sup>. The total amount of chemiluminescence was calculated by integrating the area under the curve and subtracting it from the background level during the 10 min counting period.

### **Tissue tumor necrosis factor alpha and interleukin-6 contents**

Tissues were prepared as described previously<sup>[25]</sup>. The supernatant was subjected to tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-6 (IL-6) assays using commercial rat ELISA kits (R and D Systems, Minneapolis, MN,

United States).

### **Tissue malondialdehyde content**

Lipid oxidation of the liver and pancreas was detected by a commercial malondialdehyde (MDA) assay kit (Cayman Chemical Co., Ann Arbor, MI, United States). In brief, MDA, the breakdown product of oxidative degradation of lipids, reacted with thiobarbituric acid (TBAR) to form MDA-TBAR, and its levels were expressed as nmol/g protein.

### **Tissue glutathione content**

The total glutathione in the liver was measured by using commercial glutathione assay kit (Cayman Chemical Co., Ann Arbor, MI, United States). In brief, the liver was homogenized in RIPA buffer (0.5 mol/L Tris-HCl, pH 7.4, 1.5 mol/L NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mmol/L EDTA) by using a Polytron homogenizer to obtain tissue lysates and centrifuged at 10000 g for 10 min. Subsequently, the supernatant was collected and added HPO<sub>3</sub> (v/v = 1/1) to deproteinize. The glutathione (GSH) content of deproteinized supernatant of liver (50 L) was measured by the reductive rate of 5,5'-dithio-bis-2-nitrobenzoic acid to 5-thio-2-nitrobenzoic acid according to the instructions with absorption at 405 nm. The GSH level was expressed as  $\mu$ mol/g protein.

### **Toll-like receptor 4 gene expression in the liver and pancreas**

RNA was extracted from the liver and pancreas of experimental rats using Trizol (Ambion, Austin, TX, United States) at the end of the study. TaqMan gene expression assay kits for toll-like receptor 4 (TLR4) (Rn00569848\_m1) and the TATA box binding protein (Rn01455648\_m1) were used in conjunction with a universal master mix in a 7500 real-time polymerase chain reaction system (Applied Biosystems, Foster City, CA, United States). Gene expression was normalized to the TATA box binding protein expression level measured in each sample and expressed as fold increases or decreases from control values for each gene of interest.

### **Hematoxylin and eosin stain**

The third set of rats ( $n = 5-6$  per group) was grouping as the hyperglycemic clamp experiment. The perfused liver and pancreas were then fixed in 5% zinc-formalin solution and embedded in paraffin. These tissue slices were then prepared for staining with hematoxylin and eosin (HE) (liver, pancreas) to evaluate pathological changes.

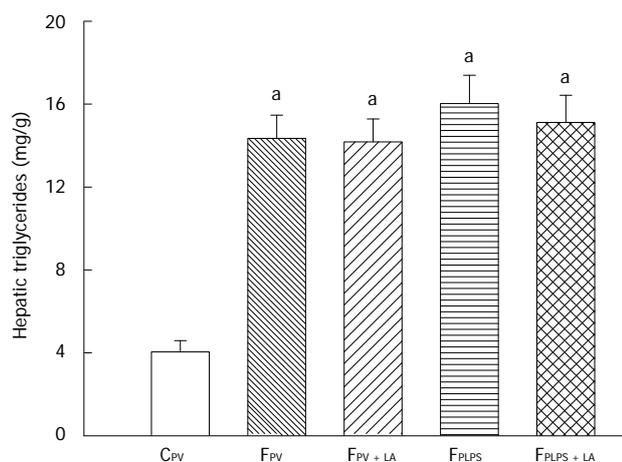
### **Immune cell immunohistochemistry and scoring**

Tissue samples were fixed in formalin, and cut into 4  $\mu$ m sections. Immunohistochemistry was performed on an automated stainer (Autostainer; Dako, Glostrup, Denmark). Then, sections were incubated with anti-CD68 antibody (ED1 mouse anti-rat CD68, Serotec, 1:100, MorphoSys UK Ltd., Oxford, United Kingdom), followed by incubation with goat anti-mouse secondary antibody and

**Table 1** Metabolic and hemodynamic parameters during pump infusion period in rats

|                               | Pump infusion (wk) | Cpv         | Fpv                      | Fpv + LA               | FPLPS                     | FPLPS + LA               |
|-------------------------------|--------------------|-------------|--------------------------|------------------------|---------------------------|--------------------------|
| Body weight (g)               | 4                  | 349 ± 19    | 383 ± 8                  | 351 ± 29               | 358 ± 6                   | 371 ± 6                  |
|                               | 8                  | 451 ± 7     | 474 ± 8                  | 445 ± 29               | 436 ± 11                  | 423 ± 7                  |
| Glucose (mmol/L)              | 4                  | 5.9 ± 0.3   | 5.9 ± 0.1                | 6.62 ± 1.2             | 6.08 ± 0.1                | 6.1 ± 0.1                |
|                               | 8                  | 5.9 ± 0.2   | 6.4 ± 0.2                | 7.8 ± 0.7              | 6.76 ± 0.2                | 6.43 ± 0.1               |
| Insulin (ng/mL)               | 4                  | 0.5 ± 0.1   | 0.7 ± 0.1                | 0.7 ± 0.1              | 0.6 ± 0.1                 | 0.6 ± 0.1                |
|                               | 8                  | 0.6 ± 0.1   | 2.5 ± 0.6 <sup>a</sup>   | 2.2 ± 0.3 <sup>a</sup> | 1.9 ± 0.5 <sup>a</sup>    | 2.4 ± 0.4 <sup>a</sup>   |
| TG (mg/dL)                    | 4                  | 58 ± 4      | 180 ± 14 <sup>a</sup>    | 185 ± 13 <sup>a</sup>  | 185 ± 24 <sup>a</sup>     | 163 ± 16 <sup>a</sup>    |
|                               | 8                  | 52 ± 4      | 324 ± 38 <sup>a</sup>    | 286 ± 22 <sup>a</sup>  | 308 ± 24 <sup>a</sup>     | 249 ± 44 <sup>a</sup>    |
| AST (U/L)                     | 4                  | 65 ± 5      | 76 ± 1                   | 82 ± 6                 | 70 ± 2                    | 77 ± 2                   |
|                               | 8                  | 58 ± 4      | 72 ± 2                   | 73 ± 6                 | 68 ± 2                    | 70 ± 2                   |
| ALT (U/L)                     | 4                  | 53 ± 2      | 57 ± 1                   | 56 ± 0                 | 52 ± 1                    | 51 ± 2                   |
|                               | 8                  | 56 ± 2      | 49 ± 1                   | 44 ± 6                 | 46 ± 1                    | 51 ± 1                   |
| Albumin (mmol/L)              | 4                  | 40 ± 1      | 35 ± 1                   | 37 ± 1                 | 35 ± 0                    | 36 ± 0                   |
|                               | 8                  | 41 ± 1      | 35 ± 0                   | 34 ± 1                 | 35 ± 0                    | 35 ± 0                   |
| CRP (μg/mL)                   | 8                  | 397 ± 10    | 504 ± 10 <sup>a</sup>    | 555 ± 39               | 583 ± 13 <sup>c</sup>     | 529 ± 22 <sup>e</sup>    |
| Amylase (U/L)                 | 8                  | 1278 ± 138  | 1750 ± 23 <sup>a</sup>   | 1753 ± 58              | 2149 ± 31 <sup>c</sup>    | 1744 ± 16 <sup>e</sup>   |
| Superoxide (count/min)        | 8                  | 1337 ± 68   | 3169 ± 24 <sup>a</sup>   | 3860 ± 52              | 6069 ± 60 <sup>c</sup>    | 3234 ± 33 <sup>e</sup>   |
| WBC count (/mm <sup>3</sup> ) | 8                  | 14168 ± 586 | 20290 ± 390 <sup>a</sup> | 22622 ± 630            | 24357 ± 1008 <sup>c</sup> | 21442 ± 767 <sup>e</sup> |
| Plasma endotoxin (EU/mL)      | 8                  | < 0.02      | < 0.02                   | < 0.02                 | < 0.02                    | < 0.02                   |

Rats were on regular (C) or high-fructose enriched diet (F) for 4 wk and then further divided into those with intraportal infusion of saline or lipopolysaccharides (LPS), combined with vehicle or  $\alpha$  lipoic acid (LA) for additional 4 wk (C<sub>PV</sub>, F<sub>PV</sub>, F<sub>PV</sub> + LA, F<sub>PLPS</sub>, F<sub>PLPS</sub> + LA). Values are expressed as mean  $\pm$  SE,  $n = 6$  per group. <sup>a</sup> $P < 0.05$  vs C<sub>PV</sub>; <sup>c</sup> $P < 0.05$  vs F<sub>PV</sub>; <sup>e</sup> $P < 0.05$  vs F<sub>PLPS</sub> for each corresponding time point. TG: Triglycerides; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; CRP: C-reactive protein; WBC: White blood cell.



**Figure 1** Effects of  $\alpha$ -lipoic acid on hepatic triglyceride contents. Hepatic triglyceride levels were measured from the blood of each group of fructose-fed rats and the control group. Values are expressed as mean  $\pm$  SE,  $n = 6$  per group. <sup>a</sup> $P < 0.05$  vs C<sub>PV</sub>. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides.

visualized with HRP substrate (REAL EnVision Detection System; Dako).

A total of  $100 \pm 25$  islets per experimental group were blindly scored for CD68-positive cells around the periphery and/or within islets from five or six different animals by two investigators. Islet area was measured as the area of islet capsule in pancreatic section with HE stain and computed using AxioVision LE 4.8.2.0 software.

### Statistical analysis

Statistical analysis was performed according to the repeated measurements of one-way analysis of variance fol-

lowed by Bonferroni test. A probability of  $P < 0.05$  was taken to indicate a significant difference between means. Values are expressed as mean  $\pm$  SE.

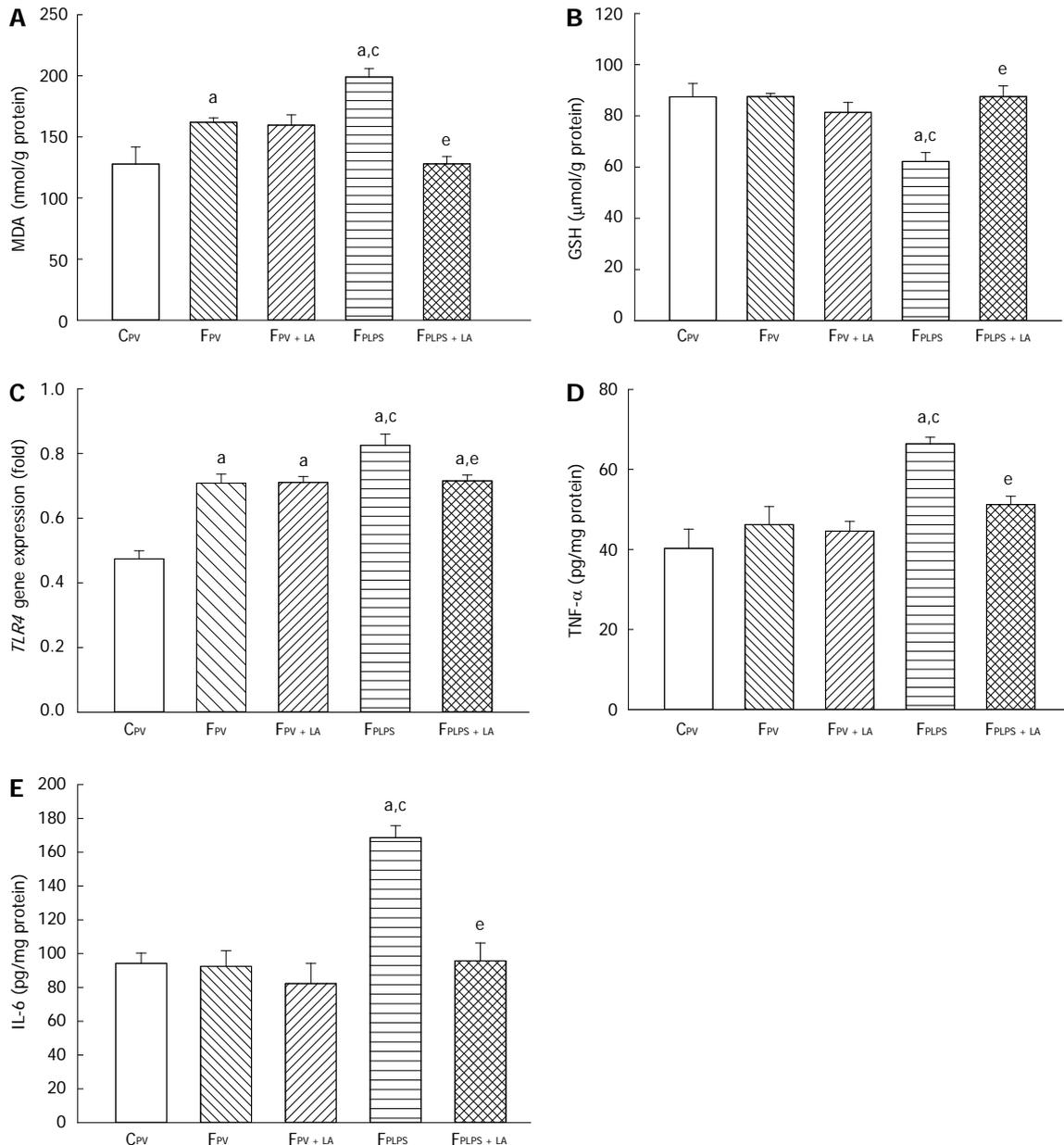
## RESULTS

### Metabolic and hemodynamic parameters

We first measured the effect of fructose feeding and LPS treatment on rats with and without LPS infusion. After high-fructose feeding for 4 wk, fasting plasma insulin levels in fructose-fed groups were significantly increased as compared with controls, but not different among fructose-fed groups. Plasma CRP, amylase, superoxide and white blood cells were also significantly increased in fructose-fed rats and further increased after intraportal LPS infusion for 4 wk. The above LPS-induced responses were suppressed in those co-treated with LA to levels similar to those in fructose-fed untreated controls. On the other hand, there were no significant differences in fasting plasma glucose, AST, ALT, albumin and endotoxin concentrations among experimental groups (Table 1).

### Triglyceride contents, oxidative parameters and inflammatory parameters in hepatic tissue

To measure the liver response to our conditions, we measured triglyceride levels, oxidative parameters, and inflammatory parameters. Fructose feeding resulted in elevated triglyceride levels in all groups when compared to the control groups. Administration of LA had no effect on these levels (Figure 1). As shown in Figure 2A, MDA levels in the liver were significantly increased in fructose-fed rats as compared with controls and further elevated in animals treated with LPS. However, the increase of



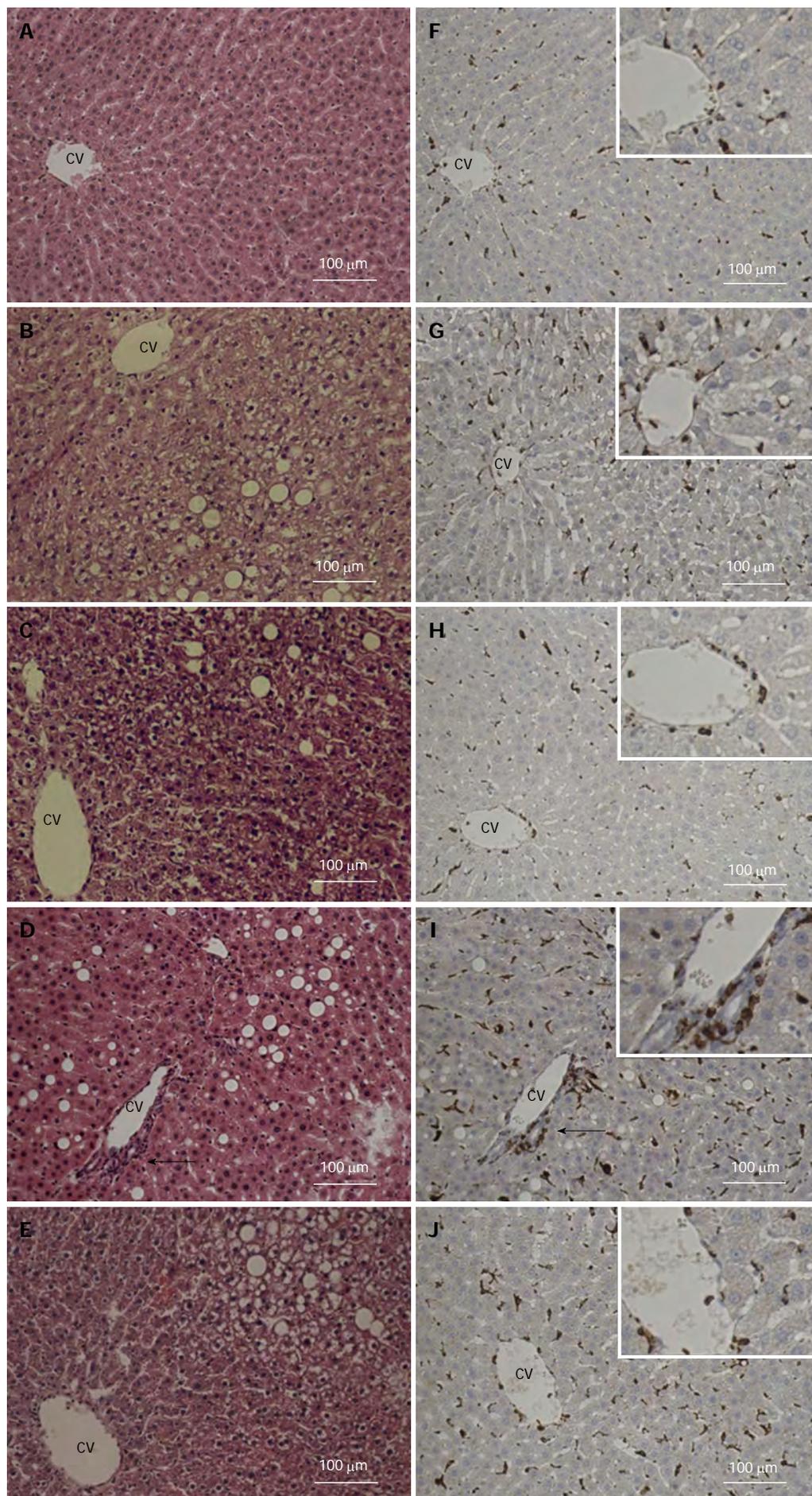
**Figure 2** Effect of  $\alpha$ -lipoic acid on portal endotoxemia-induced changes in hepatic oxidative and inflammatory markers. The following parameters were measured from liver of each group of fructose-fed rats and the control group: (A) Malondialdehyde (MDA) content; (B) Glutathione (GSH) content; (C) Toll-like receptor 4 (*TLR4*) gene expression; (D) Tumor necrosis factor alpha (TNF- $\alpha$ ) protein; and (E) Interleukin-6 (IL-6) protein. Values are expressed as mean  $\pm$  SE,  $n = 6$  per group. <sup>a</sup> $P < 0.05$  vs Cpv; <sup>b</sup> $P < 0.05$  vs Fpv; <sup>c</sup> $P < 0.05$  vs FPLPS. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides.

hepatic MDA contents in fructose-fed rats following intraportal LPS infusion was significantly reversed in those with LA administration. Liver GSH levels were significantly decreased following LPS infusion, but were significantly reversed in those treated with LA (Figure 2B). Additionally, the enhanced hepatic *TLR4* gene expression by chronic high-fructose feeding was further increased in those with intraportal LPS infusion. LA administration suppressed the augmentation of *TLR4* gene expression induced only by mild portal endotoxemia and not by high-fructose feeding (Figure 2C). The significant increase of TNF- $\alpha$  and IL-6 protein levels in the liver of LPS-infused rats was reflective of the hepatic inflammatory response. These levels were significantly suppressed with LA treat-

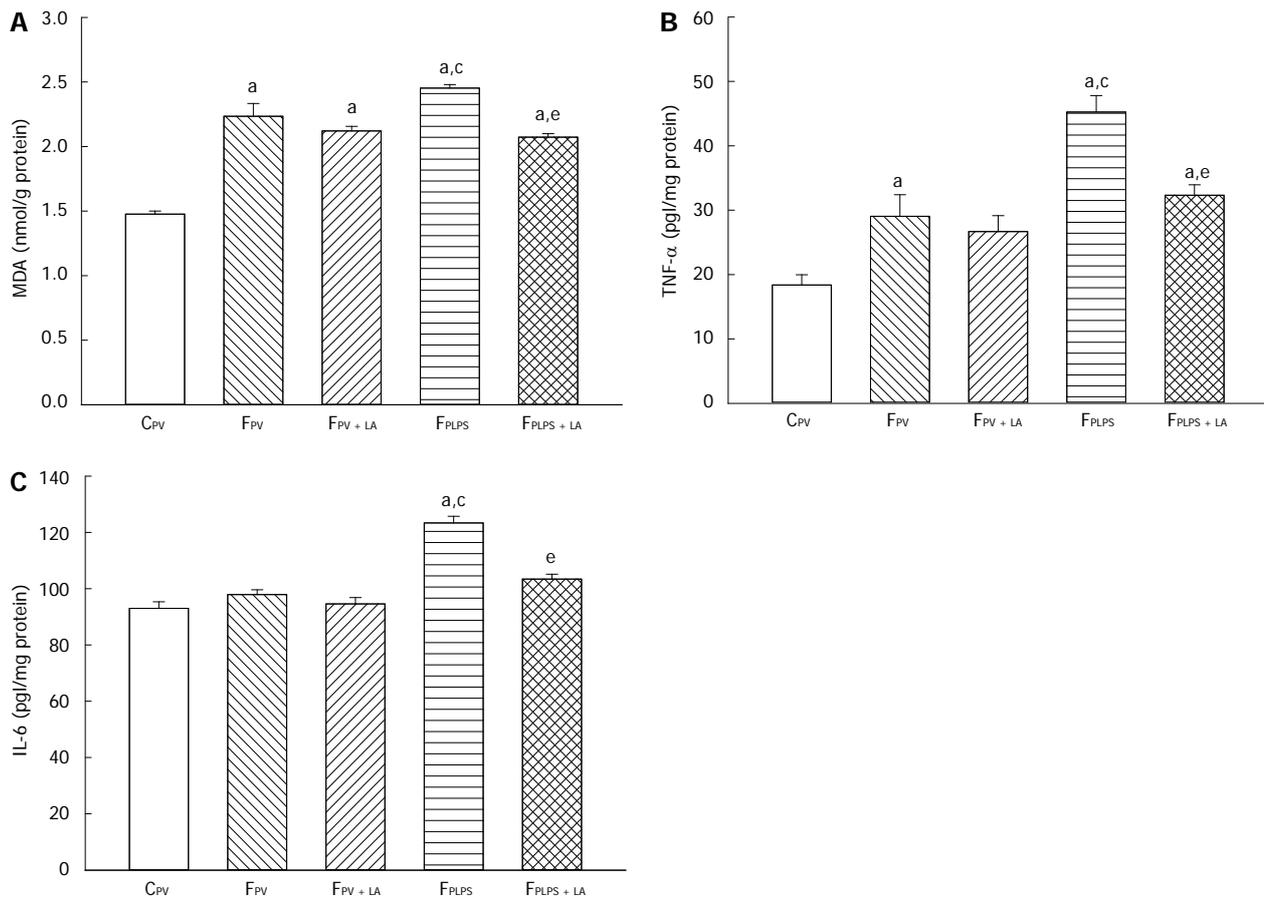
ment (Figure 2D and E). LPS infusion had no effect on triglyceride levels, but did have an effect on MDA, GSH, *TLR4*, TNF- $\alpha$  and IL-6. LA administration significantly reversed these effects.

#### Histopathological changes in the liver

Following analysis of the liver response to our treatments, we sought to examine the liver for histopathological changes. Not surprisingly, steatosis was noted in fructose-fed groups but was not different between groups. On the other hand, the infiltration of monocytes in liver was markedly exhibited in LPS-treated animals (Figure 3D), a phenotype not seen in any other treatment group (Figure 3A-C), and alleviated by the administration of LA



**Figure 3** Histopathological examination of liver in rats with regular or high-fructose feeding. Lipid accumulation in: (A) C<sub>PV</sub> rats; (B) F<sub>PV</sub> rats; (C) F<sub>PV+LA</sub>; (D) F<sub>PLPS</sub>; and (E) F<sub>PLPS+LA</sub> rats. CD-68 positive cell infiltration in: (F) C<sub>PV</sub> rats; (G) F<sub>PV</sub> rats; (H) F<sub>PV+LA</sub>; (I) F<sub>PLPS</sub>; and (J) F<sub>PLPS+LA</sub> rats. Slides were stained with hematoxylin and eosin (A-E) and immunostained with an anti-CD68 antibody (F-J). Arrows indicate CD68 positive cells. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides; CV: Central vein.



**Figure 4** Effect of  $\alpha$ -lipoic acid on portal endotoxemia-induced changes in the pancreas. The pancreata from experimental animals were harvested and tested for: (A) Malondialdehyde (MDA) content; (B) Tumor necrosis factor alpha (TNF- $\alpha$ ) protein level; and (C) Interleukin-6 (IL-6) protein level. Values are expressed as mean  $\pm$  SE,  $n = 6$  per group. <sup>a</sup> $P < 0.05$  vs CPV; <sup>c</sup> $P < 0.05$  vs FPLPS; <sup>e</sup> $P < 0.05$  vs FPLPS + LA. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides.

(Figure 3E). Accordingly, immunohistochemical staining showed that the observed CD68-positive cell infiltration was observed in areas around the central vein in LPS infused animals (Figure 3I), but significantly attenuated in those with LA administration (Figure 3J). Therefore, LPS treatment resulted in marked histopathological changes in the liver of fructose-fed animals, a phenotype that was reversed by the administration of LA.

#### Content of MDA and inflammatory markers in the pancreas

To measure the pancreas response to our conditions, we tested for MDA, TNF- $\alpha$  and IL-6 levels. Fructose feeding increased all three parameters, while fructose feeding with infusion of LPS significantly increased each (Figure 4A-C). Administration of LA resulted in each parameter returning back to original fructose-fed levels. *TLR4* gene expression was not detected in pancreas in experimental groups. Therefore, immunological markers including TNF- $\alpha$  and IL-6, but not *TLR4*, are increased upon fructose feeding and LPS infusion, a phenotype that can be reversed with administration of LA.

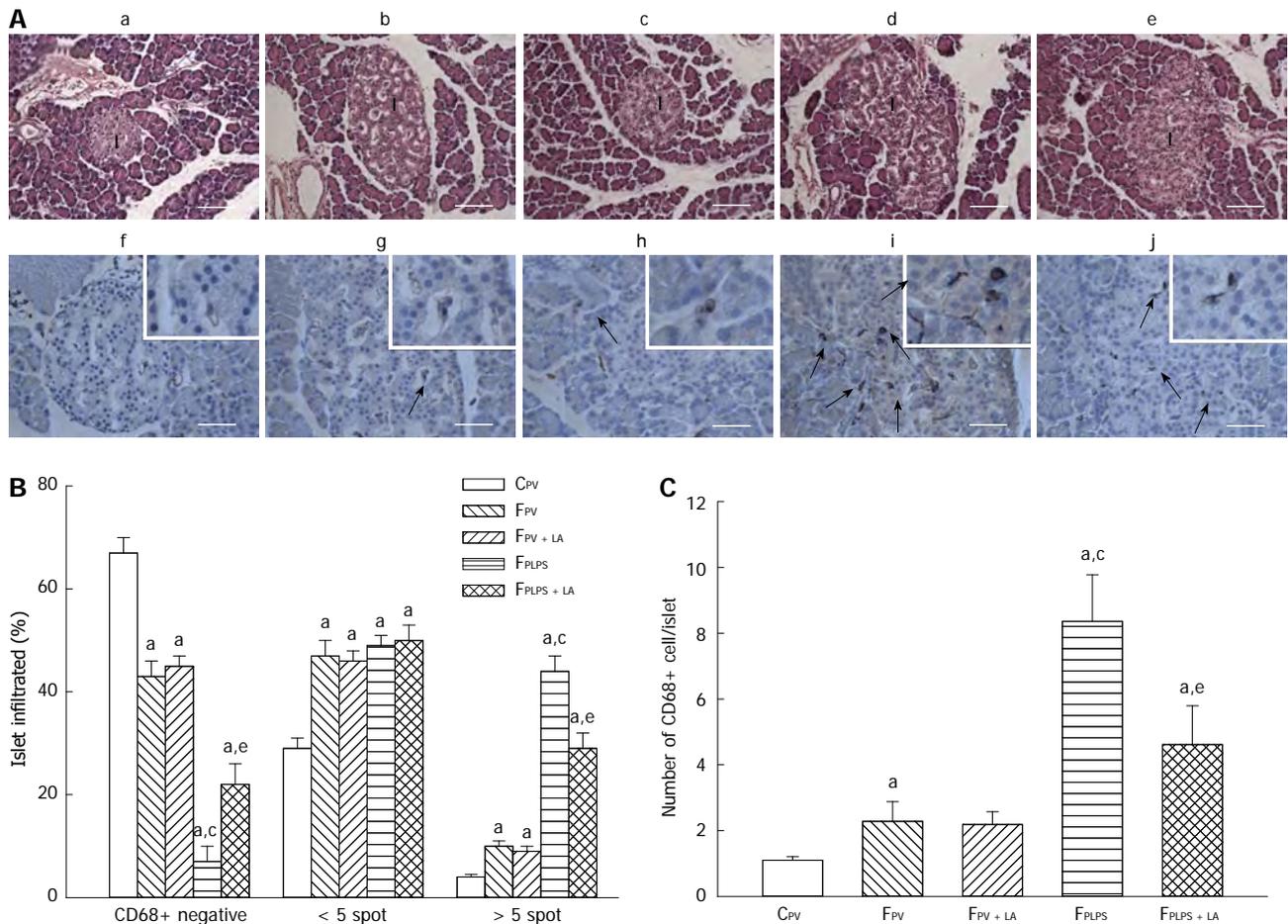
#### Histopathologic changes in the pancreatic islets

We then examined the histopathological response to our

treatments in the pancreas. Following LPS treatment, pancreatic islets exhibited damage that was attenuated with LA administration (Figure 5, top panels). Consistently, the frequency and CD68-positive cell infiltration in fructose-fed animals was only slightly increased as compared to controls. However, they were markedly increased in following LPS infusion and attenuated with LA administration (Figure 5, bottom panels). Quantitation of this data shows the frequency of CD68-positive islets and numbers of CD68-positive cells/islet in fructose-fed rats were further elevated following LPS infusion and partially suppressed in those with LA administration.

#### Glucose-stimulated insulin secretion

Hyperglycemic clamp is the gold-standard method to evaluate glucose-stimulated insulin secretion *in vivo*<sup>[26]</sup>. Plasma glucose levels were not different during the basal period and maintained similar hyperglycemia during the clamp periods among experimental groups (Figure 6A). Increases in plasma insulin levels from baseline in fructose-fed animals were significantly lower than those in controls and further decreased in those infused with LPS under similar hyperglycemic conditions (Figure 6B). The diminished glucose-stimulated insulin secretion shown in fructose-fed controls was not significantly changed with



**Figure 5** Histopathological examination of pancreatic islets. A: Tissue samples from each subset of animals were analyzed by hematoxylin and eosin (HE) staining (a-CpV, b-FpV, c-FpV + LA, d-FpLPS, e-FpLPS + LA) and immunostaining with an antibody against CD68 (f-CpV, g-FpV, h-FpV + LA, i-FpLPS, j-FpLPS + LA); This data was then quantitated as (B) percentage of islets infiltrated and (C) number of CD68+ cells per islet. Arrows indicated CD68-positive cells. I: Islet. A total of 100 ± 25 islets per experimental group were blindly scored for CD68-positive cells around the periphery and/or within islets from 5 to 6 different animals. Islet area was measured by the area of islet capsule in pancreatic section with HE stain and computed using AxioVision LE 4.8.2.0 software. Values are expressed as mean ± SE, n = 6 per group. Line bar: 50 μm. <sup>a</sup>P < 0.05 vs CpV; <sup>c</sup>P < 0.05 vs FpV; <sup>e</sup>P < 0.05 vs FpLPS. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides.

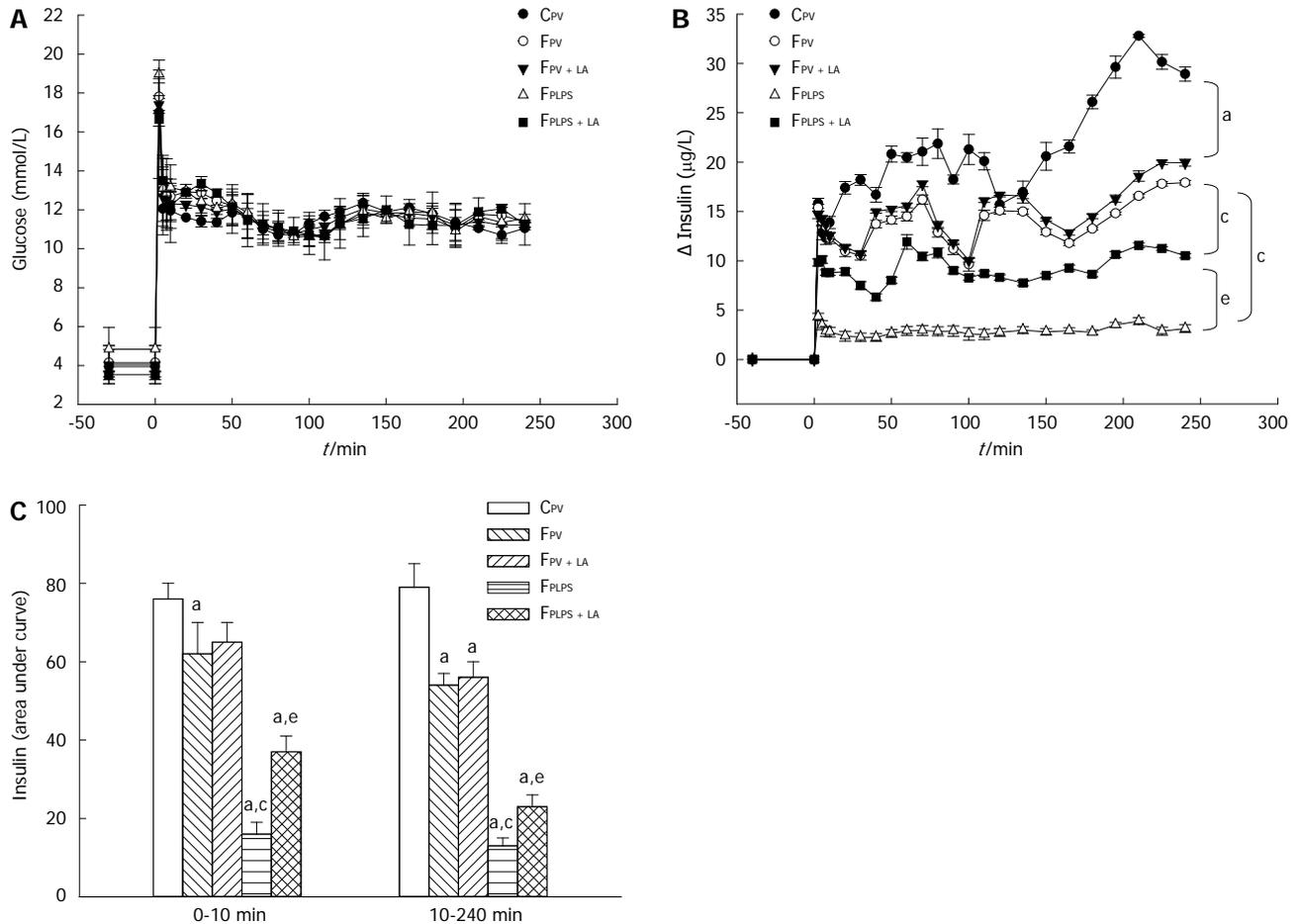
LA administration. However, intraportal LPS infusion significantly impaired the glucose-stimulated insulin secretion shown in fructose-fed controls, which was significantly reversed in those with LA treatment (Figure 6B). In addition, the first-phase insulin secretion (0-10 min post glucose treatment) and the second-phase insulin secretion (10-240 min post glucose treatment) were significantly lower in fructose-fed animals when compared with controls. This was further exacerbated following intraportal LPS infusion, a phenotype that was partially reversed when treated with LA (Figure 6C).

## DISCUSSION

Nonalcoholic steatohepatitis is not only highly correlated with the development of metabolic syndrome and type 2 diabetes mellitus, but is also a crucial factor in progression to cirrhosis and hepatocellular carcinoma<sup>[5]</sup>. In addition, mild portal endotoxemia has been speculated as a crucial risk factor to induce hepatic inflammation in the state of steatosis and impaired pancreatic β cell func-

tion<sup>[4]</sup>. This study explored the potential therapeutic role of the potent antioxidant α-LA in liver disease and associated pancreatic abnormalities. Using the animal model for metabolic steatohepatitis, fructose-fed rats, we found administration of LA reversed mild portal endotoxemia-induced inflammation. Further, portal endotoxemia also decreased glucose-stimulated insulin secretion, a phenotype that was reversed following LA treatment.

LA has been reported to scavenge free radicals, chelate metals and restore intracellular GSH, all factors associated with increasing age<sup>[13]</sup>. In addition, it is used as a therapeutic agent in several liver-related diseases such as alcohol-induced liver damage<sup>[27]</sup> and fatty liver disease<sup>[16]</sup> through multiple mechanisms. Our observations further establish LA as a therapeutic for liver disease. Specifically, our animal model shows LA improves endotoxin-induced hepatic disorders in addition to its established role as a treatment for chronic high-fructose feeding. These antioxidant and anti-inflammatory characteristics may be in the ability of LA to restore tissue GSH-dependent antioxidant defenses during portal endotoxin attack.



**Figure 6** Effect of  $\alpha$ -lipoic acid on plasma glucose and insulin levels. A hyperglycemic clamp technique was used to evaluate glucose-stimulated insulin secretion. A: Plasma glucose levels during the clamp period; B: Change in insulin levels during clamp period; C: The insulin level averages during the first phase (0-10 min) and the second phase (10-240 min) of the clamp period. Values are expressed as mean  $\pm$  SE,  $n = 6$  per group. <sup>a</sup> $P < 0.05$  vs CpV; <sup>b</sup> $P < 0.05$  vs FpV; <sup>c</sup> $P < 0.05$  vs FpLPS. C: Regular diet; F: High-fructose enriched diet; LA: Lipoic acid; LPS: Lipopolysaccharides.

Furthermore, these data show LA administration significantly suppressed the augmented *TLR4* gene expression induced by mild portal endotoxemia but not chronic fructose feeding. Hepatic *TLR4* is activated by gut-derived LPS and attributed to the pathogenesis of liver inflammation<sup>[28]</sup>. In addition, *TLR4* signaling is involved in the development of fructose-induced steatosis in mice<sup>[29]</sup> and also required for liver steatosis, inflammation and a fibrogenic response after chronic alcohol treatment in *TLR4* transgenic mice<sup>[30]</sup>. In addition to LPS, other potential agents including saturated fatty acids (*i.e.*, palmitate) and alarmins (*i.e.*, HMGB1) have also been shown to stimulate the inflammatory response in a *TLR4*-dependent manner<sup>[31,32]</sup>. Hepatic *TLR4* signaling occurs not only in Kupffer cells but also in hepatic non-immune cell populations including hepatocytes, biliary epithelial cells, endothelial cells and hepatic stellate cells<sup>[33]</sup>. In brief, these observations implicate *TLR4*-mediated inflammatory signaling in hepatic cell populations are crucially involved in the development of NAFLD, affected individually by diet components and portal endotoxemia.

Our observations showed that LA not only significantly improved the inflammatory changes in fructose-

induced NASH but also diminished the detrimental inflammatory response of the pancreas. Consistent with our results, LA has a protective effect on cholecystokinin-octapeptide induced acute pancreatitis in rats<sup>[15]</sup>. LA was reported to have a dose-related cytoprotective effect on hydrogen peroxide-induced oxidative stress on pancreatic beta cells. On the other hand, LA could also directly suppress insulin secretion in pancreatic beta cells at high concentrations by inducing AMP-activated protein kinase activation<sup>[34]</sup>. Taken together, data from these studies combined with our observations implicate a beneficial effect of LA on impaired pancreatic insulin secretion. This effect might be attributed to its anti-oxidative and anti-inflammatory actions on mild portal endotoxemia-induced liver inflammation and subsequent pancreatic damage, but not its direct effect on pancreatic beta cell secretion.

Nevertheless, the selected dose of intraportal LPS infusion was based on evaluating its effects on the mortality rate and systemic endotoxin levels during 4-wk infusion period in our previous study<sup>[11]</sup>. Consistently, this low-dose LPS infusion caused subacute hepatic inflammation that could be almost completely be cleared by Kupffer cells once it passed through the liver, so that the arterial

plasma endotoxin levels were not different among experimental groups.

The present study showed that administration of LA could protect liver from LPS-induced oxidative stress and inflammation while reversing the subsequent impairment of the pancreas in rats with fructose-associated fatty liver disease. However, LA had no effect on oxidative stress induced by high-fructose feeding. Clinical implications of this observation suggest both the suppression of portal endotoxemia-induced oxidative stress and dietary interventions are crucial for improving symptoms of NASH.

Although both oxidative stress induced by low-dose intraportal LPS infusion and high-fructose feeding have been demonstrated to contribute to the development of steatohepatitis in fructose-fed rats, the possible involvement of TLR4 signaling in individual hepatic cell populations remain elusive in the present study.

In conclusion, the present study demonstrates that LA can significantly reduce intraportal LPS-induced liver inflammation and associated deterioration of insulin secretion function in this metabolic syndrome rodent model. In addition, it is also implicated that hepatic TLR4 signaling might not only play a significant role in chronic-fructose-feeding induced hepatic steatosis but also in its subacute inflammatory change induced by mild portal endotoxemia and associated extrahepatic disorders in this model.

## ACKNOWLEDGMENTS

The authors also gratefully acknowledge the research assistance of Ms. Kai-Chil Hsieh and Mr. Hung-Che Chien.

## COMMENTS

### Background

$\alpha$ -lipoic acid (LA), a potent antioxidant and also an inducer of endogenous antioxidants has been reported to protect the liver and pancreas from injury.

### Research frontiers

Chronic stress such as portal endotoxemia has been documented to activate hepatic Kupffer cells and cause the release of reactive oxygen species, potentially inducing inflammatory changes in the liver and impairing pancreatic functions. The potential therapeutic effect of LA on mild portal endotoxemia and fructose-induced inflammatory changes of fatty liver and impaired pancreatic insulin secretion in rats remain controversial.

### Innovations and breakthroughs

LA has been documented to have a protective effect on hepatopancreatic damage. However, the effect of LA on lipopolysaccharide-induced non-alcoholic steatohepatitis (NASH) remains unclear. The present result in this manuscript has a significant contribution to clarify this issue.

### Applications

The present study implicates that LA might suppress inflammatory change of steatosis and associated deterioration of insulin secretion in the patients with metabolic syndrome.

### Terminology

Alpha LA is a potent antioxidant and an inducer of endogenous antioxidants. It also has a protective effect on hepatic and pancreatic injury.

### Peer review

The authors found that LA quenches inflammation and oxidative stress, leading to attenuation of NASH. So far a few reports describe the effect of LA on lipopolysaccharide-induced NASH. The manuscript represents a major effort to fill the gap.

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P- Reviewer Yi XW S- Editor Song XX L- Editor A  
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## Oncogene *GAEC1* regulates *CAPN10* expression which predicts survival in esophageal squamous cell carcinoma

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Supported by The General Research Fund, offered by Research Grant Council of Hong Kong to Tang JCO and Lam AKY, PolyU 5627/08M; Griffith Health Institute Project Grant

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Received: September 7, 2012 Revised: November 3, 2012

Accepted: February 5, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To identify the downstream regulated genes of *GAEC1* oncogene in esophageal squamous cell carcinoma and their clinicopathological significance.

**METHODS:** The anti-proliferative effect of knocking down the expression of *GAEC1* oncogene was stud-

ied by using the RNA interference (RNAi) approach through transfecting the *GAEC1*-overexpressed esophageal carcinoma cell line KYSE150 with the pSilencer vector cloned with a *GAEC1*-targeted sequence, followed by MTS cell proliferation assay and cell cycle analysis using flow cytometry. RNA was then extracted from the parental, pSilencer-*GAEC1*-targeted sequence transfected and pSilencer negative control vector transfected KYSE150 cells for further analysis of different patterns in gene expression. Genes differentially expressed with suppressed *GAEC1* expression were then determined using Human Genome U133 Plus 2.0 cDNA microarray analysis by comparing with the parental cells and normalized with the pSilencer negative control vector transfected cells. The most prominently regulated genes were then studied by immunohistochemical staining using tissue microarrays to determine their clinicopathological correlations in esophageal squamous cell carcinoma by statistical analyses.

**RESULTS:** The RNAi approach of knocking down gene expression showed the effective suppression of *GAEC1* expression in esophageal squamous cell carcinoma cell line KYSE150 that resulted in the inhibition of cell proliferation and increase of apoptotic population. cDNA microarray analysis for identifying differentially expressed genes detected the greatest levels of downregulation of calpain 10 (*CAPN10*) and upregulation of trinucleotide repeat containing 6C (*TNRC6C*) transcripts when *GAEC1* expression was suppressed. At the tissue level, the high level expression of calpain 10 protein was significantly associated with longer patient survival (month) of esophageal squamous cell carcinoma compared to the patients with low level of calpain 10 expression ( $37.73 \pm 16.33$  vs  $12.62 \pm 12.44$ ,  $P = 0.032$ ). No significant correction was observed among the *TNRC6C* protein expression level and the clinicopathological features of esophageal squamous cell carcinoma.

**CONCLUSION:** *GAEC1* regulates the expression of

*CAPN10* and *TNRC6C* downstream. Calpain 10 expression is a potential prognostic marker in patients with esophageal squamous cell carcinoma.

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**Key words:** Esophageal squamous cell carcinoma; Oncogene; RNA interference; Calpain 10; Tissue microarray

Chan D, Tsoi MYT, Liu CD, Chan SH, Law SYK, Chan KW, Chan YP, Gopalan V, Lam AKY, Tang JCO. Oncogene *GAEC1* regulates *CAPN10* expression which predicts survival in esophageal squamous cell carcinoma. *World J Gastroenterol* 2013; 19(18): 2772-2780 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2772.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2772>

## INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) has a multifactorial etiology which involves environmental and/or genetic factors<sup>[1,2]</sup>. The incidence of ESCC also shows marked variation in its geographic distribution and occurs at relatively high frequency in Asian regions including China<sup>[3]</sup>. Current modalities of therapy for this disease offer relatively poor survival and cure rates<sup>[4]</sup>, thus more investigations at the molecular level are essential for a better understanding the molecular pathogenesis of this disease and for making further improvements in diagnosis and treatment of ESCC.

Gene amplification and overexpression have been suggested as the major genomic aberrations involved in the pathogenesis of ESCC<sup>[5,6]</sup>. We previously employed the method of comparative DNA fingerprinting using inter-simple sequence repeat polymerase chain reaction (ISSR-PCR) and revealed that amplifications or deletions of chromosomal sequences are common events in both the preneoplastic lesions and carcinomas<sup>[7]</sup>. An analysis of the frequency of amplification or loss of individual ISSR-PCR profile bands led to the identification of a novel expressed sequence tag database entry of a cDNA clone from a chromosome 7 placental cDNA library<sup>[7,8]</sup>. Moreover, the ISSR-PCR fragment also showed 98% homology to a Homo sapiens chromosome 7 P1-derived artificial chromosome clone (approximately 125 kb) which has been mapped to chromosome band 7q22<sup>[9]</sup>. The amplification of chromosomal segment 7q22 has been implicated in many types of cancer. Reported examples include ESCC<sup>[10]</sup>, breast carcinoma<sup>[11]</sup>, pancreatic carcinoma<sup>[12]</sup>, renal-cell carcinomas<sup>[13]</sup> and T-cell leukemia<sup>[14]</sup>. Thus, further investigation on the newly identified ESCC-related genomic and expressed sequences mapped to chromosomal region 7q22 can be a fruitful approach for identifying new candidate genes crucial to the disease. We subsequently identified and characterized the role of a novel oncogene *GAEC1* which is located at 7q22 region, encodes a nuclear protein and shows a high frequency of gene amplification and overexpression in ESCC cell

lines and primary tumors<sup>[15]</sup>, as well as in colorectal adenocarcinoma<sup>[16]</sup>. Overexpression of *GAEC1* in 3T3 mouse fibroblasts caused increased cell proliferation, foci formation and colony formation in soft agar, comparable to *H-ras* overexpression. Further, injection of *GAEC1*-transfected 3T3 cells into athymic nude mice formed undifferentiated sarcoma *in vivo*, providing the first evidence about the oncogenic nature of *GAEC1*<sup>[15]</sup>. An increased *GAEC1* DNA copy number was also reported in 79% of colorectal adenocarcinomas and the copy numbers were significantly different among colorectal adenocarcinomas, adenomas, and non-neoplastic colorectal tissues<sup>[16]</sup>.

In this report, *GAEC1* was further characterized by identifying the downstream partners using cDNA microarray analysis on *GAEC1*-suppressed human esophageal carcinoma cell line KYSE150 which shows *GAEC1* overexpression. The prominently downstream-regulated genes were then studied by immunohistochemistry on a tissue microarray (TMA) of ESCC to determine their clinicopathological significance.

## MATERIALS AND METHODS

### ESCC specimens and cell lines

One hundred and thirty-two paired non-tumor and tumor fresh tissue samples were collected after esophagectomy with patients' consent at the Department of Surgery, Queen Mary Hospital, Hong Kong from 2001 to 2006. They were collected consecutively from esophagectomy specimens performed on patients who had received no prior treatment directed to the primary ESCC. The histopathological features were reported by specialist pathologists of the Department of Pathology, Queen Mary Hospital, Hong Kong. The clinicopathological parameters of the patients were collected prospectively and they included age, gender, tumor-node-metastasis pathological stages and histological grades. The actuarial survival rate of the patients was calculated from the date of surgical resection of the ESCC to the date of death or last follow-up. Management was by a pre-agreed standardized multidisciplinary protocol supervised by a senior specialist surgeon. The ESCC cell line KYSE150 is of Japanese origin. It was purchased from DSMZ (Braunschweig, Germany) and cultured as described<sup>[17]</sup>. The non-tumor esophageal epithelial cell line NE1 was used as the control to confirm the overexpression of *GAEC1* in KYSE150 and was cultured as previously described<sup>[18]</sup>.

### Preparation of small interfering RNA expression vector

A vector based RNA interference (RNAi) approach was used for suppressing the expression of *GAEC1* in KYSE150 ESCC cells. The pSilencer2.1-U6 neo vector (Ambion) was used to express the siRNA which is specific for targeting *GAEC1* expression. The pSilencer2.1-U6 neo Negative Control vector (Ambion) was used as the negative control which expressed a hairpin small interfering RNA (siRNA) with limited homology to any known sequences in the human, mouse and rat genomes. The siRNA target sequence of *GAEC1*

and the insert sequence were determined by the programs siRNA Target Finder and Insert Design Tool for the pSilencer™ Vectors (Ambion). The top strand of the insert sequence (P3-4) is 5'-GATCCGAAGTGGCTTCTGGATTAATTCAAGAGATTAATC-CAGAAGCCACTTCTTTTGGAAA-3' and the bottom strand is 5'-AGCTTTTCCAAAAAGAAGTGGCTTCTGGATTAATCTCTTGAATTAATC-CAGAAGCCACTTCG-3'. The top and bottom strands were annealed and cloned into the pSilencer2.1-U6 neo vector according to the manufacturer's instruction. The vectors were transfected into the KYSE150 cells as previously described<sup>[15]</sup> using FuGene HD (Roche Diagnostics GmbH) with G418 selection.

#### **RNA extraction and reverse transcription-polymerase chain reaction analysis**

RNA was extracted from the parental, pSilencer-P3-4 and pSilencer-negative control vectors transfected KYSE150 cells using the RNeasy mini Kit (Qiagen) after 2 mo selection under G418. About 2 µg DNA-free RNA from each sample was used for the multiplex semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis with β-actin as the internal control to show the expression level of *GAEC1* as previously described<sup>[15]</sup>. Densitometry analysis was performed to compare the intensity of the PCR products after agarose gel electrophoresis under UV using Quantity-One program (Bio-Rad).

#### **Cell proliferation assay**

The cell proliferation of the parental, pSilencer-P3-4 and pSilencer-negative control vectors transfected KYSE150 cells was determined by MTS assay using the CellTiter 96 Aqueous One Solution (Promega) as previously described<sup>[15]</sup>.

#### **Cell cycle analysis**

The parental, pSilencer-P3-4 and pSilencer-negative control vectors transfected KYSE150 cells were resuspended in 500 L 1 × phosphate buffered saline and fixed with 500 L 70% ethanol. The cells were then suspended in 1 mL PI (20 µg/mL)/Triton X-100 (0.1% v/v) staining solution with RNase A (200 µg/mL) and then analyzed by the BD FACSCalibur flow cytometer. Different fractions of cell cycles were analyzed using the Modfit LT software (Verity Software House).

#### **cDNA microarray analysis**

The differentially expressed genes of the pSilencer-P3-4 vector transfected KYSE150 cells with suppressed *GAEC1* expression were identified using cDNA microarray analysis by making comparisons between the parental cells, pSilencer-negative control vectors transfected cells, and pcDNA3.1-*GAEC1* transfected cells with *GAEC1* overexpression<sup>[15]</sup>. The cDNA microarray analysis and the associated quality control using Human Genome U133 Plus 2.0 arrays (Affymetrix) were performed in the Genome Research Centre of the University of Hong Kong according to the Affymetrix's protocol. Briefly, to-

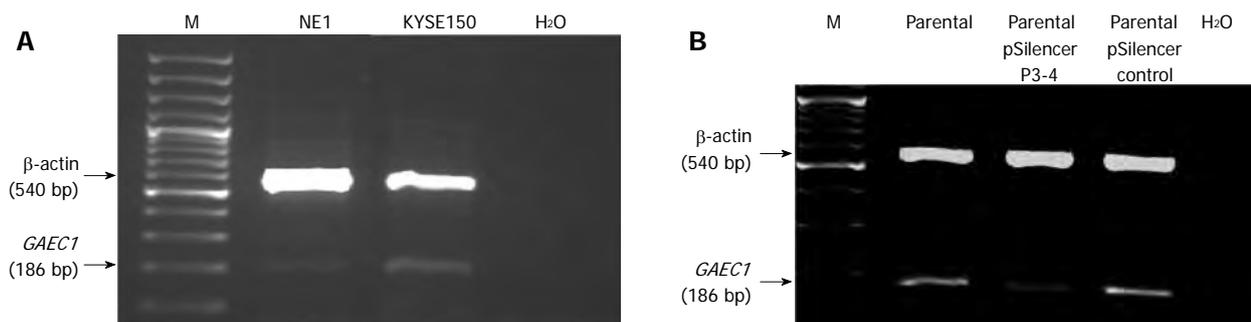
tal RNA was extracted from  $2 \times 10^6$  cells of each sample using RNeasy mini Kit (Qiagen). The RNA integrity was measured by the ratio of 28S/18S ribosomal RNA using Agilent 2100 Bioanalyzer. One microgram total RNA from each source was then reverse transcribed to the first-stranded cDNA by using oligo-dT linked-T7 RNA polymerase promoter sequence and the double-stranded cDNA was synthesized by using RT Kit (Invitrogen). The biotin labelled-cRNA was produced by *in vitro* transcription kit (Invitrogen) and purified by RNeasy mini columns (Qiagen). About 15 µg denatured cRNA was hybridized to each Human Genome U133 Plus 2.0 array (Affymetrix) and then stained with a streptavidin-phycoerythrin conjugate and the signals were detected with GeneArray scanner (Agilent). The microarray signals were analyzed by using Agilent Genespring GX and Affymetrix GeneChip Operating Softwares. The signal of each differentially expressed gene in the pSilencer-P3-4 transfected cells was determined by comparing with the parental cells and normalized with the pSilencer-negative control vector transfected cells. The threshold level of the corresponding up- or down-regulated genes with transfected pcDNA3.1-*GAEC1* vector is  $\geq 2$  folds.

#### **Tissue microarray and immunohistochemical staining**

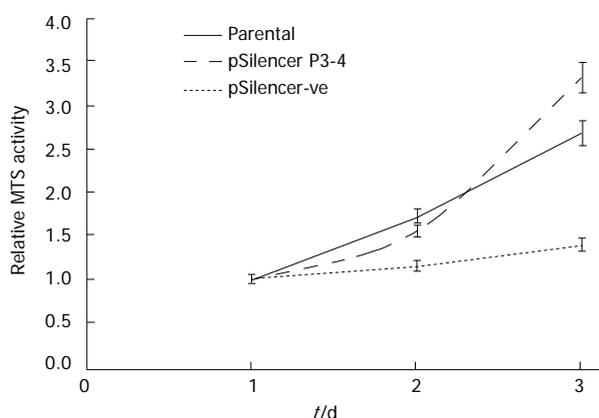
A TMA containing the 132 paired non-tumor esophageal epithelia and ESCC specimens were constructed as described previously<sup>[19]</sup>. The archival paraffin-embedded ESCC tissues were used under the ethical guidelines in the Department of Pathology of The University of Hong Kong. Immunohisto-chemical staining on the TMA sections was performed using the calpain 10 (0.03 mg/mL; Sigma-Aldrich) rabbit polyclonal antibody and *TNRC6C* (1 mg/mL; Abnova) mouse monoclonal antibody using the previously described methodology<sup>[19]</sup>. The dilution factors for the calpain 10 and *TNRC6C* antibodies were 1:50 and 1:150 respectively. The percentage of tumor cells positively stained formed the basis of grading as follows: Grade 0: less than 5%, Grade I: 5% to less than 25%, Grade II: 25% to less than 50% and Grade III: more than 50%. For each tissue sample, the tissue core with the highest grade was selected for subsequent statistical analysis. The high expression group combined those tumors with Grade II or III, and the low expression group combined those tumors with Grade 0 or I.

#### **Statistical analysis**

The Student's *t* test was used to evaluate the statistical significance of the differences in calpain 10 and *TNRC6C* expression between tumor and non-tumor tissues. The  $\chi^2$  test and *t* test were used to examine the statistical significance of the correlations between calpain 10 and *TNRC6C* expression with clinicopathological parameters. Kaplan-Meier plots and Cox multi-variant analysis were produced for overall patient survival, and statistical significance was evaluated by using Wilcoxon's signed-rank test. Statistical analysis were performed using SPSS Ver. 20.0 (SPSS, Chicago, IL, United States). Differences were



**Figure 1** Expression level of *GAEC1* in KYSE150 cells. A: Multiplex reverse transcription-polymerase chain reaction (RT-PCR) analysis showed the overexpression of *GAEC1* in KYSE150 compared with the non-tumor esophageal epithelial cell line NE1; B: Multiplex RT-PCR analysis demonstrated the down-regulation of *GAEC1* expression in KYSE150 cells transfected with pSilencer P3-4 vector compared with the parental cells and those transfected with pSilencer control vector. The amount of RNA in each lane was normalized with the amplification of  $\beta$ -actin. M: 100 bp ladder marker; H<sub>2</sub>O: Water control.



**Figure 2** MTS cell proliferation assays for esophageal squamous cell carcinoma cell line KYSE150. Cells were transfected with pSilencer vector cloned with the P3-4 sequence (pSilencer P3-4) or control vector (pSilencer-ve). MTS assays were then performed every 24 h for 3 d on each type of transfected cells and the parental cells. The respective MTS activities on each day were compared with the corresponding activities of day 1. Representative data from 3 independent experiments are shown.

considered statistically significant when the relevant *P* values were < 0.05.

## RESULTS

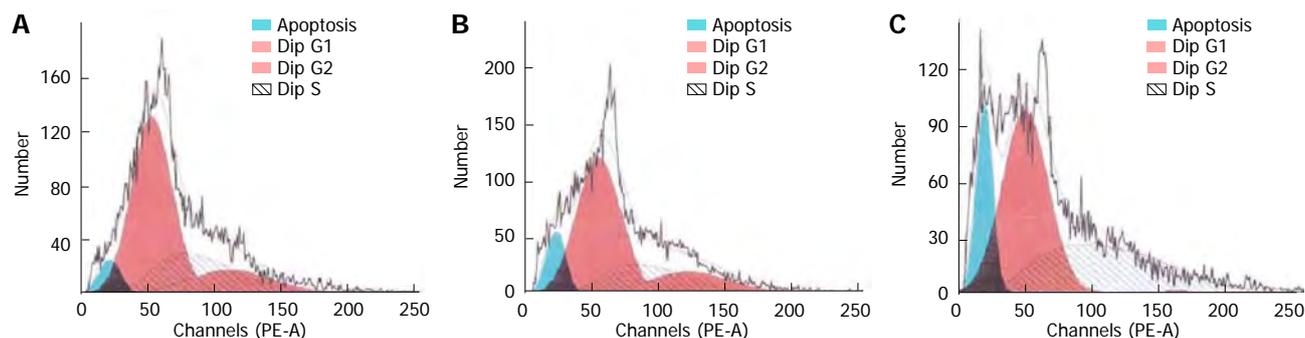
The overexpression of *GAEC1* in KYSE150 over NE1 was confirmed by multiplex semi-quantitative RT-PCR and densitometry analysis (Figure 1A). The expression level of *GAEC1* in pSilencer-P3-4 transfected KYSE150 cells was also determined by comparing with the parental and pSilencer-negative control vector transfected cells using densitometry measurement. The results indicated that the pSilencer-P3-4 transfected KYSE150 cells showed a down-regulation of *GAEC1* expression compared with the parental cells and the control. The comparison of the band intensities among the samples by densitometry measurement showed that the *GAEC1* expression level was down-regulated in the pSilencer-P3-4 transfected cells by about three folds (Figure 1B).

To assess the effect on cell proliferation with suppressed *GAEC1* expression, a comparison was made

between the MTS activities generated from the parental cells and the cells transfected with the pSilencer-negative control vector. The results indicated that the KYSE150 cells with down-regulated *GAEC1* showed an obvious reduction in proliferation rate compared with the parental and control-vector transfected cells (Figure 2). Further analysis on the cell cycle related changes using flow cytometry demonstrated an approximately 50% increase in apoptotic population with suppressed *GAEC1* expression, compared with the parental and control-vector transfected cells (Figure 3).

To identify the downstream candidate genes which are regulated by the suppressed *GAEC1* expression, cDNA microarray analysis was performed using the Human Genome U133 Plus 2.0 array (Affymetrix) which comprises of more than 47000 transcripts and variants in each chip. The results of the identified lists of more than 5-fold down-regulated (total 10 genes) and more than 3-fold up-regulated (total 9 genes) targets were shown in Table 1 respectively. All the listed genes were selected based on more than 2-fold expression signals of the corresponding up- or down-regulation of the respective genes when the cells were transfected with the pcDNA3.1-*GAEC1* vector and no significant fold change was detected with transfected pSilencer-negative control vector compared with parental cells. With suppressed *GAEC1* expression, calpain 10 (*CAPN10*) was identified to have the highest level (> 15 folds) of down-regulation (Table 1), while trinucleotide repeat containing 6C (*TNRC6C*) was shown to have the highest level (> 7 folds) of up-regulation (Table 1). These two *GAEC1*-regulated target genes with the greatest changes in expression level were followed up by the immunohistochemical analysis using the ESCC tissue microarray.

The expression of *CAPN10* and *TNRC6C* proteins in TMA sections sampling 132 paired tumor and non-tumor tissues from ESCC specimens was investigated using immunohistochemistry. Fourteen out of 132 tumors (10.61%) were found to belong to the high expression group of *CAPN10* expression. However, *TNRC6C* did not show any significant expression signals in all the ESCC cases analyzed except eight non-tumor esopha-



**Figure 3** Flow cytometry analyses for KYSE150 cells. KYSE150 transfected with pSilencer cloned with P3-4 sequence demonstrated an increased apoptotic population by approximately 50% (C) compared with the parental cells (A) and cells transfected with pSilencer-ve control vector (B).

**Table 1** List of more than 5-fold and 3-fold down-regulated genes induced by stable *GAEC1* knockdown in KYSE150 cells compared with the parental cells

| Probe set ID | Gene title   | Down-regulation with transfected pSilencer P3-4 | Up-regulation with transfected pcDNA3.1- <i>GAEC1</i> | pSilencer-ve control |
|--------------|--|---|---|----------------------|
| 221040_at    | Calpain 10   | 15.3033010                                      | 2.1643467   | 1.0476209            |
| 1561417_x_at | Not assigned   | 12.1628650                                      | 2.0130675   | 1.1347373            |
| 1562828_at   | Not assigned   | 9.9816000                                       | 2.6808436   | 1.1661105            |
| 229929_at    | splA/ryanodine receptor domain and SOCS box containing 4   | 8.3420770                                       | 2.2365010   | 1.1751518            |
| 235209_at    | Chromosome 8 open reading frame 84                         | 7.6294910                                       | 2.1356385   | 1.1450081            |
| 220090_at    | Cornulin   | 7.6125007                                       | 2.3401918   | 1.1664450            |
| 242713_at    | Not assigned   | 7.3507795                                       | 2.0578532   | 1.1959343            |
| 224499_s_at  | Activation-induced cytidine deaminase                      | 5.8917794                                       | 3.3146940   | 1.1664389            |
| 229543_at    | Not assigned   | 5.3493247                                       | 2.0206234   | 1.0065930            |
| 242064_at    | Sidekick homolog 2 (chicken)                               | 5.0322995                                       | 2.8469403   | 1.0170712            |
| 1561041_at   | Trinucleotide repeat containing 6C                         | 7.5979643                                       | 2.1234870   | 1.0152589            |
| 216787_at    | Not assigned   | 5.3369575                                       | 2.2657390   | 1.1658608            |
| 206725_x_at  | Bone morphogenetic protein 1                               | 4.8828310                                       | 2.7046654   | 1.1015952            |
| 206276_at    | Lymphocyte antigen 6 complex, locus D                      | 4.7652740                                       | 2.7379642   | 1.0686288            |
| 1560482_at   | Not assigned   | 4.2348604                                       | 3.0122058   | 1.1875614            |
| 211362_s_at  | Serpin peptidase inhibitor, clade B (ovalbumin), member 13 | 4.0584164                                       | 2.4208739   | 1.0186443            |
| 216491_x_at  | Immunoglobulin heavy constant mu                           | 3.4819565                                       | 2.8060850   | 1.0186309            |
| 238415_at    | Not assigned   | 3.2034543                                       | 2.9523630   | 1.0138865            |
| 241028_at    | RPGRIPI-like   | 3.0331728                                       | 2.3752263   | 1.0001514            |

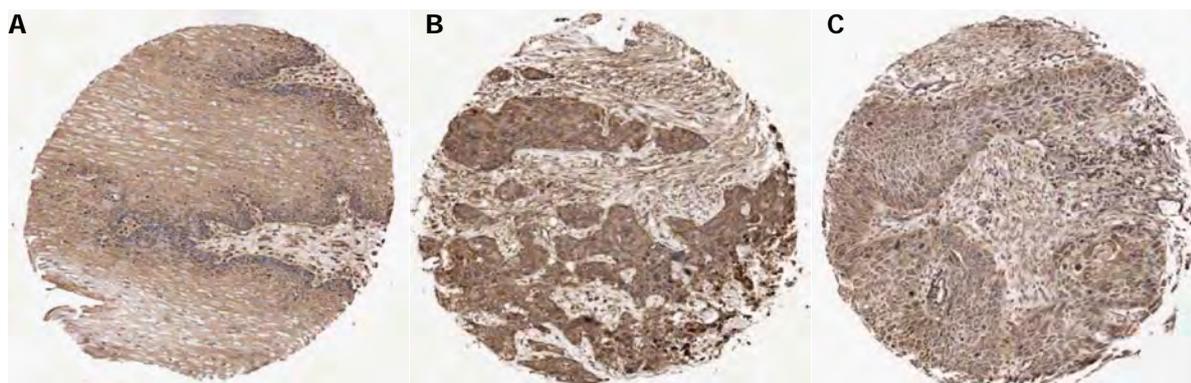
All the listed genes were selected based on more than 2-fold of the corresponding up-regulation when *GAEC1* was overexpressed with transfected pcDNA3.1-*GAEC1* vector and no significant fold change with transfected pSilencer-negative control vector compared with parental cells.

geal tissues which also served as the positive controls. Representative examples of immunohistochemical staining of *CAPN10* and *TNRC6C* are shown in Figure 4. Correlation between expression level of *CAPN10* and clinicopathological features are summarized in Table 2. There was no significant correlation of any clinicopathological features with the expression level of *CAPN10*. The median survival of patients with high expression level of *CAPN10* was 38 mo whereas that of low expression level was 13 mo, and the survival range is from 0.72 to 65.15 mo. The difference was significant on both univariate and multi-variate analysis ( $P = 0.032$  and  $0.035$  respectively; Figure 5).

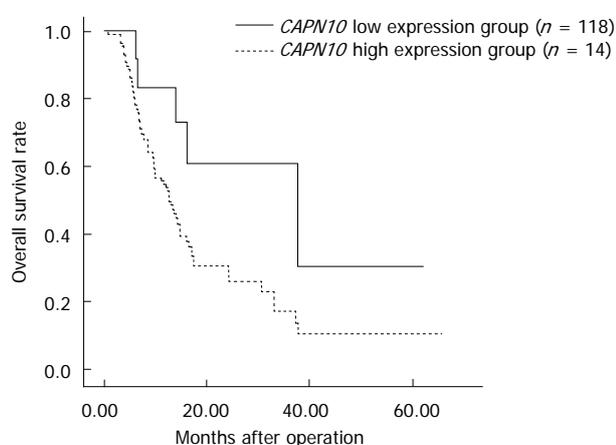
## DISCUSSION

Our previous study reported the oncogenic role of *GAEC1* in esophageal carcinogenesis and high expres-

sion level of *GAEC1* caused malignant transformation of normal cells<sup>[15]</sup>. High DNA copy number of *GAEC1* was also observed in colorectal adenocarcinoma and significant difference was reported in cancer sub-sites and tumor types<sup>[16]</sup>. From our previous study<sup>[15]</sup>, however, no significant correlation was observed between *GAEC1* amplification and clinicopathological parameters and prognosis in ESCC tumors, and thus the DNA amplification study of *GAEC1* is not included in this report. In the present study, an attempt was made to investigate the downstream-regulated genes when *GAEC1* expression was suppressed in an ESCC cell line KYSE150. Our group also investigated the ESCC cell lines which showed overexpression of *GAEC1* as we reported previously<sup>[15]</sup>. KYSE150 showed the more stable and consistent overexpression with time compared among the ESCC cell lines. Reduction of proliferation rate and increase in apoptotic population were observed in association with reduced



**Figure 4** Immunohistochemical staining of *CAPN10*. A: In normal esophageal epithelial tissue showing weak *CAPN10* staining; B: esophageal squamous cell carcinoma (ESCC) tissue showing strong *CAPN10* staining; C: ESCC tissue showing weak *CAPN10* staining in tumor. *CAPN10* was mainly localized in the cytoplasm of the cancer cells (original magnification, ×100).



**Figure 5** Overall 5-year survival rates as determined by the expression level of *CAPN10* in esophageal squamous cell carcinoma patients. Low expression group of *CAPN10* in ESCC patients showed a significantly lower 5-year survival rate than those of high expression group.

**Table 2** Relationship between *CAPN10* expression and clinicopathological features

| Characteristics       | Patients | Low expression | High expression | P value |
|-----------------------|----------|----------------|-----------------|---------|
| Age, yr (mean ± SD)   | 132      | 65.64 ± 10.55  | 63.50 ± 13.39   | 0.572   |
| Gender                |          |                |                 | 0.086   |
| Male                  | 102      | 94             | 8               |         |
| Female                | 30       | 24             | 6               |         |
| TNM stage             |          |                |                 | 0.762   |
| 0/ I / II             | 18       | 15             | 9               |         |
| III / IV              | 83       | 72             | 11              |         |
| Tumor depth           |          |                |                 | 0.729   |
| T1-3                  | 79       | 67             | 12              |         |
| T4                    | 22       | 20             | 2               |         |
| Lymph node metastasis |          |                |                 | 0.363   |
| N0                    | 32       | 26             | 6               |         |
| N1                    | 69       | 61             | 8               |         |
| Distant metastasis    |          |                |                 | 1       |
| M0                    | 66       | 57             | 9               |         |
| M1                    | 35       | 30             | 5               |         |
| Differentiation       |          |                |                 | 0.459   |
| Well                  | 16       | 15             | 1               |         |
| Moderate              | 59       | 51             | 8               |         |
| Poor                  | 26       | 21             | 5               |         |

*GAEC1* expression in ESCC cells. Thus our study is the first report to demonstrate the significance of suppressing *GAEC1* as a target of reducing the malignant properties of ESCC. In order to assess whether the tumors are more proliferative, the use of other histological markers for assessing proliferation, such as Ki-67<sup>[20]</sup> and AgNOR<sup>[21]</sup>, in parallel to *CAPN10* is suggested in future studies to determine whether the *CAPN10* level is associated with progression of the disease. Similar targeting approach against potential oncogenes is now being explored intensively in the direction of gene therapy for various types of cancers<sup>[22]</sup>. Examples include the suppression of *MTA1* in esophageal carcinoma<sup>[23]</sup>, alpha-actinin-4 in oral carcinoma<sup>[24]</sup>, osteopontin in colon carcinoma<sup>[25]</sup> and *EGFR* in hepatocellular carcinoma<sup>[26]</sup>. The application of RNAi approach has been recognized as having high potential for the clinical application of targeted cancer therapy<sup>[27]</sup>. To date, clinical trials at different stages were reported and they targeted against various oncogenic components in various cancers, including metastatic melanoma, liver cancer, chronic myelogenous leukemia, pancreatic cancer and colon cancer<sup>[22]</sup>. Moreover, the

RNAi approach for targeting specifically on transforming growth factor-β has been developed as a “cancer vaccine” against ovarian cancer<sup>[22]</sup>. Thus our present study offers a new direction for exploring the application of RNAi-based method for suppressing the oncogenic target *GAEC1* as a novel gene therapy approach in our future investigations.

Calpain 10 (*CAPN10*) is a member of the mitochondrial calpain system<sup>[28]</sup>. Mitochondrial calpain system has been shown to promote caspase-independent programmed cell death *via* the apoptotic inducing factor-mediated mechanism<sup>[28]</sup> and its expression has been correlated to insulin-stimulated glucose uptake<sup>[29]</sup> and type 2 diabetes<sup>[30]</sup>. However, the correlation and functional roles of *CAPN10* in tumorigenesis are still not fully understood, although *CAPN10* has been linked to laryngeal<sup>[31]</sup>, colorectal<sup>[32]</sup> and pancreatic cancers<sup>[33]</sup>. In the present study, the RNAi-based suppression of *GAEC1*

in KYSE150 resulted in the suppression of *CAPN10* expression (approximately 15-fold compared with the parental cells). For those ESCC tumors belonging to the *CAPN10* low expression group, the 5-year survival rate is significantly lower than those belonging to the *CAPN10* high expression group. From the study of Moreno-Luna *et al.*<sup>[31]</sup>, *CAPN10* genotype 12 was reported to be related with a worse prognosis in laryngeal cancer, which is similar to our present study which is newly described in ESCC. Our observation from the low *CAPN10* expression group implied the possibility that the oncogene *GAEC1* overexpression within this group might involve more prominently at the initial stage of molecular carcinogenesis, so that the expression level of *CAPN10* was lower in ESCC at the time of operation. Similar pattern of oncogenic expression happening at the earlier stage of carcinogenesis was also observed from fibroblast growth factor-2 in melanoma<sup>[34]</sup> and *KLF4* in cutaneous squamous epithelial neoplasia<sup>[35]</sup>. The verification of this hypothesis can be followed up with the future development of *GAEC1*-specific antibody, which is still unavailable in market, for the future analysis of *GAEC1* expression in various stages in ESCC. This important finding also paves the path for the further investigation for the roles of *CAPN10* in the molecular pathogenesis of esophageal carcinoma. Moreover, in the present study, no significant correlation of any other clinicopathological features with the expression level of *CAPN10* was found, but *CAPN10* predicted the poor survival of ESCC patients. Similar results were also reported previously in which the overexpression of a chemokine *CXCL12* in ovarian cancer<sup>[36]</sup> and a protein Rad51 for homologous recombination in ESCC<sup>[37]</sup> also showed a correlation to the survival of patients, but no correlation to other clinicopathological features was found. The level of *CAPN10* is also not associated with local lymph node and distant metastasis in the ESCC cases, implying the possibility that *GAEC1* expression may not be relevant to the control of metastasis in ESCC.

*TNRC6C* has been reported to be the miRNA regulation-related genes and their mutation was correlated to cancer development through deregulating the miRNA regulation<sup>[38]</sup>. *TNRC6C* was also shown in the present study to undergo up-regulation with the suppression of *GAEC1* expression by the RNAi approach, but there was no significant expression of *TNRC6C* in the ESCC cases studied. This may be due to the down-regulation of *TNRC6C* in ESCC by other unknown mechanisms which are subjected to further investigation. Future study of *TNRC6C* mutation in ESCC is required to investigate the possible roles of *TNRC6C* in carcinogenesis. Moreover, among the down-regulated genes identified by cDNA microarray with suppressed *GAEC1* expression, activation-induced cytidine deaminase (AID) was reported to show overexpression in Barrett's esophagus and Barrett's adenocarcinoma<sup>[39]</sup>, but there was no investigation on the roles of AID in molecular pathogenesis in ESCC. AID has been shown to induce somatic mutations in host genes and implicated in the carcinogenesis of lung<sup>[40]</sup>,

colorectal<sup>[41]</sup> and gastric cancers<sup>[42]</sup>. Therefore, our findings provide a new evidence for prompting future study on the role of AID in the development of ESCC.

In conclusion, the suppression of *GAEC1* expression resulted in reduced tumor cell proliferation, increased apoptotic population in ESCC cells and also regulated *CAPN10* and *TNRC6C* expression. The low expression of *CAPN10* predicted the poor survival of ESCC patients.

## ACKNOWLEDGMENTS

Special thanks are given to Professor George SW Tsao of the Department of Anatomy of The University of Hong Kong for giving us the cell line NE1 as the control. Tang JCO would also like to thank the Griffith University of Australia for awarding the Visiting Research Fellowship (2012).

## COMMENTS

### Background

Esophageal squamous cell carcinoma (ESCC) has a multifactorial etiology which involves environmental and/or genetic factors. More investigations at the molecular level are essential for a better understanding the molecular pathogenesis of this disease. Authors subsequently identified and characterized the role of a novel oncogene *GAEC1* which is located at 7q22 region. In this report, *GAEC1* was further characterized by identifying the downstream partners. The prominently downstream-regulated genes were then studied by immunohistochemistry on ESCC tissues to determine their clinicopathological significance.

### Research frontiers

The anti-proliferative effect of knocking-down the expression of *GAEC1* in ESCC cells was studied. The research hotspot is to find out the target genes which are most regulated by *GAEC1* and to determine their clinicopathological significance in ESCC.

### Innovations and breakthroughs

The RNA interference (RNAi) approach showed effective suppression of *GAEC1* expression in ESCC cells to inhibit cell proliferation and increase apoptosis. cDNA microarray analysis for differentially expressed genes identified the greatest levels of downregulation of calpain 10 (*CAPN10*) and upregulation of trinucleotide repeat containing 6C when *GAEC1* expression was suppressed. High level expression of calpain 10 was significantly associated with longer patient survival. This is the first study to explore the regulatory roles of *GAEC1* on the downstream targets and to report the association of *CAPN10* to the survival of ESCC patients.

### Applications

This study suggested that the potential use of *CAPN10* as a prognostic marker to predict the survival of ESCC patients after operation. The findings of the present study pave the path for the future related studies in other human cancers.

### Terminology

Squamous cell carcinoma: It is a cancer of a kind of epithelial cell called squamous cell. Squamous cells also occur in the lining of the digestive tract, such as the esophagus; Oncogene: An oncogene is a gene that has the potential to cause cancer. In tumor cells, they are often mutated or expressed at high levels.

### Peer review

This is a good study in which the authors employed *GAEC1* RNAi to knockdown the expression of *GAEC1*, investigated its effects on *GAEC1*-overexpressed esophageal carcinoma cell line KYSE150, and then explored the possible mechanisms. The study design is reasonable, statistical methods are appropriate.

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**P- Reviewers** Ghigna C, Guo YM, Takeno S, Ding MX, Guerra C, Lin CH **S- Editor** Gou SX **L- Editor** A **E- Editor** Li JY



## MAWBP and MAWD inhibit proliferation and invasion in gastric cancer

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Supported by The National Bio-Tech 863 Program, No. 2006AA02A402; and National Natural Science Foundation of China, No. 30901717

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Received: February 5, 2013 Revised: March 27, 2013

Accepted: April 10, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To investigate role of putative mitogen-activated protein kinase activator with WD40 repeats (MAWD)/MAWD binding protein (MAWBP) in gastric cancer (GC).

**METHODS:** MAWBP and MAWD mRNA expression level was examined by real-time reverse transcriptase-polymerase chain reaction and semi-quantitative polymerase chain reaction in six GC cell lines. Western blotting was used to examine the protein expression levels. We developed GC cells that stably overexpressed MAWBP and MAWD, and downregulated expression by RNA interference assay. Proliferation and migration

of these GC cells were analyzed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT), soft agar, tumorigenicity, migration and transwell assays. The effect of expression of MAWBP and MAWD on transforming growth factor (TGF)- $\beta$ 1-induced epithelial-mesenchymal transition (EMT) was examined by transfection of MAWBP and MAWD into GC cells. We detected the levels of EMT markers E-cadherin, N-cadherin and Snail in GC cells overexpressing MAWBP and MAWD by Western blotting. The effect of MAWBP and MAWD on TGF- $\beta$  signal was detected by analysis of phosphorylation level and nuclear translocation of Smad3 using Western blotting and immunofluorescence.

**RESULTS:** Among the GC cell lines, expression of endogenous MAWBP and MAWD was lowest in SGC7901 cells and highest in BGC823 cells. MAWBP and MAWD were stably overexpressed in SGC7901 cells and knocked down in BGC823 cells. MAWBP and MAWD inhibited GC cell proliferation *in vitro* and *in vivo*. MTT assay showed that overexpression of MAWBP and MAWD suppressed growth of SGC7901 cells ( $P < 0.001$ ), while knockdown of these genes promoted growth of BGC823 cells ( $P < 0.001$ ). Soft agar colony formation experiments showed that overexpression of MAWBP and MAWD alone or together reduced colony formation compared with vector group in SGC7901 ( $86.25 \pm 8.43$ ,  $12.75 \pm 4.49$ ,  $30 \pm 6.41$  vs  $336.75 \pm 22.55$ ,  $P < 0.001$ ), and knocked-down MAWBP and MAWD demonstrated opposite effects ( $131.25 \pm 16.54$ ,  $88.75 \pm 11.12$ ,  $341.75 \pm 22.23$  vs  $30.25 \pm 8.07$ ,  $P < 0.001$ ). Tumorigenicity experiments revealed that overexpressed MAWBP and MAWD inhibited GC cell proliferation *in vivo* ( $P < 0.001$ ). MAWBP and MAWD also inhibited GC cell invasion. Transwell assay showed that the number of traverse cells of MAWBP, MAWD and coexpression group were more than that in vector group ( $84 \pm 16.57$ ,  $98.33 \pm 9.8$ ,  $29 \pm 16.39$  vs  $298 \pm 11.86$ ,  $P < 0.001$ ). Coexpression of MAWBP and MAWD significantly decreased the cells traversing the matrix membrane. Conversely, knocked-down MAWBP

and MAWD correspondingly promoted invasion of GC cells ( $100.67 \pm 14.57$ ,  $72.66 \pm 8.51$ ,  $330.67 \pm 20.55$  vs  $27 \pm 11.53$ ,  $P < 0.001$ ). More importantly, coexpression of MAWBP and MAWD promoted EMT. Cells that coexpressed MAWBP and MAWD displayed a pebble-like shape and tight cell-cell adhesion, while vector cells showed a classical mesenchymal phenotype. Western blotting showed that expression of E-cadherin was increased, and expression of N-cadherin and Snail was decreased when cells coexpressed MAWBP and MAWD and were treated with TGF- $\beta$ 1. Nuclear translocation of p-Smad3 was reduced by attenuating its phosphorylation.

**CONCLUSION:** Coexpression of MAWBP and MAWD inhibited EMT, and EMT-aided malignant cell progression was suppressed.

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**Key words:** Gastric cancer; Mitogen-activated protein kinase activator with WD40 repeats binding protein; Mitogen-activated protein kinase activator with WD40 repeats; Invasion; Transforming growth factor- $\beta$ ; Epithelial-mesenchymal transition

**Core tip:** Our previous study revealed that mitogen-activated protein kinase activator with WD40 repeats (MAWD) and MAWD binding protein (MAWBP), acting as a complex, were differentially expressed in gastric cancer (GC) tissues compared with that in normal gastric tissues. The present study provided direct evidence that MAWBP and MAWD inhibited proliferation and migration of GC cells. Importantly, interaction of MAWBP and MAWD influenced expression of epithelial-mesenchymal transition (EMT) markers induced by transforming growth factor (TGF)- $\beta$ 1 in GC cells. It indicated that coexpression of MAWBP and MAWD inhibited TGF- $\beta$ 1-induced EMT, thus suppressing EMT-aided GC malignant progression.

Li DM, Zhang J, Li WM, Cui JT, Pan YM, Liu SQ, Xing R, Lu YY. MAWBP and MAWD inhibit proliferation and invasion in gastric cancer. *World J Gastroenterol* 2013; 19(18): 2781-2792 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2781.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2781>

## INTRODUCTION

Gastric cancer (GC) is the second most common cause of cancer death worldwide and is especially common in China<sup>[1]</sup>. Multiple factors are involved in the development of GC carcinogenesis. Molecular genetic studies have addressed accumulation of multiple genes and proteins alteration involved in GC development<sup>[2,3]</sup>. Investigation of GC biomarkers has focused on discovering differential protein signatures and has explored their biology

mechanisms. The genes involved include activation of *c-myc*, *erbB-2*, *c-met*, and *k-ras*<sup>[4-7]</sup> oncogenes and inactivation of tumor suppressor genes *p53*, *APC*, *E-cadherin* and *RUNX3*<sup>[8-10]</sup>.

Our laboratory previously found (using 2D gel electrophoresis and mass spectrometry) that expression of mitogen-activated protein kinase activator with WD40 repeats (MAWD) and MAWD binding protein (MAWBP) were differentially expressed in GC tissues. MAWD interacts with MAWBP and forms complexes in GC cell lines<sup>[11]</sup>, which suggests that these proteins are involved in GC carcinogenesis. Combined analysis of MAWBP and MAWD expression would provide useful information in uncovering their roles in GC.

The proteins MAWBP and MAWD were discovered in 2000 and 2001, respectively<sup>[12,13]</sup>. MAWD is widely expressed in many tissues and sequence analysis has indicated that MAWD contains a WD40 repeat domain. Datta *et al*<sup>[14]</sup> have shown that MAWD-homolog protein serine-threonine kinase receptor-associated protein (STRAP) recruits Smad7, forming a complex that increases inhibition of transforming growth factor (TGF)- $\beta$  signaling. Iriyama *et al*<sup>[13]</sup> tried to detect MAWD-related protein using a conventional two-hybrid technique and found MAWBP had an affinity for MAWD. The effects of MAWD in cancer have been reported in breast, colon and lung cancer but views about its role in cancer are divergent<sup>[15]</sup>. However, there is no current report on the function of MAWD in GC, and little is known about MAWBP other than its affinity for MAWD.

We hypothesize that MAWBP and MAWD interactions have a key role in GC tumorigenesis, and therefore investigated their biological function in GC cell lines. We found that these two proteins inhibited cell proliferation, and coexpression of MAWBP and MAWD obviously suppressed migration as well as invasive behavior of GC cells. Recent evidence implies that epithelial-mesenchymal transition (EMT) contributes to cancer progression, invasion and metastasis in various cancers<sup>[16,17]</sup>. TGF- $\beta$  is the main and best-characterized inducer of EMT during embryogenesis and cancer pathogenesis<sup>[18]</sup>. MAWBP and MAWD are involved in the TGF- $\beta$  signaling pathway<sup>[14]</sup>. We further sought to determine whether coexpression of MAWBP and MAWD could inhibit TGF- $\beta$ 1-induced EMT.

The canonical EMT program is characterized by complex proteome changes, leading to loss of epithelial markers such as E-cadherin, and expression of mesenchymal markers such as vimentin and N-cadherin<sup>[19]</sup>. Transcriptional regulator Snail is also activated in EMT. TGF- $\beta$  signaling regulates expression of Snail, SOX2 and SOX4<sup>[20]</sup>.

In this study, we analyzed the effect of MAWBP and MAWD on expression of E-cadherin, N-cadherin and Snail. We further demonstrated the relationship of this effect with TGF- $\beta$  signalling pathway via detection of the phosphorylation level and nuclear translocation of Smad3. Our findings suggest that coexpression of MAWBP and MAWD inhibits TGF- $\beta$ 1-induced EMT

and suppresses EMT-aided GC cell invasion.

## MATERIALS AND METHODS

### Cell lines and cell culture

GC cell lines BGC823, MGC803, SGC7901, AGS, N87 and MKN45 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Gaithersburg, MD, United States), supplemented with 5% fetal bovine serum (FBS). All cell lines were maintained at 37 °C in 5% CO<sub>2</sub> as previously described<sup>[21,22]</sup>.

### Plasmid construction

We constructed MAWBP and MAWD expression plasmids using pcDNA3.1B(-). Total RNA was extracted from 19-wk-old fetal liver. MAWBP and MAWD cDNA was produced by reverse-transcriptase polymerase chain reaction (RT-PCR). The reaction was initiated by 5-min incubation at 94 °C; 35 cycles of 94 °C for 45 s, 56 °C for 45 s, 72 °C for 60 s; and terminated after a 10-min extension at 72 °C. Products were purified by gel extraction. Recombinant plasmids were transferred into *Escherichia coli* DH5 $\alpha$ , and identified by restriction enzymes digestion and sequencing analysis. Then, we constructed MAWBP and MAWD short hairpin RNA (shRNA) plasmids. Oligonucleotides were annealed and ligated to pSilencer3.1-H1-Neo. All of the primers are shown on Table 1.

### Real-time PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, United States), and subjected (5  $\mu$ g) to RT-PCR (Table 1). The internal control,  $\beta$ -actin, was processed with all specimens simultaneously. Real-time PCR was performed using Applied Biosystem 7500 Real-Time PCR System (Foster City, CA, United States). Data were analyzed using the relative standard curve method.

### Western blotting

Proteins were extracted from cells for western blotting. Proteins (50  $\mu$ g) were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (Bio-Rad, Hercules, CA, United States). Immunoreactivity was tested with anti-MAWD (1:500, our laboratory), anti-MAWBP (1:500, our laboratory)<sup>[11]</sup>, E-cadherin (1:500, BD, Franklin Lakes, NJ, United States), N-cadherin (1:500, BD), Snail (1:500, Cell Signaling, Danvers, MA, United States), diluted in blocking buffer at 4 °C overnight. The signal was detected by Super Signal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, United States).

### Transfection studies

SGC7901 cells were transfected with overexpression plasmids, while BGC823 cells were transfected with shRNA plasmids. Cells were cultured at 60%-70% confluence in 35-mm plates and were transfected using Lipofectamine 2000 (Invitrogen). Except that mono-plasmids and empty vector were transfected into GC cells, overexpressed

**Table 1** List of oligonucleotide primers

| Target gene             | Primer ID | Sequence (5'-3')   |
|-------------------------|-----------|--|
| MAWBP (132 bp)          | Forward   | GGGTCTGCACACCGCTGTTTC  |
|                         | Reverse   | TAATGTCAACCCTTCCGTCT   |
| MAWD (162 bp)           | Forward   | GGGACAGGATAAACTTTAGC   |
|                         | Reverse   | AGCATGATCCCAAAGTCGAAC  |
| MAWBP (867 bp)          | Forward   | AACTTGGTCGACCAGCTTGAAGG<br>AAAAATG   |
|                         | Reverse   | ATAACTCGAGCTAGGCTGTCAGTGT<br>GCC   |
| MAWD (1053 bp)          | Forward   | CGCGGATCCATGGCAATGAGACAG<br>ACG  |
|                         | Reverse   | CCCAAGCTTTTCAAGCCTTAACATCA<br>GG   |
| $\beta$ -actin (510 bp) | Forward   | CGGAAATCGTGCCTGACATT   |
|                         | Reverse   | CTAGAAGCATTTCGGTGGAC   |
| $\beta$ -actin (150 bp) | Forward   | TTAGTTGCGTTACACCCCTTTC   |
|                         | Reverse   | ACCTTCACCGTTCAGTTT   |
| MAWD (shRNA)            | Ps-F1     | GATCCGCTTATGGACGATCTATTCG<br>TTCAAGAGAGCAATAGATCGTCCAT<br>AAGTTTTTTGGAAA     |
|                         |           | AGCTTTTCCAAAAAACCITATGGACG<br>ATCTATTGCTCTCTTGAAGCAATAG<br>ATCGTCCATAAGCG    |
|                         | Ps-R1     | GATCCGCTTATGGACGATCTATTCG<br>TTCAAGAGAGACAAAACGTGACGCGT<br>GCTATTTTTGGAAAAGC |
|                         |           | AGCTTGGCGTAATCATGGTCATAGC<br>TGTTTCTGTGTGAAAATTGTTATCCG<br>CTCACAATTCCACACA  |

MAWD: Mitogen-activated protein kinase activator with WD40 repeats; MAWBP: MAWD binding protein; shRNA: Short hairpin RNA.

plasmids of MAWBP and MAWD were cotransfected into SGC7901 cells. shRNA plasmids of MAWBP and MAWD were cotransfected into BGC823 cells. At 48 h post-transfection, cells were seeded for 21 d in selection medium containing 400  $\mu$ g/mL G418, to screen for stable clones. The efficacy of transfection was identified by RT-PCR and Western blotting.

### 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay

Stable transfected cells ( $1 \times 10^3$ ) in 200  $\mu$ L DMEM supplemented with 5% FBS were seeded in duplicate into each well of 96-well culture plates, and 10  $\mu$ L 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, GenView, Jacksonville, FL, United States) (5 mg/mL) was added at 0, 24, 48, 72 and 96 h. The MTT was removed after 4 h incubation, and 100  $\mu$ L dimethylsulfoxide (Amresco, Solon, OH, United States) was pipetted into each well and incubated for 30 min. Absorbance was measured at 570 nm using an iMark Microplate Reader (Bio-Rad, Hercules, CA, United States).

### Soft agar colony formation assay

Cells ( $3 \times 10^3$ ) were trypsinized and resuspended in 4 mL 0.3% agar in DMEM containing 10% FBS, and overlaid with 0.6% agar in 60-mm culture dishes. The dishes were incubated routinely for 21 d. Colonies were stained with 0.2% p-iodonitrotetrazolium violet, photographed,

and counted.

### **Tumorigenicity assay in nude mice**

Animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ethics Committee of Peking University. All efforts were made to minimize suffering. Transfected cells were resuspended in  $1 \times$  Hank's Buffer at a concentration of  $5 \times 10^5$  cells/mL. A 100- $\mu$ L suspension was injected subcutaneously into the left dorsal flank of 15 5-wk-old female nude mice, and the right side was inoculated with GC cells transfected by vector alone as a control. The mice were checked every 3 d for tumor appearance, and the large (a) and small (b) diameters of the palpable tumors were recorded for tumor volume calculation according to  $a \times b^2 \times 0.5$ .

### **Wound healing assay**

Cells were cultured at 80%-90% confluence in 60-mm dishes. Cells were scratched with a pipette tip to produce a straight line. The detached cells were washed three times with phosphate buffered solution (PBS) and incubated for a further 24 h. The scratched gap was inspected at 0, 2, 4, 6, 12 and 24 h. Photographs were taken at  $\times 200$  magnification, using a TS100 inverted microscope (Nikon, Tokyo, Japan).

### **Transwell assay**

The invasion assay was performed using a BD Matrigel Invasion Chamber. Cells ( $1 \times 10^5$ ) were suspended in serum-free DMEM and seeded on matrix membranes. DMEM supplemented with 10% FBS was used as a chemoattractant. After 48 h incubation, cells were fixed with methanol and stained with crystal violet for 20 min. Cells that penetrated the membrane were counted.

### **Immunofluorescence**

Cells were grown on glass slides, washed with PBS, fixed in methanol for 10 min, and processed for immunofluorescence. Cells were exposed to anti-p-Smad3 overnight at 4 °C, incubated for 1 h with rhodamine-conjugated anti-rabbit secondary antibodies, and nuclei were stained with 4',6-diamidino-2-phenylindole. Cells were studied with a confocal fluorescence imaging microscope (TCS-SP5; Leica, Mannheim, Germany).

### **Analysis of TGF- $\beta$ pathway responses**

Cells were starved for 24 h, and then incubated in 5% FBS-DMEM containing 2 or 4 ng/mL TGF- $\beta$  for 24 or 48 h. Plasminogen activator inhibitor (PAI)-1 promoter assays were used to select the optimum TGF- $\beta$  conditions. Transfected cells were cultured in 5% FBS-DMEM containing 4 ng/mL TGF- $\beta$  for 24 h. PhosphoSafe Extraction Reagent (Merck, San Diego, CA, United States) was used to extract phosphoprotein. P-Smad3 (1:1000, Abcam, Cambridge, United Kingdom) and p-Smad2 (1:500, Millipore, Temecula, CA, United States) were analyzed by western blotting as described above. Smad2

(1:500, Bioworld, Boston, MA, United States) and Smad3 (1:500, Bioworld) were detected at the same time. We then separated the cytosolic and nuclear fractions according to the protocol of the Nuclear-Cytosol Extraction Kit (Applygen Technologies Inc., Beijing, China) and detected p-Smad3 levels. Nuclear translocation ability of p-Smad3 (1:50) was analyzed by confocal microscopy as described above.

### **Ethics**

The study has been examined and approved ethically by the Ethics Committee of Beijing Cancer Hospital.

### **Statistical analysis**

Statistical analysis used SPSS version 16.0. Student's *t* test and analysis of variance were used for data measurement. Quantitative values were presented as mean  $\pm$  SD. Differences with  $P < 0.05$  were considered statistically significant.

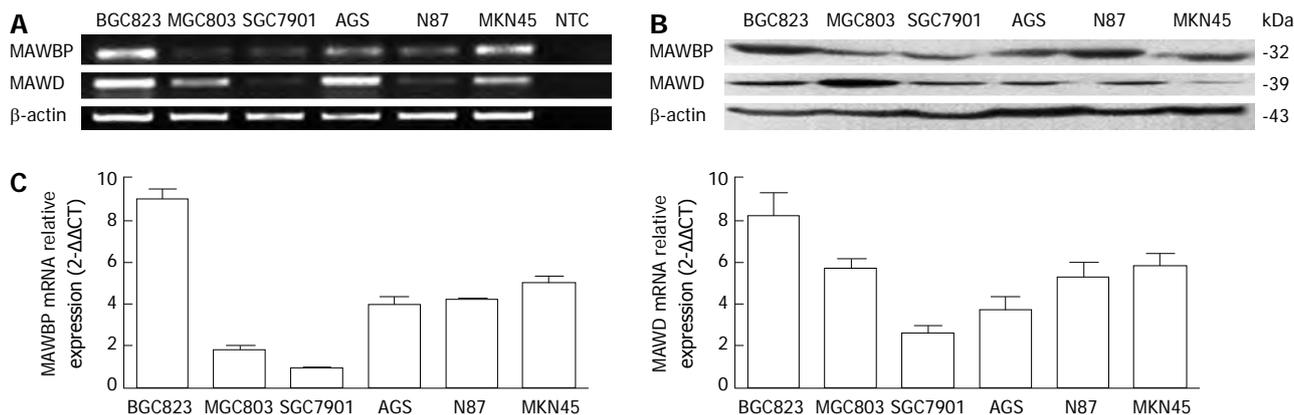
## **RESULTS**

### **MAWBP and MAWD inhibited proliferation and tumorigenicity of GC cells**

BGC823, MGC803, SGC7901, AGS, N87 and MKN45 GC cell lines were used to detect MAWBP and MAWD expression, which was found to differ between the cell types. RT-PCR and real-time PCR revealed low levels of endogenous MAWBP and MAWD mRNA in SGC7901 cells but high levels in BGC823 cells, and Western blotting confirmed these results (Figure 1). These two cell lines were selected for the following experiments.

To investigate further biological function of MAWBP and MAWD in GC cells, MAWBP-pcDNA3.1 and MAWD-pcDNA3.1, alone or in combination, and empty vector were transfected into SGC7901 cells, in which these two proteins were expressed at a lower level compared with other GC cells. G418-resistant clones were isolated, which were stably transfected cells. These cells were termed as MAWBP, MAWD, MAWBP/D (MAWBP and MAWD cotransfected cells) and vector, respectively. shRNA plasmid MAWBP-pSilencer3.1 and MAWD-pSilencer3.1, alone or in combination, and empty vector were transfected into BGC823 cells, in which they were expressed at a higher level compared with other GC cells. The stably transfected cells were termed as sh-MAWBP, sh-MAWD, sh-MAWBP/D (MAWBP and MAWD co-downregulated groups) and sh-vector, respectively. We analyzed MAWBP and MAWD expression at the mRNA and protein levels by semi-quantitative RT-PCR and Western blotting, respectively.

In the MTT assay, cell growth in the overexpressed group was suppressed in the MAWBP, MAWD and MAWBP/D groups, compared with the vector group (Figure 2A,  $P < 0.001$ ). Knockdown of MAWBP and MAWD in BGC823 cells using RNA interference (RNAi) increased cell growth (Figure 2B,  $P < 0.001$ ). The soft agar assay in overexpressed cells showed reduced colony formation for the MAWBP, MAWD, and MAWBP/D



**Figure 1** Analysis of expression of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats in six gastric cancer cell lines. A: mRNA expression for mitogen-activated protein kinase activator with WD40 repeats (MAWD)/MAWD binding protein (MAWBP) in BGC823, MGC803, SGC7901, AGS, N87 and MKN45 six gastric cancer cell lines was detected by reverse transcription-polymerase chain reaction (PCR). There was a low level of endogenous MAWBP and MAWD mRNA in SGC7901 cells, and a high level in BGC823 cells; B: Expression of MAWBP and MAWD proteins was detected by Western blotting. The expression level was lower in SGC7901 cells than in the other cell lines. Expression of MAWBP and MAWD was higher in BGC823 cells than in the other cells; C: mRNA expression for MAWBP and MAWD was detected by real-time PCR. There were lower levels of endogenous MAWD and MAWBP mRNA in SGC7901 cells, and higher levels in BGC823 cells. NTC: No template control.

groups for cell number and size compared with the vector group (Figure 2C, clones number:  $86.25 \pm 8.43$ ,  $12.75 \pm 4.49$ ,  $30 \pm 6.41$  vs  $336.75 \pm 22.55$ ,  $P < 0.001$ ). The corresponding knockdown group demonstrated the opposite effects (Figure 2D,  $131.25 \pm 16.54$ ,  $88.75 \pm 11.12$ ,  $341.75 \pm 22.23$  vs  $30.25 \pm 8.07$ ,  $P < 0.001$ ). These results suggested that expression of MAWBP and MAWD play a role in inhibiting proliferation of GC cells.

*In vivo* experiments, tumor growth appeared to be slow in nude mice injected with MAWBP, MAWD, and MAWBP/D compared with the control group. Tumors from MAWD-transfected cells were smaller than those from the other groups (Figure 2E and F,  $P < 0.001$ ).

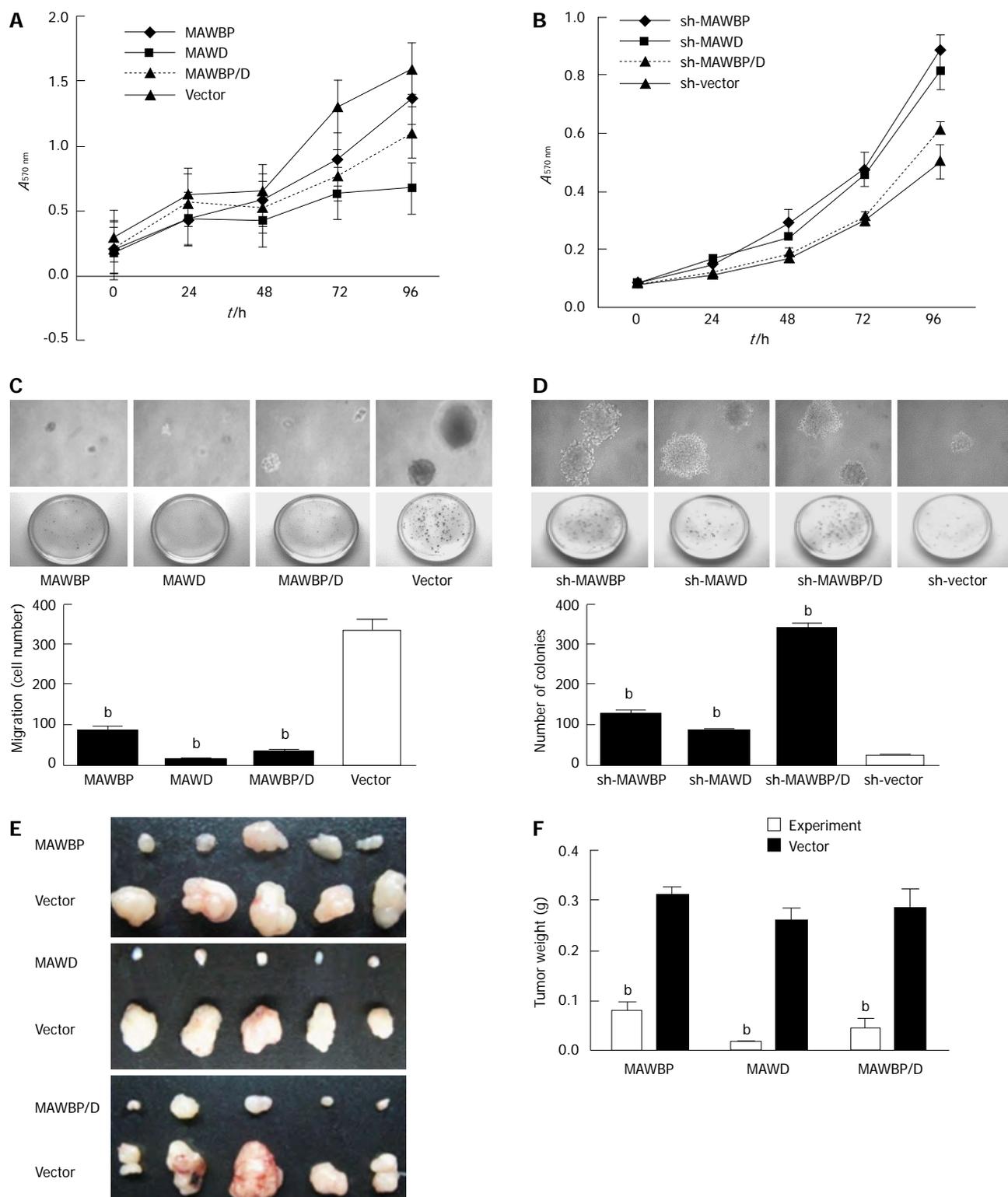
#### Co-expression of MAWBP and MAWD suppressed migration and invasion of GC cells

To explore the potential role of MAWBP and MAWD in GC metastasis and invasion, we evaluated the effects of the stably transfected cells on migration and invasion using a wound healing assay and transwell invasive activity assay, respectively. In the wound healing assay, the scratch gap of vector transfected cells was almost closed at 24 h in the overexpressed group. Cells with co-overexpression of MAWBP and MAWD showed the slowest rate of migration (Figure 3A). In the knockdown group, the cells with combined downregulation of MAWBP and MAWD expression migrated faster than the other cells. Migration of sh-vector cells was slowest (Figure 3B). In the transwell assay, there was significant difference in the number of the cells traversing the matrix membrane between the vector and other groups. Combined overexpression of MAWBP and MAWD decreased the invasive ability of GC cells (Figure 4A). The number of traverse cells of MAWBP, MAWD and co-expression group was higher than that in the vector group (Figure 4C,  $84 \pm 16.57$ ,  $98.33 \pm 9.8$ ,  $29 \pm 16.39$  vs  $298 \pm 11.86$ ,  $P < 0.001$ ).

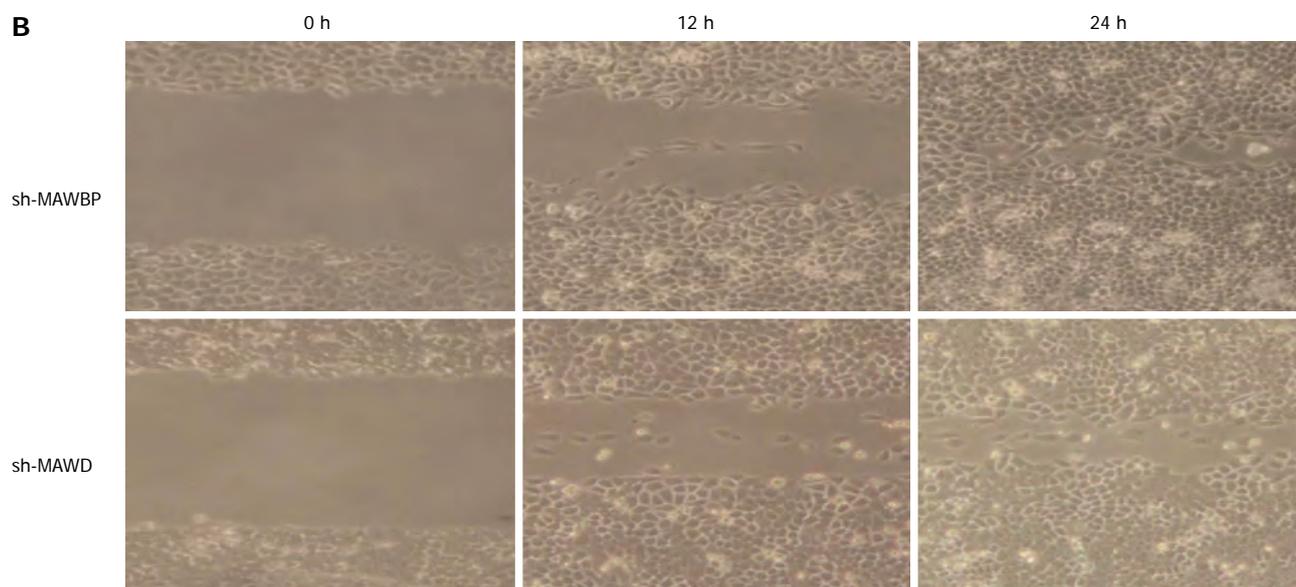
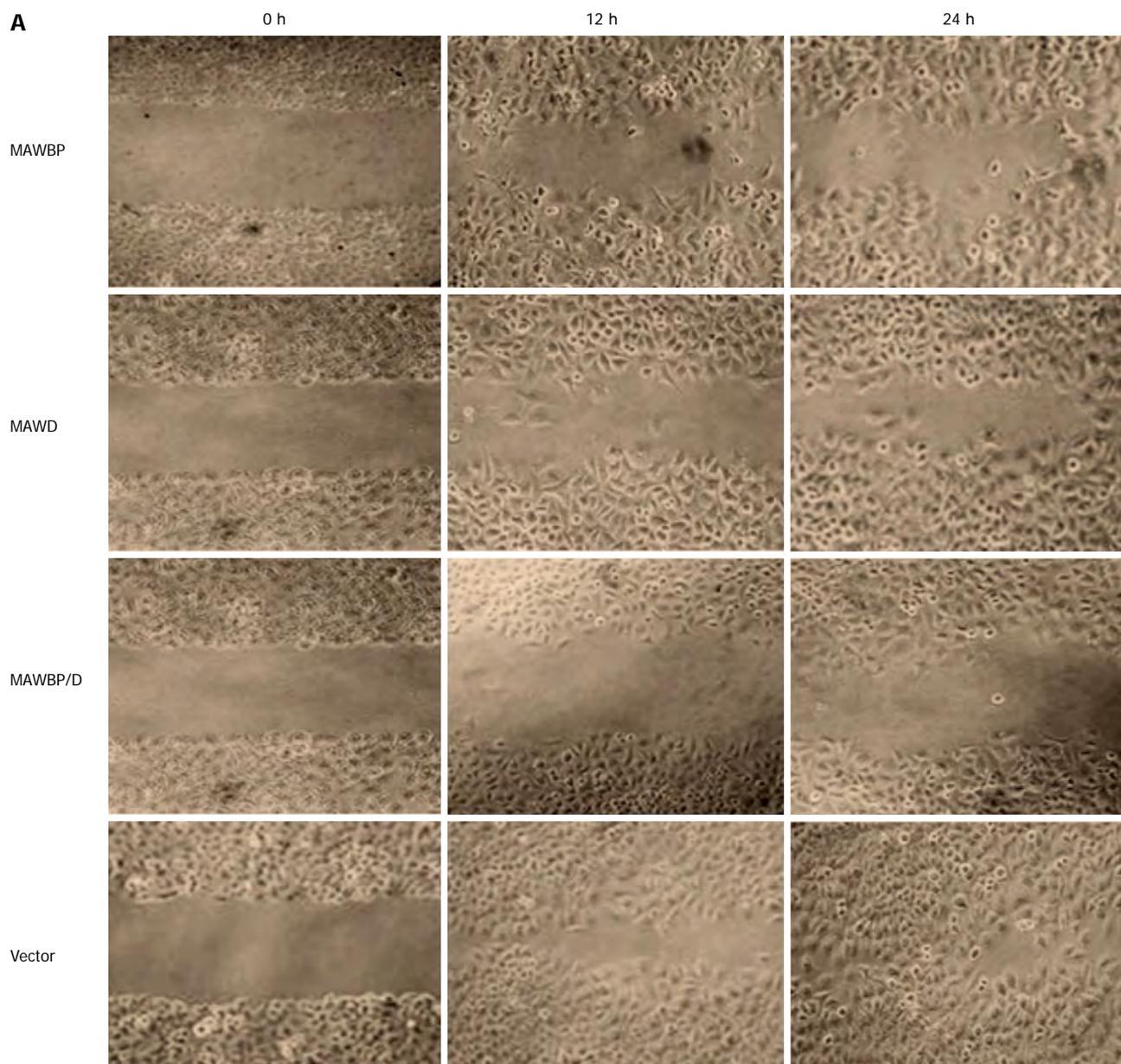
Knockdown of expression of MAWBP and MAWD increased the number of cells that traversed the matrix membrane (Figure 4B). Cells with combined downregulation of MAWBP and MAWD expression migrated faster than the other cells (Figure 4B). Invasion of sh-vector cells was slowest (Figure 4C,  $100.67 \pm 14.57$ ,  $72.66 \pm 8.51$ ,  $330.67 \pm 20.55$  vs  $27 \pm 11.53$ ,  $P < 0.001$ ). These data showed that MAWBP and MAWD inhibited migration and invasion of GC cells.

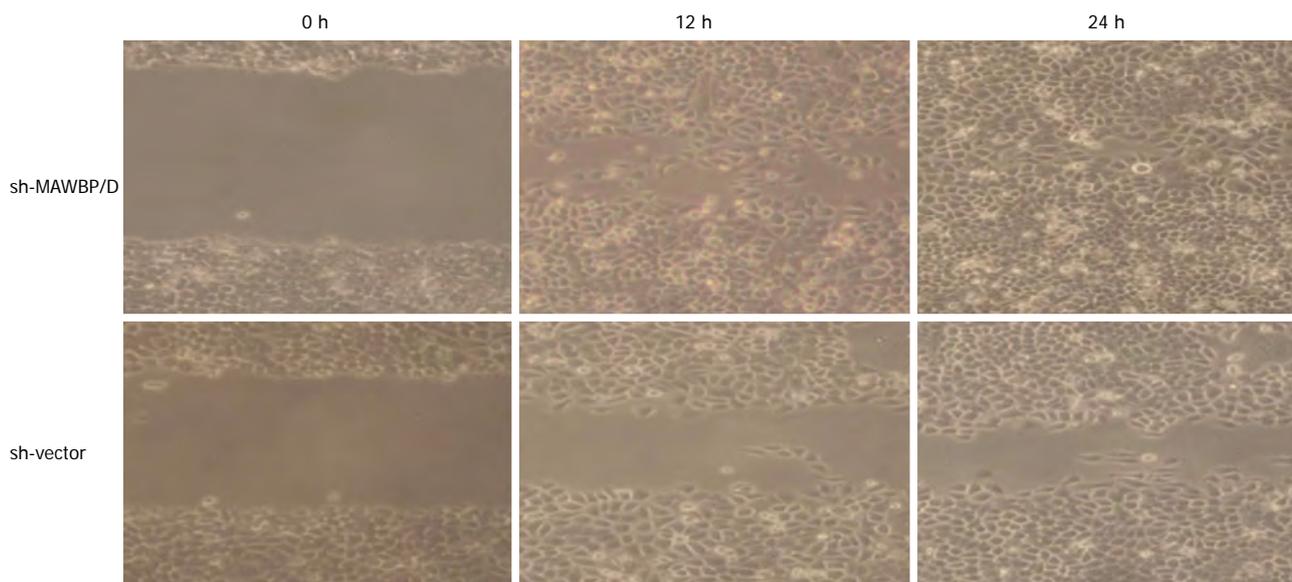
#### Co-expression of MAWBP and MAWD influenced expression of EMT markers induced by TGF- $\beta$ 1 in GC cells

EMT contributes to cancer progression and metastasis. TGF- $\beta$  is the main and best-characterized inducer of EMT. We sought to determine whether co-expression of MAWBP and MAWD inhibited TGF- $\beta$ 1-induced EMT, thus suppressing migration and transwell behavior of GC cells. We detected a relationship between the expression of MAWBP and MAWD and the EMT markers induced by TGF- $\beta$ 1, such as E-cadherin, N-cadherin, and transcription factor Snail. We established the optimum TGF- $\beta$ 1 concentration and treatment time to stimulate cells. We used the expression of TGF- $\beta$  reporter gene PAI-1 to indicate that the optimum TGF- $\beta$ 1 concentration and treatment time was 4 ng/mL for 24 h (Figure 5A). We stimulated GC cells that overexpressed MAWBP and MAWD with TGF- $\beta$ 1, and observed their morphology. We found that cells that overexpressed both MAWBP and MAWD displayed a pebble-like shape and tight cell-cell adhesion, while vector-treated cells showed a classical mesenchymal phenotype (Figure 5B). That means that co-expression of MAWBP and MAWD inhibited morphological changes of TGF- $\beta$ 1-induced EMT. We next detected expression of E-cadherin, N-cadherin and Snail in cells overexpressing MAWBP and MAWD. Ex-

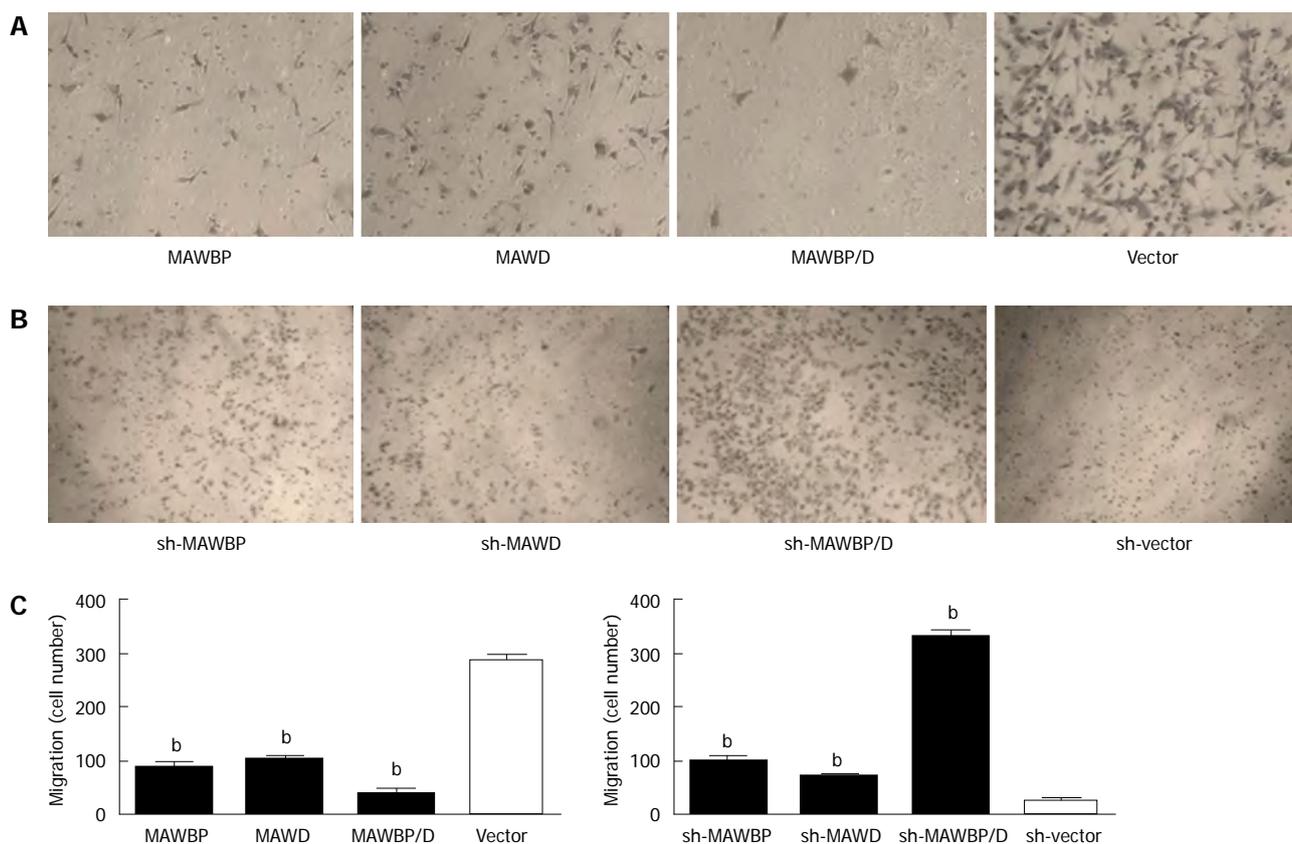


**Figure 2** Effect of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats on proliferation and tumorigenicity of gastric cancer cells. **A:** 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide assay showed that growth of SGC7901 cells overexpressing mitogen-activated protein kinase activator with WD40 repeats (MAWD)/MAWD binding protein (MAWBP) was markedly inhibited; **B:** In BGC823 cells with inhibition of expression of MAWBP and MAWD, cell growth was inversely increased; **C:** In soft agar assay, colonies of cells with overexpression of MAWBP, MAWD and MAWBP/D were reduced in number and size compared with cells transfected with vectors alone (original magnification of clones:  $\times 100$ ); **D:** Knockdown group demonstrated the opposite effects. There was an increase in the number and size of sh-MAWBP, sh-MAWD, sh-MAWBP/D cells compared with cells transfected with vectors alone; **E:** Nude mouse xenografts. Tumors induced by MAWBP, MAWD and MAWBP/D cotransfected cells were smaller than those of the vector group; **F:** Xenograft weight. The average tumor weight were calculated from five nude mice in every group. Data are presented as the mean  $\pm$  SD from three independent experiments. <sup>b</sup> $P < 0.01$  vs vector group.

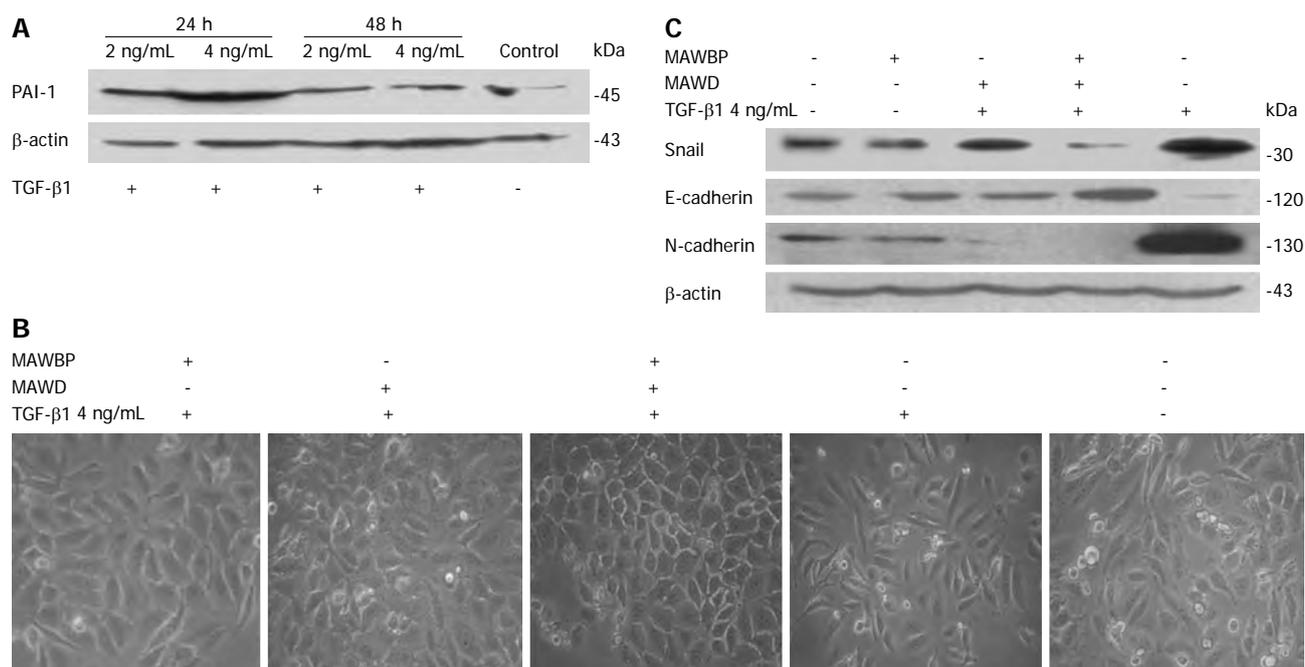




**Figure 3** Effect of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats on migration of gastric cancer cells. A: Wound healing assay showed that migration of vector-transfected cells was faster than that of cells overexpressing mitogen-activated protein kinase activator with WD40 repeats (MAWD)/MAWD binding protein (MAWBP). The scratch gap in vector group was almost closed at 24 h. The migration of MAWBP/D cotransfected cells was slowest; B: Cells with combined downregulation of MAWBP and MAWD expression migrated faster than the other cells. Migration of sh-vector cells was slowest (original magnification,  $\times 100$ ).



**Figure 4** Effect of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats on invasive ability of gastric cancer cells. A: Transwell assay showed that invasive ability of mitogen-activated protein kinase activator (MAWD) with WD40 repeats binding protein (MAWBP)/D cotransfected cells was weakest, with the lowest number of cells to cross the matrix membranes. Vector-transfected cells migrated farthest; B: Knockdown of MAWBP and MAWD increased the invasive ability of gastric cancer (GC) cells (original magnification:  $\times 100$ ); C: Number of cells that traverse the matrix membrane in the different groups. Data are presented as the mean  $\pm$  SD from three independent experiments. <sup>b</sup> $P < 0.01$  vs vector group.



**Figure 5** Effect of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats on expression of biomarkers specific for epithelial-mesenchymal transition induced by transforming growth factor- $\beta$ 1. A: According to the expression of transforming growth factor (TGF)- $\beta$  downstream reporter gene plasminogen activator inhibitor (PAI)-1, the optimum TGF- $\beta$ 1 concentration and time were confirmed as 4 ng/mL for 24 h; B: SGC7901 cells overexpressing mitogen-activated protein kinase activator with WD40 repeats (MAWD)/MAWD binding protein (MAWBP) were stimulated by 4 ng/mL TGF- $\beta$ 1 for 24 h. Cells that overexpressed both MAWBP and MAWD displayed a pebble-like shape, while vector cells showed a classical mesenchymal phenotype (original magnification,  $\times 400$ ); C: Expression of E-cadherin was strongest in the MAWBP/D cotransfection group and weakest in the vector group, using Western blotting. Snail and N-cadherin were inversely associated with E-cadherin expression.

pression of E-cadherin was strongest in the MAWBP/D group and weakest in the vector group. N-cadherin and Snail expression was inversely associated with E-cadherin expression (Figure 5C). Collectively, these data demonstrated that MAWBP and MAWD were involved in TGF- $\beta$ 1-induced EMT through upregulating E-cadherin and downregulating N-cadherin and Snail in GC cells.

#### Co-expression of MAWBP and MAWD suppressed phosphorylation and nuclear translocation of p-Smad3

Following MAWBP and MAWD overexpression in cells stimulated with TGF- $\beta$ 1, the level of p-Smad3 was lowest in the MAWBP/D group and highest in the vector group (Figure 6A). The level of p-Smad2 was also lower in the MAWBP/D group. Furthermore, we separated the proteins in the cytoplasm and nucleus and found that p-Smad3 in the nucleus had the lowest level, as shown by Western blotting (Figure 6B) and confocal microscopy (Figure 6C). That means that the nuclear translocation capability of p-Smad3 in cotransfected cells was weakest. These results imply that the MAWBP/D complex suppressed TGF- $\beta$  signaling by inhibiting downstream phosphorylation.

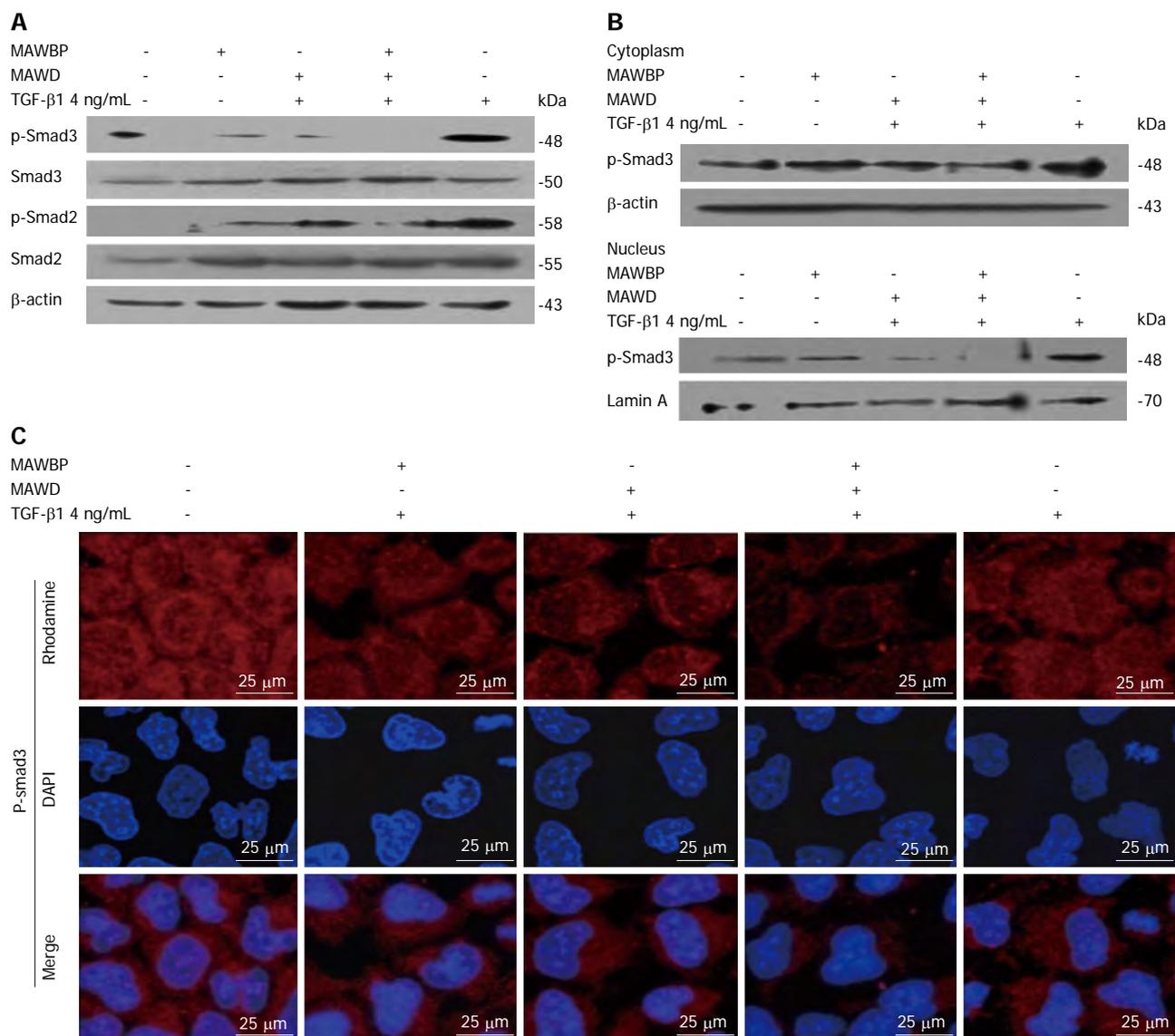
## DISCUSSION

From the outset of this study, we analyzed the biological function of MAWBP and MAWD in GC cell lines. We found that MAWBP and MAWD inhibited prolifera-

tion and migration of GC cells. Importantly, combined overexpression of MAWBP and MAWD in GC cells suppressed TGF- $\beta$ 1-induced EMT by attenuating phosphorylation of Smad3 and reducing its nuclear translocation.

In a previous study, we reported proteomic data acquired from screening protein profiles from GC tissues, including MAWBP and MAWD, and showed that they formed a complex in GC cells by co-immunoprecipitation<sup>[11]</sup>. MAWD has been reported to have divergent effects in cancer. Some researchers have suggested that MAWD promotes cancer development. Matsuda *et al.*<sup>[12]</sup> found that MAWD was overexpressed in 45.6% (21/46) of human breast tumor tissues, and promoted anchorage-independent cell growth. Kim *et al.*<sup>[15]</sup> reported MAWD upregulation in 50.8% (30/59) of adenomas and 70.7% (87/123) of colorectal cancers. Halder *et al.*<sup>[23]</sup> found that STRAP was upregulated in 60% (12/20) of colon and 78% (11/14) of lung carcinomas. However, other researchers have found that MAWD suppressed development of malignant cells. Buess *et al.*<sup>[24]</sup> reported complete or partial allelic loss of MAWD in 45.2% (75/166) of colorectal cancer patients. Jung *et al.*<sup>[25]</sup> found that MAWD was a binding partner of NM23-H1, creating a complex that interacted with and potentiated p53. Dong *et al.*<sup>[26]</sup> detected chromosomal deletions in prostate cancer that overlapped MAWD gene locations. Zhao *et al.*<sup>[27]</sup> reported that MAWBP was down-regulated in ulcerative colitis. The function of MAWBP and MAWD in GC has not been reported.

In the present study, we investigated the biological



**Figure 6** Combination of mitogen-activated protein kinase activator with WD40 repeats binding protein and mitogen-activated protein kinase activator with WD40 repeats inhibited the transforming growth factor- $\beta$  pathway. **A:** The level of p-Smad3 was lowest in the mitogen-activated protein kinase activator (MAWD) with WD40 repeats binding protein (MAWBP)/D cotransfected group and highest in the vector group. p-Smad2 was also lower in the MAWBP/D group; **B, C:** Nuclear translocation capability of p-Smad3 in cotransfected cells was weakest that means the activity of transforming growth factor (TGF)- $\beta$  pathway in co-expression group was inhibited. DAPI: 4',6-diamidino-2-phenylindole.

role of MAWBP and MAWD in GC. We first investigated expression levels of MAWBP and MAWD in six GC cell lines at the RNA and protein levels. We found that expression was lowest in SGC7901 cells and highest in BGC823 cells. Thus, we selected SGC7901 for overexpression of MAWBP and MAWD and BGC823 for RNAi assay, and constructed the eukaryotic expression plasmid and RNAi plasmid for these two proteins for cell transfection. We generated MAWBP/D-cotransfected cells to establish whether one complements the function of the other. We found that MAWBP and MAWD acted as tumor suppressors. Our results showed that overexpression of MAWBP and MAWD suppressed growth of SGC7901 cells. Knockdown of their expression enhanced proliferation of BGC823 cells. The suppressive ability of MAWD was more pronounced than that of MAWBP.

Interestingly, the results from the migration and transwell assays indicated that combined overexpression of these two proteins more obviously limited migration and invasive behavior of GC cells. The cotransfected cells showed mixed characteristics for proliferation and migration, meaning that MAWBP and MAWD had a synergetic role in regulating migration and invasion of GC cells.

EMT is thought to be a key step in the progression of tumors toward invasion and metastasis<sup>[28]</sup>. EMT is a cellular process during which epithelial polarized cells become motile mesenchymal-appearing cells. This process can lead to loss of epithelial markers - especially E-cadherin - and expression of mesenchymal markers such as vimentin and N-cadherin<sup>[29]</sup>. E-cadherin is a cell-adhesion protein that is regulated by transcription factors including Snail and Slug. Snail act as a repressor and blocks E-cad-

herin transcription, and has emerged as an essential regulator of physiological and pathological EMT processes<sup>[30]</sup>. It has been shown that TGF- $\beta$  induces changes in cell morphology that are consistent with the acquisition of the EMT phenotype<sup>[31]</sup>.

In this study, we sought to determine whether co-expression of MAWBP and MAWD inhibited TGF- $\beta$ 1-induced EMT, thus suppressing migration and transwell behavior of GC cells. We stimulated GC cells with over-expression of MAWBP and MAWD with TGF- $\beta$ 1 and detected the expression level of epithelial and mesenchymal markers and transcription factors. We found that E-cadherin was upregulated in the co-expression group and N-cadherin and Snail expression was inversely associated with E-cadherin expression. This revealed that MAWBP and MAWD had a synergetic function in inhibiting TGF- $\beta$ 1-induced EMT.

TGF- $\beta$ 1 stimulation induces upregulation of Snail and induces EMT in Smad-dependent signaling<sup>[30]</sup>. MAWD was found to recruit Smad7 and form a complex that inhibited TGF- $\beta$  signaling. To confirm whether the MAWBP and MAWD complex further suppressed TGF- $\beta$ 1 and decreased Snail expression, we evaluated TGF- $\beta$  activity in MAWBP and MAWD overexpressed cells. Phosphorylation of effector molecules is often essential for downstream receptor kinase signaling<sup>[31]</sup>. Thus, we detected the phosphorylation level and nuclear translocation of p-Smad2 and p-Smad3 to indicate TGF- $\beta$  activity. We found that the level of p-Smad3 was lowest in the combined overexpression group and highest in the vector group. Nuclear translocation of p-Smad3 was weakest in cells with combined overexpression. These results imply that the MAWBP/D complex suppresses TGF- $\beta$  signaling, and therefore downregulates Snail level and inhibits EMT.

All together, the present study demonstrates that MAWBP and MAWD have a suppressive role in progression of tumor growth and invasion of GC. Co-expression of MAWBP and MAWD inhibits TGF- $\beta$ 1-induced EMT, which suppresses EMT-assisted GC cell malignant progression. In future research, we should attempt to find the mechanisms mediating MAWBP and MAWD expression in GC. MAWBP and MAWD interaction domains will be predicted by biological information analysis and tested in cell assays.

## ACKNOWLEDGMENTS

We thank the Tissue Bank of Beijing Cancer Hospital/Institute for providing gastric specimens.

## COMMENTS

### Background

Gastric cancer (GC) is the second most common cause of cancer death worldwide. GC incidence in Asian countries, particularly in East Asia, is significantly higher than that in other parts of the world. GC creates a serious public health problem. Early diagnosis is important for therapy and prognosis of patients. Therefore, investigation of sensitive biomarkers and analysis of their function

are important.

### Research frontiers

During the past decade, a great effort has been made to define better the biological profile of GC. Molecular genetic studies have investigated the accumulation of mutations and alterations in proteins involved in GC. These include activation of c-myc, erbB-2, c-met, and k-ras oncogenes and inactivation of tumor suppressor genes *p53*, *APC*, *E-cadherin* and *RUNX3*. Their previous study found that mitogen-activated protein kinase activator with WD40 repeats (MAWD) and MAWD binding protein (MAWBP) were differentially expressed and interacted in GC.

### Innovations and breakthroughs

The present study provided direct evidence that MAWBP and MAWD inhibited proliferation and migration of GC cells. Importantly, interaction of MAWBP and MAWD influenced expression of epithelial-mesenchymal transition (EMT) markers induced by transforming growth factor (TGF)- $\beta$ 1 in GC cells. It also reduced nuclear translocation of p-Smad3. This means that co-expression of MAWBP and MAWD inhibits TGF- $\beta$ 1-induced EMT and suppresses EMT-aided GC malignant progression.

### Applications

The authors found that interaction between MAWBP and MAWD could shed new light on the carcinogenic mechanisms of GC. MAWBP and MAWD as biomarkers might be diagnostic and therapeutic targets for GC.

### Terminology

MAWD is a protein that is evolutionarily conserved and is widely expressed in many tissues. Sequence analysis indicates that the protein structure of MAWD contains a WD40 repeat domain. WD repeat proteins help to assemble macromolecular complexes, such as shown for the  $\beta$ -subunit of G proteins. The homologous protein of MAWD, serine-threonine kinase receptor-associated protein recruits Smad7 to the activated type I receptor and forms a complex. MAWBP is a MAWD binding protein. EMT is the morphological and molecular changes that occur when epithelial cells lose their characteristics, gain mesenchymal properties and become motile, which is a key event in tumor invasion and metastasis.

### Peer review

The authors analyzed MAWD and MAWBP in a series of GC cell lines. They found that co-expression of both genes is potentially involved in the suppression of migration and invasion in their selected cell lines. This study was a straightforward continuation of the authors' own work and they have recently reported differential expression of both genes in GC tissues. Now, they have analyzed the functional consequences of suppression or overexpression of both genes in an *in vitro* setting.

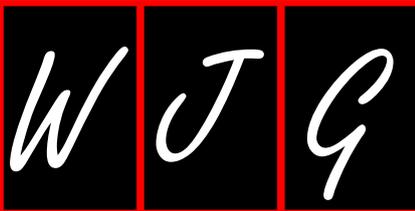
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## Sustained virological response: A milestone in the treatment of chronic hepatitis C

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Received: August 31, 2012 Revised: March 8, 2013

Accepted: March 15, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To evaluate the long-term eradication of hepatitis C virus (HCV) infection and liver-related complications in chronically infected patients that have achieved sustained virological response.

**METHODS:** One hundred and fifty subjects with chronic hepatitis C (CHC) or cirrhosis and sustained virological response (SVR) between the years of 1989 and 2008 were enrolled in a long-term clinical follow-up study at the Gastrointestinal and Liver Unit of the University Hospital of Naples "Federico II". At the beginning of the study, the diagnosis of HCV infection was made on the basis of serum positivity for antibodies to HCV

and detection of HCV RNA transcripts, while a diagnosis of chronic hepatitis was formulated using imaging techniques and/or a liver biopsy. SVR was achieved by interferon-based therapy, both conventional and pegylated, with and without ribavirin treatment. The patients were evaluated for follow-up at a median length of 8.6 years, but ranged from 2-19.9 years. Among them, 137 patients had pre-treatment CHC and 13 had cirrhosis. The patients were followed with clinical, biochemical, virological, and ultrasound assessments on a given schedule. Finally, a group of 27 patients underwent a liver biopsy at the beginning of the study and transient elastography at their final visit to evaluate changes in liver fibrosis.

**RESULTS:** The median follow-up was 8.6 years (range 2-19.9 years). HCV RNA remained undetectable in all patients, even in patients who eventually developed liver-related complications, indicating no risk of HCV recurrence. Three liver-related complications were observed: two cases of hepatocellular carcinoma and one case of bleeding from esophageal varices resulting in an incidence rate of 0.23%/person per year. Further, all three complications took place in patients diagnosed with cirrhosis before treatment began. Only one death due to liver-related causes occurred, resulting in a mortality rate of 0.077% person per year. This amounts to a 99.33% survival rate in our cohort of patients after therapy for HCV infection. Finally, of the 27 patients who underwent a liver biopsy at the beginning of the study, a reduction in liver fibrosis was observed in 70.3% of the cases; only three cases registering values of liver stiffness indicative of significant fibrosis.

**CONCLUSION:** Patients with CHC and SVR show an excellent prognosis with no risk of recurrence and a very low rate of mortality. Our data indicate that virus-eradication following interferon treatment can last up to 20 years.

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**Key words:** Antiviral therapy; Cirrhosis; Hepatitis C virus; Sustained virological response; Fibrosis

**Core tip:** This study represents one of the longest follow-up studies on the natural history of successfully treated chronically hepatitis C virus (HCV) infected individuals. The outcome of the study was very positive, as it revealed an extremely high survival rate, an extremely low rate of liver complications, and a significant reduction in liver fibrosis in patients who have achieved sustained virological response (SVR). All of the patients without cirrhosis before starting the treatment showed no signs of evolution or decompensation over the years of observation, proving that SVR positively changes the natural history in individuals with HCV-infection.

Morisco F, Granata R, Stroffolini T, Guarino M, Donnarumma L, Gaeta L, Loperto I, Gentile I, Auriemma F, Caporaso N. Sustained virological response: A milestone in the treatment of chronic hepatitis C. *World J Gastroenterol* 2013; 19(18): 2793-2798 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2793.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2793>

## INTRODUCTION

Hepatitis C virus (HCV) infection represents a serious challenge to global health, with an estimated 170 million carriers worldwide<sup>[1]</sup>. A significant complication in chronically infected individuals is the development of liver inflammation and fibrosis, which may progress to liver cirrhosis and hepatocellular carcinoma (HCC)<sup>[2-10]</sup>.

For nearly 20 years, interferon-based therapy has been the standard therapy for chronic hepatitis C (CHC), with the optimal treatment of the combination of pegylated-interferon and ribavirin dating back ten years. The primary goal of HCV treatment is to achieve a sustained virological response (SVR). SVR is defined by undetectable HCV RNA levels 24 wk after completing treatment<sup>[11]</sup>. The achievement of the SVR in patients with CHC represents a milestone because it has been associated with the eradication of the infection, improvement in liver histology, improvement in quality of life, and reduced risk of cirrhosis and hepatocellular carcinoma<sup>[12-15]</sup>. Short term SVR in patients with chronic HCV-infection is well established, however, knowledge regarding long-term SVR remains to be elucidated. There are limited long-term studies observing chronically infected patients who have achieved SVR, all with a limited number of patients<sup>[12,16]</sup>. Further, studies that do have a large cohort of patients have a fairly short follow-up period<sup>[17,18]</sup>. Finally, the data gathered thus far lack real world examples as data gathered by subjects with SVR are usually limited to those participating in pivotal clinical trials.

The aim of this study was to evaluate the long-term

outcomes of patients who obtain a SVR after antiviral therapy, regardless of the type treatment, in a monocentric cohort of patients representing a real world population with CHC during the last 20 years.

## MATERIALS AND METHODS

### Patients

From January 1989 to April 2008, 150 HCV-positive patients who achieved SVR after interferon-based therapy at the Gastroenterology Unit of the University of Naples "Federico II" were enrolled in this cohort study. There were 100 men and 50 women (mean age,  $56.37 \pm 12.05$  years; range 22-67 years). The main characteristics of our study population at baseline are reported in Table 1.

In this cohort, 137/150 (91.3%) patients had chronic hepatitis and 13 had liver cirrhosis. Before treatment, 21 patients had normal alanine transaminase (ALT) values and 129/150 (86%) had elevated ALT values ranging between 1.2 and 15.87 times the upper normal limit. A histological examination was performed in 103/150 (68.6%) of patients at baseline, while 47/150 (31.3%) patients refused the biopsy procedure and 27 patients showed comorbidities (Table 1). Nine patients had a diagnosis of arterial hypertension and two had an incomplete right bundle branch block. One patient had a medical history of previous angina pectoris and another suffered from a previous myocardial infarction, however, there was no cardiologic contraindication at the time the therapy started. Seven patients had thyroid disease; two patients had type II diabetes; two had gastroesophageal reflux disease, and two had a co-infection of hepatitis B. One patient had a history of non-Hodgkin lymphoma that had been in remission for five years since the time the antiviral therapy started.

### Modality of diagnosis

The diagnosis of HCV infection was made on the basis of serum positivity for antibodies to HCV and detection of HCV RNA transcripts. The diagnosis of chronic hepatitis was formulated by a histological assessment and scored according to the internationally accepted criteria<sup>[19]</sup>. The liver biopsy was not performed if the patients refused the procedure or had evidence of clinical, biochemical, ultrasound and endoscopic signs of liver cirrhosis. Transient elastography (TE), a non-invasive method of quantifying fibrosis developed as an alternative to liver biopsy, was carried out using FibroScan. Ultrasound elastography analyzes ultrasound frequency waves related to the elasticity (deforming capacity) of the liver. The methodology is simple, highly reproducible and can be completed in ten minutes. The diagnostic accuracy of TE is not as high as a liver biopsy, which remains the gold standard for evaluation of fibrosis. Nonetheless, in this context, TE is both highly sensitive and specific (79%-83% and 83%-89%, respectively)<sup>[20]</sup>.

### Therapy schedules

All patients were treated with interferon-based therapy,

**Table 1** Baseline characteristics of 150 chronically infected hepatitis C virus patients with sustained virological response

|   |     |
|---|-----|
| Patients  | 150 |
| Gender  |     |
| Male  | 100 |
| Female  | 50  |
| Patients with histological assessment <sup>[20]</sup> | 103 |
| Mild hepatitis (G0-G4)                                | 52  |
| Moderate hepatitis (G5-G9)                            | 44  |
| Severe hepatitis (G10-G18)                            | 5   |
| Staging S0-S1   | 44  |
| Staging S2  | 25  |
| Staging S3  | 10  |
| Staging S4-S5   | 9   |
| Cirrhosis S6  | 2   |
| Genotype  |     |
| 1a  | 14  |
| 1b  | 75  |
| 2   | 54  |
| 3   | 5   |
| 4   | 2   |
| Comorbidity   |     |
| Cardiovascular diseases                               | 13  |
| Thyroid diseases                                      | 7   |
| Crohn's disease                                       | 1   |
| GERD  | 2   |
| Diabetes mellitus type 2                              | 2   |
| HBV co-infection                                      | 2   |

GERD: Gastroesophageal reflux disease; HBV: Hepatitis B virus.

either in mono-therapy or in combination with ribavirin. Sixty-six subjects received conventional interferon mono-therapy. Twenty-five patients were treated with conventional interferon plus ribavirin and 59 patients were treated with pegylated interferon plus ribavirin. Conventional interferon was used at doses ranging between 3 and 6 MU every 2 d for a mean period of  $49 \pm 3.12$  wk. The treatment regimen with pegylated interferon was carried out by international guideline recommendations and method indicated in the relevant product data sheets (Pegasys<sup>®</sup> 180 µg once weekly, subcutaneously; Peginteron<sup>®</sup> 1.5 µg/kg per week, subcutaneously; ribavirin 800, 1000 or 1200 mg/week orally, depending on body weight and virus genotype). The mean duration of the therapy was  $47 \pm 13$  wk.

### Follow-up

All patients were followed and analyzed for clinical, biochemical, virological and ultrasound parameters. All assessments were performed every 6 mo for the first two years of observation. In patients with cirrhosis at baseline, we continued to perform sequential clinical, biochemical and ultrasound follow-up every 6 mo, while the same panel of tests was conducted once a year in patients without cirrhosis. HCV RNA assessment was performed once per year during follow-up for all patients and whenever a decompensation or a progression of the liver disease occurred. At each medical visit, life-sign assessments and potential therapy-adverse effects were investigated. Biochemical evaluations measuring haemocrome (blood count), transaminases, bilirubin,

alkaline phosphatase and renal function were performed. All patients underwent abdominal ultrasound every 6 mo. If a new space-occupying lesion was suspected, it was first analyzed by ultrasonography to determine if it was unquestionably benign (*e.g.*, cysts or hemangioma). If necessary, the lesion was further examined by computed tomography, magnetic resonance imaging, arteriography, or liver biopsy. In a group of 27 patients with liver biopsy at baseline, liver stiffness was assessed during the last visit. During this follow-up, cirrhotics were considered to have progression if they showed any of the following findings: ascites, bleeding varices, hepatic encephalopathy and HCC. The diagnosis of HCC was formulated using imaging techniques and/or biopsy, according to the Barcelona Clinical Liver Cancer standardized staging system for hepatocellular carcinoma<sup>[21]</sup>.

### Laboratory analysis

Serum was collected for detection of HCV RNA once per year after SVR was obtained. Qualitative detection of HCV-RNA was performed by a standardized qualitative assay, Cobas AmpliPrep/Cobas Taqman (CAP/CTM). This assay is based on the reverse transcription-polymerase chain reaction (RT-PCR) method, followed by HCV RNA real-time fluorescent detection from 850 µL of serum. HCV RNA concentration was read in IU/mL. The CAP/CTM assay has a sensitivity of 15 IU/mL, with a linear quantification range of  $43-6.9 \times 10^7$  IU/mL.

## RESULTS

### Follow-up

The median duration of follow-up was 8.6 years (range 2-19.9 years). One hundred and fifteen/150 (76.6%) patients were followed for more than 4 years. Fifty-two/150 (34.6%) patients were followed for more than 10 years.

### Virological outcomes

Zero of the 150 patients had a recurrence of HCV infection during the follow-up period. HCV RNA was evaluated in all patients with CAP/CTM assay, using at least four blood samples taken in different times (including the last sample available for each patient). No patient had detectable HCV-RNA in serum by RT-PCR in any sample, even in the patients who developed liver-related complications.

### Survival and complication rates

The results from this study indicate the risk of liver-related death was only 0.67%, as only one patient died from liver-related causes. In addition, liver-related complications were observed in only three patients with a global complication incidence rate of 2%, while the complication incidence/person per year was 0.08%. These three complications included two patients with HCCs and one patient experiencing bleeding from esophageal varices. All three patients who developed complications had pre-treatment cirrhosis. Both patients who developed HCC were males, aged 61 developing the complication

**Table 2** Liver fibrosis with paired assessment at baseline *vs* the end of follow-up

| Patients | Baseline           |                                      | End of follow-up               |                     |                                   |
|----------|--------------------|--------------------------------------|--------------------------------|---------------------|-----------------------------------|
|          | METAVIR score      | Corresponding value of LS (21) (kPa) | Mean value (range) of LS (kPa) | Mean follow-up (yr) | Patients with fibrosis regression |
| 1        | F0                 | < 6                                  | 5.9                            | 10.6                | 0                                 |
| 18       | F1                 | 6.5 ± 1.1                            | 5.4 (2.8-6.3)                  | 9.48                | 13                                |
| 6        | F2                 | 7.3 ± 1.4                            | 6.2 (4.0-8.8)                  | 6.02                | 4                                 |
| 0        | F3                 | 10.2 ± 1.9                           | -                              | -                   | -                                 |
| 1        | F4                 | 15 ± 4.1                             | 6.8                            | 8.3                 | 1                                 |
| 1        | Clinical cirrhosis | 10.3                                 | 19.9                           | 1                   | 0                                 |

LS: Liver stiffness.

five years after follow-up, and aged 65 developing the complication nine years after follow-up, respectively. The 65-year-old male eventually died from liver-related complications. A 66-year-old female patient developed esophageal 17 bleeding years after the follow-up.

### Regression of liver fibrosis

During the final visit, we performed TE on 27 patients who underwent a liver biopsy at baseline. The histological staging at baseline and the mean values of liver stiffness at the end of follow-up are reported in Table 2. Only in three cases were the values of liver stiffness higher than 7.3 kPa, the threshold indicative of significant fibrosis (F2 according to Metavir score). None of the patients had a liver stiffness score higher than 14.5 kPa, suggestive of cirrhosis. We observed regression of the fibrosis in 19/27 patients (70% of the cases).

## DISCUSSION

The aim of this study was to assess the long-term effect of antiviral treatment in a large cohort of patients chronically infected with hepatitis C who had achieved SVR. This is one of the largest and longest studies on the natural history of successfully treated patients with chronic HCV infection in a real world setting. In the majority of the cases reported in the literature, the median follow-up duration is less than 5 years. In our study however, we observed 50 patients for up to 10 years and 21 patients for up to 15 years, with the median duration of the follow-up being 8.6 years. Overall, the clinical outcome of this study was very positive, indicating that prognosis in patients who obtained SVR is extremely promising.

The overall hepatic complication rate in our population was only 2%; a figure that is in agreement with other studies. Veldt *et al*<sup>[22]</sup> reported 3 cases of HCC in a population of 142 patients (2%) with SVR and a baseline fibrosis score that ranged between 4 and 6 according to the Ishak index<sup>[19]</sup>. Turner *et al*<sup>[23]</sup> reported 2 cases of HCC in a population of 152 patients (1.3%) with SVR and no cirrhosis. Ikeda *et al*<sup>[24]</sup> reported 30 cases of HCC in a population of 1097 patients (2.7%), of which 97 had cirrhosis and obtained SVR; but it was a retrospective, multicentric study with a median follow-up of 4.6 years. The high survival rate observed in our study (99.3%) is very consistent with values observed by George *et al*<sup>[18]</sup>

(99.4%) and Imazeki *et al*<sup>[25]</sup> (99.97%).

We could not detect HCV-specific RNA transcripts in any of the patients at each follow-up, confirming that a durable SVR can be achieved with both conventional and pegylated interferon treatment<sup>[7-9]</sup>. Although a low rate of HCV-recurrence detected through RT-PCR assays has been reported, these data are in agreement with more recent studies on the topic<sup>[26-28]</sup>. The sensitivity of laboratory assays for HCV RNA detection has significantly increased throughout the years, and we can hypothesize that low serum levels of HCV RNA may not have been detected by a low sensitivity assay in the past, leading to a misclassification of SVR subjects. In fact, 5%, 9.7%, and 8% rates of HCV recurrence have been observed in previous studies<sup>[26-28]</sup>. In our study, HCV RNA was assessed through the most sensitive assay available (RT-PCR with sensitivity < 50 IU/mL). This sensitivity adds validity to our hypothesis that the hepatic complications observed in patients in our study were not directly related to ongoing HCV replication, but rather other complicating factors.

Of the 27 patients where fibrosis was evaluated, no patient with pre-treatment chronic hepatitis showed progression to cirrhosis, including those with advanced fibrosis (Ishak score 3-4/6). Moreover, a regression of fibrosis was reported in 70% of the cases. These results correlate well with the work by Poynard *et al*<sup>[29]</sup> showing that SVR (regardless of the therapy with which it is obtained) not only stops liver damage progression, but can also induce its regression. In this cohort, 1904 patients with CHC and SVR and with paired pre-treatment and post-treatment biopsies were observed; 86% of patients showed an improvement in fibrosis stage while 12% showed no change, even when the mean time between biopsies was only 20 mo<sup>[29]</sup>. Our data also comply with two recent studies with mean follow-up times of 5 and 6.5 years reporting 83% and 61% rates of fibrosis regression, respectively<sup>[18,30]</sup>. In these studies, the regression of fibrosis was assessed through liver biopsy. Although a liver biopsy is considered the gold standard; it does have limitations including sampling errors and interpretation of results influenced by intra- and inter-observer variation, implying that distinguishing real changes in fibrosis, longitudinally, is a difficult task<sup>[31]</sup>. Ellis *et al*<sup>[32]</sup> suggested that the most convincing evidence for regression of liver fibrosis derives from large-scale studies on post-treatment natural history. In fact, long-term follow-up studies indicate that regression of liver fibrosis is

associated with improved clinical outcomes, strengthening the perception that histological regression is a real phenomenon. Even though there is no universal parameter to define fibrosis regression, it is suggested that a long term assessment of clear clinical outcomes combined with non-invasive testing for fibrosis could help with the interpretation of the results<sup>[32]</sup>.

In conclusion, our study documents that progression of liver damage and HCV infection relapse are virtually non-existent in patients with chronic hepatitis C infection who have achieved sustained virological response. Further, patients with pre-treatment evidence of cirrhosis show a rate of complications that cannot be neglected, but are hypothesized to occur due to complicating factors separate from HCV infection.

## COMMENTS

### Background

Sustained virological response (SVR) is by defined by undetectable hepatitis C virus (HCV) RNA levels 24 wk after completing treatment. The achievement of the SVR in patients with chronic hepatitis C (CHC) represents a milestone because it has been associated with the eradication of the infection, improvement in liver histology, improvement in quality of life, and reduced risk of cirrhosis and hepatocellular carcinoma. Short term SVR in patients with chronic HCV-infection is well established, however, knowledge regarding long-term SVR remains to be elucidated. There are limited long-term studies observing chronically infected patients who have achieved SVR, all with a limited number of patients.

### Innovations and breakthroughs

This study represents one of the longest follow-up studies on the natural history of successfully treated chronically HCV infected individuals. The outcome of the study was very positive, as it revealed an extremely high survival rate, an extremely low rate of liver complications, and a significant reduction in liver fibrosis in patients who have achieved SVR.

### Applications

The aim of this study was to evaluate the long-term outcomes of patients who obtain a SVR after antiviral therapy, regardless of the type treatment, in a monocentric cohort of patients representing a real world population with CHC during the last 20 years.

### Peer review

It is a well planned study with sound methodology.

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P- Reviewer Zhao HT S- Editor Song XX L- Editor A  
E- Editor Li JY



## Long-term efficacy of endoscopic coagulation for different types of gastric vascular ectasia

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Received: November 24, 2012 Revised: February 1, 2013

Accepted: March 6, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To examine the long-term therapeutic efficacies of endoscopic cauterization for gastric vascular ectasia, according to the type of lesion.

**METHODS:** Thirty-eight patients with hemorrhagic gastric vascular ectasia (VE) were treated by endoscopic cauterization: 13 by heater probe coagulation and 25 by argon plasma coagulation. Depending on the number of lesions, 14 and 24 patients were classified into localized VE ( $\leq 10$ ; LVE) and extensive VE ( $> 10$ ; EVE), respectively. The patients were followed-up by repeated endoscopic examinations after the therapy, and the incidences of VE recurrence and re-bleeding from the lesions were evaluated.

**RESULTS:** Although the VE lesions disappeared initially in all the patients after the therapy, the recurrence of VE developed in 25 patients (66%) over a mid-term observation period of 32 mo, and re-bleeding occurred in 15 patients (39%). The recurrence of VE was found in

all patients with EVE, with re-bleeding occurring in 14 patients (58%). In contrast, only 1 patient (7%) with LVE showed recurrence of the lesions and complicating hemorrhage. Both the cumulative recurrence-free rates and cumulative re-bleeding-free rates were significantly lower in the EVE group than in the LVE group ( $P < 0.001$  and  $P < 0.001$ , respectively). Moreover, the cumulative re-bleeding-free rate in the EVE group was 47.6% at 1 year and 25.4% at 2 years in patients with chronic renal failure, which were significantly lower than the rates in the patients without chronic renal failure (83.3% and 74.1%, respectively) ( $P < 0.05$ ).

**CONCLUSION:** The recurrence of VE and re-bleeding from the lesions was more frequent in the patients with EVE, especially in those with complicating renal failure.

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**Key words:** Gastric vascular ectasia; Heater probe coagulation; Argon plasma coagulation; Renal failure; Recurrence

**Core tip:** The aim of this study was to examine the long-term therapeutic efficacies of endoscopic cauterization for gastric vascular ectasia (VE), according to the type of lesion. Depending on the number of lesions, 14 and 24 patients were classified into localized vascular ectasia ( $\leq 10$ ) and extensive VE ( $> 10$ ; EVE), respectively. The incidences of VE recurrence and re-bleeding from the lesions were evaluated. The recurrence of VE and re-bleeding from the lesions was more frequent in the patients with EVE, especially in those with complicating renal failure, even after the initial successful arrest of bleeding and disappearance of the lesions by the endoscopic therapy.

Imai Y, Mizuno Y, Yoshino K, Watanabe K, Sugawara K, Motoya D, Oka M, Mochida S. Long-term efficacy of endoscopic

coagulation for different types of gastric vascular ectasia. *World J Gastroenterol* 2013; 19(18): 2799-2805 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2799.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2799>

## INTRODUCTION

Vascular ectasia (VE) of the stomach has been recognized as a rare cause of hemorrhage from the upper gastrointestinal tract<sup>[1-4]</sup>. Patients may present with either evidence of chronic occult bleeding, *i.e.*, iron deficiency anemia, or with acute hematemesis or melena. VE is a term that encompasses a broad spectrum of lesions visualized on endoscopic examination, including angiodysplasia, watermelon stomach, diffuse antral VE (DAVE) and telangiectasis associated with Osler-Weber-Rendu disease<sup>[5]</sup>. In general, angiodysplasia is defined as VE with a limited number of red flat spots or reticulated vascular areas in the gastric mucosa on endoscopic examination. In contrast, gastric antral VE (GAVE) is diagnosed as watermelon stomach when the lesions are visualized as stripes radiating outwards from the pylorus and as DAVE in cases with diffuse red spots in the gastric antrum.

Various therapeutic procedures have been employed for hemorrhagic gastric VE. Surgical resection of the stomach is the most curative therapeutic procedure, but it is a highly invasive procedure that maybe associated with high mortality, especially in elderly patients with underlying diseases such as metabolic syndrome. Thus, endoscopic cauterization has generally been performed for the treatment of gastric VE. Several types of coagulation procedures may be employed for endoscopic cauterization, including heater probe coagulation<sup>[6]</sup>, laser coagulation<sup>[7-10]</sup> and argon plasma coagulation<sup>[10-16]</sup>, and these devices have been reported to be useful for the temporary arrest of bleeding from any type of gastric VE. However, the long-term prognosis of patients undergoing endoscopic cauterization has to be elucidated. In the present paper, we examined the long-term therapeutic efficacies of endoscopic cauterization, including the recurrence rate of the lesions and the rates of re-bleeding, in patients with hemorrhaging from gastric VE, according to the type of lesion.

## MATERIALS AND METHODS

### Patients

The subjects included 42 patients with gastrointestinal bleeding from gastric VE lesions, which were diagnosed by gastrointestinal endoscopic examination between October 1996 and December 2007. Among them, 4 patients, who moved to a different institution for follow-up following discharge from our hospital after the successful arrest of bleeding from the VE, were excluded from the analysis. The remaining 38 patients consisted of 21 men and 17 women, with a median age of 69 years (range, 48-85 years). Twenty patients (53%) had underlying liver cirrhosis, and 15 patients (39%) were undergoing mainte-

nance hemodialysis for chronic renal failure.

On endoscopic examination, bleeding from the gastric VE lesions was observed in 21 patients (55%). In the remaining 17 patients, other gastrointestinal lesions that could serve as the possible source of the bleeding were not detectable by the endoscopic examinations, including small intestinal endoscopy, despite the patient presenting with hematemesis or melena. The patients were classified into 2 groups: 14 patients with  $\leq 10$  VE lesions (localized VE, the LVE group) and 24 patients with  $> 10$  VE lesions (extensive VE, the EVE group). The LVE group comprised 9 patients with a single lesion and 5 patients with multiple lesions (range, 3 to 10 lesions), including one patient with Osler-Weber-Rendu disease showing 3 lesions in the stomach. In contrast, the EVE group consisted of 5 patients with watermelon stomach and 19 patients with DAVE, including one patient with radiation-induced DAVE.

All patients were treated by endoscopic cauterization. Thereafter, follow-up endoscopic examinations were performed at intervals ranging from 1 to 6 mo, depending on the clinical features of each patient, and the rate of the recurrence of VE with or without re-bleeding from the lesions was evaluated. The recurrence of VE was defined as the presence of  $> 10$  lesions in the EVE group, and of at least one lesion in the LVE group. The diagnosis of re-bleeding was conducted when the patients showed hematemesis or melena and/or hemorrhagic features of the gastric VE lesions on endoscopic examination irrespective of the number of lesions. Fifteen and twenty patients received treatment with a proton pump inhibitor and H<sub>2</sub>-receptor antagonist, respectively, during the observation period. Written informed consent for the endoscopic procedures was obtained from all the patients. This study was retrospectively performed with the approval of the Institutional Review Board of the Hospital.

### Endoscopic cauterization therapies

Thirteen patients seen between October 1996 and March 2000 were treated by heater probe coagulation, and twenty-five patients seen after April 2000 were treated by argon plasma coagulation. The therapies were repeated until the VE lesions completely disappeared from the stomach following the arrest of hemorrhaging. Heater probe coagulation was performed using a heater probe unit (Olympus Co., Tokyo, Japan) at 15 J for patients with bleeding VE and/or those with VE lesions with a diameter of  $> 3$  mm and at 10 J for those with non-bleeding VE lesions with a diameter of  $\leq 3$  mm. Argon plasma coagulation was performed using a high-frequency generator (Erbotom ICC200; ERBE, Tübingen, Germany) with an automatically regulated argon source (APC300; ERBE) and a flexible APC probe (ERBE) with a high-frequency output at 60 W. Argon gas was delivered at a flow rate of between 1.0 and 2.0 L/min.

### Statistical analysis

The differences in the characteristics between the 2 groups

**Table 1** Demographic and clinical features of the patients with gastrointestinal bleeding caused by gastric vascular ectasia

|   | Total     | Groups <sup>2</sup> |           | P value  |
|---|-----------|---------------------|-----------|----------|
|   |           | LVE                 | EVE       |          |
| No. of patients                                   | 38        | 14                  | 24        |          |
| Sex: male/female <sup>1</sup>                     | 21 / 17   | 9/5                 | 12/12     | NS       |
| Age, yr (medium)                                  | 69        | 67.5                | 72        | NS       |
| Bleeding from lesions at examination <sup>1</sup> | 21        | 6                   | 15        | NS       |
| Cauterization <sup>3</sup>                        |           |                     |           |          |
| Method: HPC/APC <sup>1</sup>                      | 13 / 25   | 6/8                 | 7/17      | NS       |
| No. of treatment sessions (mean ± SD)             | 2.3 ± 1.6 | 1.1 ± 0.3           | 3.0 ± 1.6 | < 0.0001 |
| Observation period, mo (medium)                   | 32        | 68.5                | 29.5      | < 0.01   |
| Medication during observation <sup>1</sup>        |           |                     |           |          |
| Proton pump inhibitors                            | 15        | 4                   | 11        | NS       |
| H <sub>2</sub> -receptor antagonists              | 20        | 9                   | 11        |          |
| None  | 3         | 1                   | 2         |          |
| Underlying diseases <sup>1</sup>                  |           |                     |           |          |
| Chronic renal failure                             | 15        | 3                   | 12        | NS       |
| Liver cirrhosis                                   | 20        | 5                   | 15        | NS       |

<sup>1</sup>Number of the patients; <sup>2</sup>Localized vascular ectasia (LVE) denotes localized vascular ectasia and extensive vascular ectasia (EVE) denotes extensive vascular ectasia; <sup>3</sup>Heater probe coagulation (HPC) denotes heater probe coagulation and argon plasma coagulation (APC) denotes argon plasma coagulation. NS: Not significant.

were analyzed by Mann-Whitney's *U* test, Fisher's exact test and the  $\chi^2$  test. The cumulative recurrence-free and rebleeding-free rates were analyzed by the Kaplan-Meier method. Factors associated with the type of VE and the treatment methods were compared by the log-rank test. *P* values of less than 0.05 were considered to be statistically significant.

## RESULTS

### Demographic and clinical features of the patients in the LVE and EVE groups

As shown in Table 1, there were no significant differences in the sex distribution or age of the patients between the LVE and EVE groups. Hemorrhaging from the VE lesions was observed in 43% (6/14) and 63% (15/24) of the patients in the LVE and EVE groups, respectively. Additionally, 21% (3/14) and 36% (5/14) of the patients in the LVE group and 50% (12/24) and 63% (15/24) of the patients in the EVE group had underlying liver cirrhosis and chronic renal failure, respectively, but the prevalences of the 2 underlying diseases were not significantly different between the two groups.

### Therapeutic efficacies of the endoscopic coagulation procedures

Among the 21 patients in whom active bleeding from the VE lesions was observed, heater probe coagulation was performed in 3 LVE and 4 EVE patients, and argon plasma coagulation in 3 LVE and 11 EVE patients. Hemostasis was obtained in all patients after either endoscopic coagulation procedure. Although the VE lesions diminished

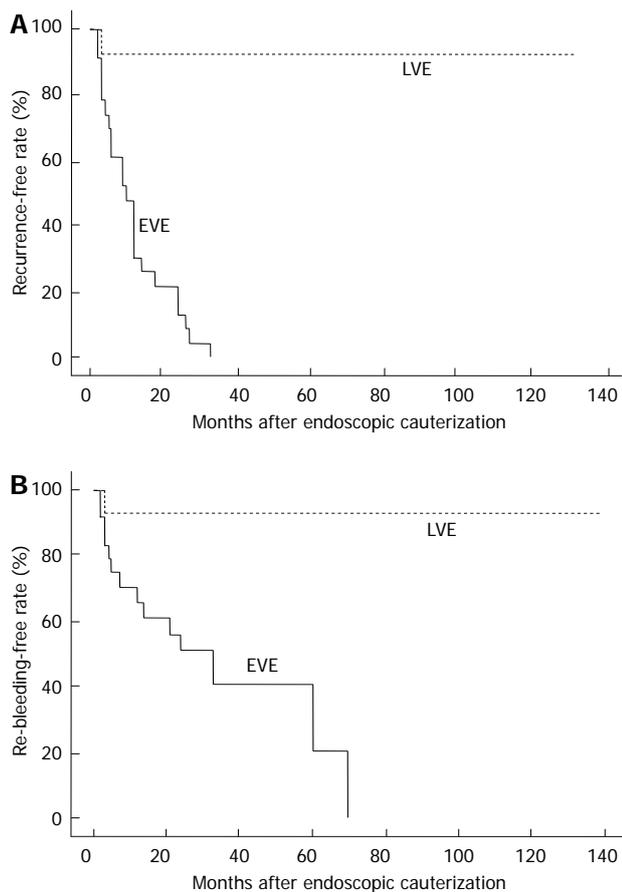
**Table 2** Long-term efficacies of endoscopic cauterization therapies in the patients with gastrointestinal bleeding caused by gastric vascular ectasia *n* (%)

| Groups      | Recurrence rate | Re-bleeding rate |
|-------------|-----------------|------------------|
| LVE         | 1 (7.1)         | 1 (7.1)          |
| Without CRF | 0 (0.0)         | 0 (0.0)          |
| With CRF    | 1 (33.3)        | 1 (33.3)         |
| EVE         | 24 (100.0)      | 14 (58.3)        |
| Without CRF | 12 (100.0)      | 6 (50.0)         |
| With CRF    | 12 (100.0)      | 8 (66.7)         |

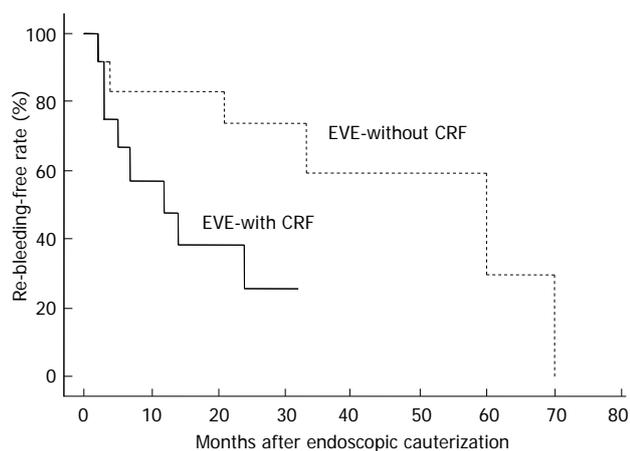
LVE: Localized vascular ectasia, EVE: Extensive vascular ectasia; CRF: Chronic renal failure.

after the procedures in all patients, the number of treatment sessions required for the complete disappearance of the lesions in the stomach differed between the LVE and EVE groups (Table 1). A single treatment session was sufficient to achieve the complete disappearance of the VE lesions in all the patients of the LVE group, except one, who required 2 sessions of heater probe coagulation. In contrast, the patients in the EVE group needed a mean of 3.0 treatment sessions (range, 2-7 treatment sessions) for the complete disappearance of the lesions. No complications of the endoscopic coagulation treatments were encountered in either treatment group.

In all patients, the recurrence of VE and of re-bleeding was examined by repeated follow-up endoscopic examinations for more than 6 mo after the final session of endoscopic cauterization therapy; the median observation period was 32 mo (range, 6-139 mo). As shown in Table 1, however, the observation periods were significantly longer in the LVE group than in the EVE group (*P* < 0.01). The medications prescribed for the patients during the observation period were similar between the two groups. The endoscopic examination revealed recurrence of the VE in 25 of 38 patients (66%), and re-bleeding from the recurrent gastric VE lesions in 15 of these patients (39%) over a median observation period of 5 mo (range, 2-70 mo) after the final session of the coagulation therapy. Particularly in the EVE group, the VE recurred in all the patients, and re-bleeding from the recurrent lesions developed in 14 of these patients (58%) (Table 2). No patients in the EVE group developed re-bleeding with the re-appearance of  $\leq 10$  VE lesions. All of the patients showing re-bleeding in the EVE group had underlying diseases: 8 with chronic renal failure, 5 with liver cirrhosis and 1 with radiation-induced mucosal damage of the gastrointestinal tract. In contrast, in the LVE group, the recurrence of the gastric VE was found in only 1 patient (7%), who had a single lesion that was found before the first session of argon plasma coagulation therapy. This patient also had underlying renal failure and was under long-term maintenance hemodialysis. Consequently, re-bleeding from the recurrent VE lesions developed in 60% (9/15) of the patients with chronic renal failure, which was significantly higher than the percentage in the patients without chronic renal failure (26%; 6/23) (*P* = 0.036).

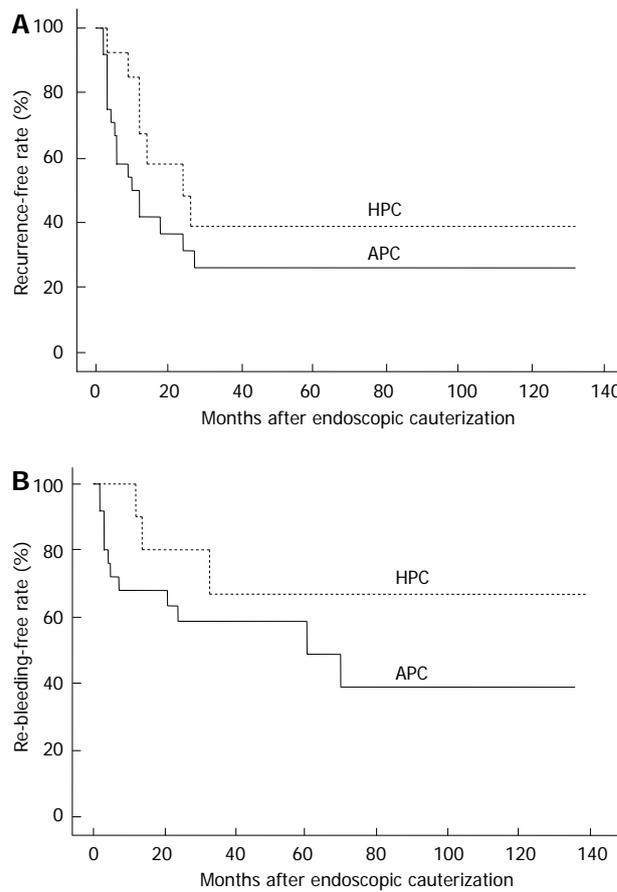


**Figure 1** Long-term outcomes of the patients with hemorrhaging gastric vascular ectasia treated by endoscopic cauterization, depending on the number of lesions. A: Cumulative recurrence-free rates; B: Cumulative re-bleeding-free rates. Both rates were significantly higher in the localized vascular ectasia (LVE) group than in the extensive vascular ectasia (EVE) group ( $P < 0.001$ ).



**Figure 2** Cumulative re-bleeding-free rates in the patients with extensive vascular ectasia treated by endoscopic cauterization. The rates were significantly higher in the patients without chronic renal failure (CRF) than in those with CRF ( $P < 0.05$ ). EVE: Extensive vascular ectasia.

Kaplan-Meier analysis revealed that the cumulative recurrence-free rates of the patients with VE were 29.2% and 0% at 1 and 3 years, respectively, after the endoscopic coagulation therapy in the EVE group (Figure 1A), and



**Figure 3** Long-term outcomes of the patients with hemorrhaging gastric vascular ectasia treated by endoscopic cauterization, depending on the type of procedure. A: Cumulative recurrence-free rates; B: Cumulative re-bleeding-free rates. Both the rates were comparable between the patients treated by heater probe coagulation (HPC) and those treated by argon plasma coagulation (APC).

the cumulative re-bleeding-free rates in these patients were 65.9%, 40.8% and 20.4% at 1, 3 and 5 years, respectively, after the therapy (Figure 1B). Both the recurrence-free rates and re-bleeding-free rates were significantly lower in the EVE group than in the LVE group. Moreover, the cumulative re-bleeding-free rate in the EVE group was 47.6% at 1 year and 25.4% at 2 years after the therapy in patients with chronic renal failure, which were significantly lower than the rates in the patients without chronic renal failure (83.3% and 74.1%, respectively) (Figure 2). There were no significant differences in either the recurrence-free rates or re-bleeding-free rates between the patients treated by heater probe coagulation and those treated by argon plasma coagulation, as shown in Figure 3.

## DISCUSSION

In the present study, we examined the long-term prognosis of 38 patients with hemorrhagic gastric VE treated by endoscopic coagulation procedures, with a medium observation period of 32 mo after the therapies. Although both heater probe coagulation and argon plasma coagulation were useful to achieve the arrest of bleeding and

disappearance of the VE lesions, the recurrence of VE and re-bleeding from the recurrent lesions were noted in 66% and 39% of the patients, respectively. Additionally, we found that the recurrence of VE and/or re-bleeding from the recurrent lesions were more frequent in patients with EVE, especially those with underlying chronic renal failure undergoing maintenance hemodialysis, than in those with LVE. We also found that neither the lesion recurrence rate nor the re-bleeding rate differed significantly between the patients treated by heater probe coagulation and those treated by argon plasma coagulation, although the number of patients evaluated in the study was relatively small.

In Japan, endoscopic laser therapy has not been widely employed for the treatment of VE in the gastrointestinal tract because of the high risk of severe complications, such as perforation<sup>[8]</sup>. Thus, endoscopic cauterization either with heater probe coagulation or argon plasma coagulation is the standard therapeutic strategy used for gastric VE with or without bleeding. However, few studies have been performed to determine the long-term outcomes of such therapies. Olmos *et al.*<sup>[13]</sup> reported that argon plasma coagulation was effective for the prevention of recurrent bleeding from VE in the gastrointestinal tract. Although they showed that re-bleeding did not occur from recurrent lesions in 83% of the patients over a median observation periods of 18 mo, only 10 patients with hemorrhagic gastric VE were included in their study after the exclusion of patients with chronic renal failure, liver cirrhosis and GAVE, including patients with the lesions caused by radiation. Moreover, Zushi *et al.*<sup>[14]</sup> examined the outcomes in 16 patients with liver cirrhosis with hemorrhagic GAVE treated by endoscopic cauterization and reported that 25% of the patients showed re-bleeding from recurrent lesions. In their study, however, the mean observation period was only 10 mo, which is too short to evaluate the long-term outcomes of therapy in these patients. In contrast, Nakamura *et al.*<sup>[15]</sup> evaluated the long-term efficacy of argon plasma coagulation in 22 patients with GAVE and reported that the cumulative re-bleeding rates at 1, 2 and 3 years after the therapy were 50.3%, 64.5% and 64.5%, respectively, over a mean observation period of 23.5 mo. Herrera *et al.*<sup>[17]</sup> examined the therapeutic efficacy of argon plasma coagulation in patients with VE depending on the type of lesion, and reported that there were no cases of re-bleeding from recurrent lesions among the patients with LVE. Our results regarding the EVE group were in line with those reported by Nakamura *et al.*<sup>[15]</sup> but not with those by Herrera *et al.*<sup>[17]</sup>, in which re-bleeding after the therapies developed only in 1 of 8 patients with GAVE. In our study, re-bleeding from recurrent VE was especially frequent in patients with EVE and in those with chronic renal failure undergoing maintenance hemodialysis, whereas underlying diseases such as chronic renal failure were found only in 3 patients with GAVE in the study by Herrera *et al.*<sup>[17]</sup>. These differences in the clinical features of the patients

may have produced the discrepancies in the results by Herrera *et al.*<sup>[17]</sup>, those by Nakamura *et al.*<sup>[15]</sup> and those in the present study.

Both LVE and EVE have been reported to develop at a high frequency in association with chronic renal failure<sup>[18]</sup>. Clouse *et al.*<sup>[3]</sup> reported that 18 of 30 patients (60%) with hemorrhagic angiodysplasia had underlying renal failure, with 10 of these patients (33%) under long-term maintenance hemodialysis and/or who underwent renal transplantation. The present study demonstrated that endoscopic cauterization did not provide satisfactory long-term outcomes in patients with EVE complicated with chronic renal failure, even after the successful initial arrest of bleeding had been achieved by the endoscopic cauterization therapy. Notably, the VE developed again after the therapies in all of the patients with EVE, even in the absence of underlying chronic renal failure, followed by re-bleeding from the recurrent lesions in half of these patients, with a cumulative re-bleeding rate of approximately 40% at 3 years. These observations prompted us to postulate that the etiology and the clinical characteristics may be different between the LVE and EVE groups, and also between VE patients with and without chronic renal failure. Based on the findings, careful endoscopic follow-up after the initial successful cauterization therapy is required for patients with EVE, regardless of the presence/absence of underlying diseases, including chronic renal failure.

With respect to the endoscopic cauterization procedures available, argon plasma coagulation has been employed more frequently compared with heater probe coagulation for the treatment of gastric VE in Japan. Similarly, at our institution, almost all patients seen after the year 2000 have been treated by argon plasma coagulation. As shown in Figure 3, both the cumulative recurrence and re-bleeding rates were equivalent between patients with gastric VE treated by argon plasma coagulation and those treated by heater probe coagulation. The limitation of our evaluation is that it is a retrospective and single cohort study. A randomized controlled study would be required to confirm our results. Additionally, the safety, including the frequency of complications, convenience of instrument handling, and number of sessions required for the treatment, has been reported to not be significantly different between the patients treated by the two procedures<sup>[19-22]</sup>. Thus, the criteria for the selection of either procedure for patients with gastric ectasia need to be established in the future. Recently, endoscopic band ligation was shown to be useful for the treatment of GAVE<sup>[23-27]</sup>. Wells *et al.*<sup>[25]</sup> reported that endoscopic band ligation was superior to thermal therapies, including argon plasma coagulation, in terms of the therapeutic efficacy to arrest bleeding, the volume of blood transfusion needed after the procedure and the duration of hospitalization in 22 patients with GAVE. Sato *et al.*<sup>[26]</sup> also reported the superiority of endoscopic band ligation compared with argon plasma coagulation for GAVE as-

sociated with liver diseases. Moreover, a novel endoscopic ablation method, the HALO<sup>90</sup> system, has been reported to be useful for the treatment of GAVE in a few patients. A large-scale study would be required to compare the efficacy and safety of these novel procedures with those of argon plasma coagulation and heater probe coagulation.

In conclusion, the recurrence of VE and re-bleeding from the lesions was frequent in patients with EVE, especially in those with underlying chronic renal failure after the initial successful control of the bleeding and disappearance of the lesions by endoscopic cauterization. Therefore, careful observation by endoscopy is important for these patients even after initial successful therapy.

## COMMENTS

### Background

Vascular ectasia (VE) of the stomach has been recognized as a rare cause of hemorrhage from the upper gastrointestinal tract. Endoscopic coagulation has been reported to be useful for the temporary arrest of bleeding from any type of gastric VE. However, the long-term prognosis of patients undergoing endoscopic coagulation has not yet been elucidated.

### Innovations and breakthroughs

The recurrence of VE and re-bleeding from the lesions was frequent in patients with extensive VE, especially in those with underlying chronic renal failure, after the initial successful control of the bleeding and disappearance of the lesions by endoscopic cauterization.

### Applications

The findings of this study may help establish the treatment and follow-up strategy for the patients with bleeding gastric VE.

### Terminology

VE is a term encompassing a broad spectrum of lesions visualized on endoscopic examination, including angiodysplasia, watermelon stomach and diffuse antral VE. In this study, VE lesions were classified into 2 subtypes: localized VE with ≤ 10 VE lesions and extensive VE with > 10 VE lesions.

### Peer review

This is a paper on the treatment of VE of the stomach with some novel findings. The cohort is large and well described. It is worth publishing to demonstrate the novel finding of worse outcomes in those patients with chronic renal failure.

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**P- Reviewer** Selinger CP **S- Editor** Gou SX **L- Editor** A  
**E- Editor** Li JY



## Extremely high prevalence of *Helicobacter pylori* infection in Bhutan

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Supported by A Grant from the grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 23790798; A Grant from the National Research University Project of the Thailand Office of Higher Education Commission; The National Institutes of Health (DK62813) and the grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, No. 22390085 and No. 22659087

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Received: November 16, 2012 Revised: February 13, 2013

Accepted: February 28, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To revealed the prevalence of *Helicobacter pylori* (*H. pylori*) infection in the Bhutanese population.

**METHODS:** We recruited a total of 372 volunteers (214 females and 158 males; mean age of  $39.6 \pm 14.9$  years) from three Bhutanese cities (Thimphu, Punaka, and Wangdue). The status of *H. pylori* infection was determined based on five different tests: the rapid urease test (CLO test), culture, histology, immunohistochemistry (IHC), and serum anti *H. pylori*-antibody.

**RESULTS:** The serological test showed a significantly higher positive rate compared with the CLO test, culture, histology and IHC ( $P < 0.001$ ,  $P < 0.001$ ,  $P = 0.01$ , and  $P = 0.01$ , respectively). When the subjects were considered to be *H. pylori* positive in the case of at least one test showing a positive result, the overall prevalence of *H. pylori* infection in Bhutan was 73.4%. The prevalence of *H. pylori* infection significantly decreased with age ( $P < 0.01$ ). The prevalence of *H. pylori* infection was lower in Thimphu than in Punakha and Wangdue ( $P = 0.001$  and  $0.06$ , respectively). The prevalence of *H. pylori* infection was significantly higher in patients with peptic ulcers than in those with gastritis ( $91.4\%$  vs  $71.3\%$ ,  $P = 0.003$ ).

**CONCLUSION:** The high incidence of gastric cancer in Bhutan may be attributed to the high prevalence of *H. pylori* infection.

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**Key words:** *Helicobacter pylori*; Bhutan; Prevalence

**Core tip:** The high prevalence of *Helicobacter pylori* (*H. pylori*) infection in Bhutan may contribute to the high incidence of peptic ulcers and gastric cancer. The prevalence of *H. pylori* infection in the capital city, Thimphu, was significantly lower than that of other rural areas. Therefore, performing eradication therapy of *H. pylori* and improving the sanitary conditions to decrease the rate of *H. pylori* infection in Bhutan can contribute to decreasing *H. pylori*-related diseases such as peptic ulcers and gastric cancer.

Vilaichone R, Mahachai V, Shiota S, Uchida T, Ratanachu-ek T, Tshering L, Tung NL, Fujioka T, Moriyama M, Yamaoka Y. Extremely high prevalence of *Helicobacter pylori* infection in Bhutan. *World J Gastroenterol* 2013; 19(18): 2806-2810 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2806.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2806>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a spiral, Gram-negative bacterium that chronically colonizes the human stomach and is currently recognized to play a causative role in the pathogenesis of various gastroduodenal diseases, including gastritis, peptic ulcers, gastric cancer, and mucosa-associated lymphoid tissue lymphoma<sup>[1,2]</sup>. Infection with *H. pylori* almost always results in chronic gastritis, but only a small population of infected patients develop more severe diseases, such as peptic ulcers and gastric cancer<sup>[2,3]</sup>. In Asia, gastric cancer is still a significant health problem, and the incidence of gastric cancer geographically varies greatly. Based on the age-standardized incidence rate (ASR) of gastric cancer, Asian countries can be categorized as high-risk (e.g., Japan, South Korea and China), intermediate-risk (e.g., Vietnam) or low-risk (e.g., Thailand and Indonesia) countries for gastric cancer<sup>[4]</sup>. Although the association between *H. pylori* infection and gastric cancer has been well-established<sup>[5,6]</sup>, a high prevalence of *H. pylori* infection is not always associated with a high incidence of gastric cancer. For example, despite the high infection rate in India, the incidence of gastric cancer there is low, which is known as an "Asian enigma"<sup>[7]</sup>.

Bhutan is a small landlocked country in South Asia, located at the eastern end of the Himalayas and bordered on the South, East and West by India and on the North by China. The ASR of gastric cancer in Bhutan was reported to be 24.2/100000, which is relatively high among Asian countries<sup>[4]</sup>. Although several studies focused on the prevalence of *H. pylori* infection have been conducted in many countries with different socio-economic, cultural, and racial groups<sup>[8-10]</sup>, the prevalence of *H. pylori* infection in Bhutan has not been investigated yet. In this study, we first disclosed the infection rate of *H. pylori* in Bhutan, and the findings from this study can be used as baseline epidemiological data for further research to understand the epidemiology of *H. pylori* infection in Bhutan and other Asian countries.

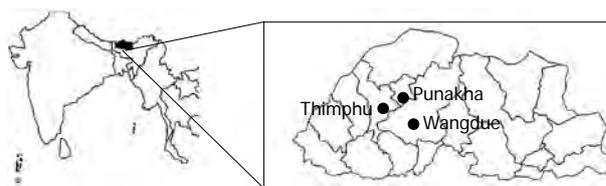


Figure 1 Geographic location in Bhutan.

## MATERIALS AND METHODS

### Study population

We recruited a total of 372 volunteers with dyspeptic symptoms (214 females and 158 males; mean age of  $39.6 \pm 14.9$  years) during four days (December 6 to 9) in December 2010. The survey was conducted in Thimphu ( $n = 194$ ), the capital city, and in two other cities within a 200 km radius of the capital, Punakha ( $n = 119$ ) and Wangdue ( $n = 59$ ) (Figure 1). Written informed consent was obtained from all participants, and the protocol was approved by the Ethics Committee of Jigme Dorji Wangchuk National Referral Hospital, Bhutan.

During each endoscopy session, four gastric biopsy specimens were obtained (three from the antrum and one from the corpus). The three specimens from the antrum were used for *H. pylori* culture, rapid urease test and histological examination. The specimen from the corpus was used for histological examination. Peptic ulcers and gastric cancer were identified by endoscopy, and gastric cancer was further confirmed by histopathology. Gastritis was defined as *H. pylori* gastritis in the absence of a peptic ulcer or gastric malignancy.

### Status of *H. pylori* infection

To maximize the diagnostic accuracy, 5 different methods were combined for the diagnosis of *H. pylori* infection, including culture, histology, immunohistochemistry (IHC), the rapid urease test and serum *H. pylori* antibody. For the *H. pylori* culture, one biopsy specimen from the antrum was homogenized in saline and inoculated onto Mueller Hinton II Agar medium (Becton Dickinson, NJ, United States) supplemented with 7% horse blood without antibiotics. The plates were incubated for up to 10 d at 37 °C under microaerophilic conditions (10% O<sub>2</sub>, 5% CO<sub>2</sub> and 85% N<sub>2</sub>). *H. pylori* was identified on the basis of colony morphology, Gram staining and positive reactions for oxidase, catalase, and urease. Isolated strains were stored at -80 °C in Brucella Broth (Difco, NJ, United States) containing 10% dimethylsulfoxide and 10% horse serum. For histology, all biopsy materials were fixed in 10% buffered formalin for 24 h and then embedded in paraffin. Serial sections were stained with hematoxylin and eosin and with May-Giemsa stain. The state of the gastric mucosa was evaluated according to the updated Sydney system<sup>[11]</sup>. The degree of the bacterial load was classified into four grades: 0, "normal"; 1, "mild"; 2, "moderate"; and 3, "marked". A bacterial load grade greater than or equal to 1 was defined as *H. pylori* positive. *H. pylori* seropositivity was evaluated with a commercially available ELISA kit (Eiken Co., Ltd.,

**Table 1** Prevalence of *Helicobacter pylori* infection in each diagnostic test *n* (%)

| Diagnostic test | Age (yr)               |                        |                        |                        |                       | Total ( <i>n</i> = 372) |
|-----------------|------------------------|------------------------|------------------------|------------------------|-----------------------|-------------------------|
|                 | ≤ 29 ( <i>n</i> = 107) | 30-39 ( <i>n</i> = 96) | 40-49 ( <i>n</i> = 80) | 50-59 ( <i>n</i> = 45) | ≥ 60 ( <i>n</i> = 44) |                         |
| Serum           | 80 (74.8)              | 80 (83.3)              | 47 (58.8)              | 30 (66.7)              | 24 (54.5)             | 261 (70.2)              |
| CLO             | 68 (63.6)              | 59 (61.5)              | 35 (43.8)              | 24 (53.3)              | 17 (38.6)             | 203 (54.6)              |
| Culture         | 72 (67.3)              | 61 (63.5)              | 38 (47.5)              | 23 (51.1)              | 16 (36.4)             | 210 (56.5)              |
| Histology       | 77 (72.0)              | 64 (66.7)              | 41 (51.3)              | 28 (62.2)              | 19 (43.2)             | 229 (61.6)              |
| IHC             | 77 (72.0)              | 64 (66.7)              | 41 (51.3)              | 28 (62.2)              | 19 (43.2)             | 229 (61.6)              |
| Final           | 86 (80.4)              | 80 (83.3)              | 49 (61.3)              | 32 (71.1)              | 26 (59.1)             | 273 (73.4)              |

CLO: The rapid urease test; IHC: Immunohistochemistry.

Tokyo, Japan) according to the manufacturer's instructions. Patients were considered to be *H. pylori*-negative when all five tests were negative, and a *H. pylori*-positive status required at least one positive test result.

### Immunohistochemistry

IHC was performed as described previously<sup>[12]</sup>. Briefly, after antigen retrieval and inactivation of endogenous peroxidase activity, tissue sections were incubated with the  $\alpha$ -*H. pylori* Ab (DAKO, Denmark) overnight at 4 °C. After washing, the sections were incubated with biotinylated goat anti-rabbit IgG (Nichirei Co., Japan), followed by incubation with a solution of avidin-conjugated horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories Inc., Burlingame, CA, United States). Peroxidase activity was detected using a H<sub>2</sub>O<sub>2</sub>/diaminobenzidine substrate solution. For all cases, we performed Giemsa staining using a serial section to identify the presence of *H. pylori*. If the *H. pylori* identified by Giemsa staining was found to be positively immunostained, we judged the case to be positive.

### Statistical analysis

The statistical analysis were conducted using the chi-square test to compare discrete variables and Cochran-Armitage analysis to compare the prevalence of *H. pylori* infection. Differences in the prevalence in each group were analyzed using the Mantel-Haenszel method. To match age and sex, multiple backward stepwise logistic regression analyses were used to examine the associations of peptic ulcers with the main predictor variables. The predictor variables for peptic ulcers were age, sex and *H. pylori* status. For each variable, the OR and 95%CI were calculated. Differences at  $P < 0.05$  were regarded as statistically significant. The data analysis was performed using JMP® 9 statistical software (SAS Institute Inc., Cary, NC, United States) and SPSS version 19 (SPSS Inc., Chicago, IL, United States).

## RESULTS

A total of 372 subjects were recruited, comprising 107 who were ≤ 29 years old, 96 who were 30-39 years old, 80 who were 40-49 years old, 45 who were 50-59 years old, and 44 who were ≥ 60 years old. Table 1 shows the *H. pylori*-positive rate for each test. The serological test showed a significantly higher positive rate compared with the CLO

test, culture, histology and IHC ( $P < 0.001$ ,  $P < 0.001$ ,  $P = 0.01$ , and  $P = 0.01$ , respectively). The prevalence of *H. pylori* infection by the serological test was as follows: 74.8% (80/107) for the ≤ 29 years old group, 83.3% (80/96) for the 30-39 years old group, 58.8% (47/80) for the 40-49 years old group, 66.7% (30/45) for the 50-59 years old group, and 54.5% (24/44) for the ≥ 60 years old group. When the subjects were considered to be *H. pylori* positive in the case of at least one positive test, the prevalence of *H. pylori* was 80.4% (86/107) for the ≤ 29 years old group, 83.3% (80/96) for the 30-39 years old group, 61.3% (49/80) for the 40-49 years old group, 71.1% (32/45) for the 50-59 years old group, and 59.1% (26/44) for the ≥ 60 years old group; thus, the prevalence was significantly decreased with age ( $P < 0.01$ ). This phenomenon was significant in the Thimphu area. Because the number of subjects older than 50 years old in Punakha and Wangdue was too small for the statistical analysis, this trend was not observed in these two areas. Overall, the prevalence of *H. pylori* infection in Bhutan was 73.4% (273/372). Figure 2 shows the prevalence of *H. pylori* infection according to the various range age groups. There was no significant difference between men and women (data not shown).

The prevalence of *H. pylori* infection in the three cities was analyzed by the Mantel-Haenszel method to adjust for age. It differed among the three cities, with the highest in Punakha (102/119, 85.7%), followed by Wangdue (44/59, 74.5%) and Thimphu (127/194, 65.4%). The prevalence of *H. pylori* infection was significantly lower in Thimphu than in Punakha even after the adjustment for age ( $P = 0.001$ ). Although there was no significant difference, the prevalence tended to be lower in Thimphu than in Wangdue even after the adjustment for age ( $P = 0.06$ ).

In the endoscopic diagnosis, gastritis was the most common finding (307/372, 82.5%). Gastric and duodenal ulcers were found in 25 (6.7%) and 22 (5.9%) cases, respectively. Gastric cancer was found in 5 cases (1.3%). Duodenal erosion, duodenal tumor, and reflux esophagitis were found at 5, 1 and 7 cases, respectively. Table 2 shows the prevalence *H. pylori* infection in each diagnosis. A high infection rate was detected among patients with gastric ulcers (92.0%) and duodenal ulcers (90.9%). In addition, 71.3% of the subjects with gastritis were *H. pylori*-positive. When gastric and duodenal ulcers were defined as peptic ulcers, the prevalence of *H. pylori* infection in peptic ulcers was significantly higher than that in gastritis (91.4% *vs* 71.3%,  $P = 0.003$ ). The percentage of

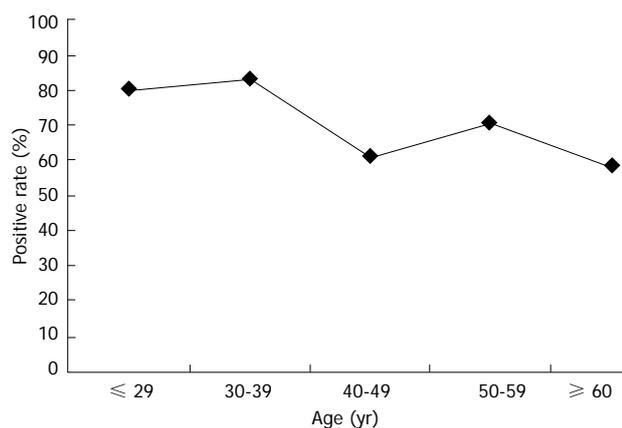


Figure 2 Prevalence of *Helicobacter pylori* infection by age group in Bhutan.

men was also significantly higher in patients with peptic ulcers than in those with gastritis (72.3% vs 37.1%,  $P < 0.001$ ). Multiple logistic analysis after adjusting for age and gender showed that *H. pylori*-positivity in addition to male gender was significantly associated with peptic ulcers (OR = 3.89, 95%CI: 1.33-11.33).

## DISCUSSION

We first revealed that the prevalence of *H. pylori* in Bhutan was 73.4%. In contrast with developed countries, *H. pylori* infections occur earlier in life and with a higher frequency in the developing world<sup>[8]</sup>. The prevalence of infection with *H. pylori* infection exceeds 50% by 5 years of age, and by adulthood, infection rates exceeding 90% are not unusual in developing countries<sup>[13]</sup>. Although the prevalence of infection has dropped significantly in many parts of North America, Western Europe and Asia, no such decline has been noted in the developing world<sup>[13]</sup>. The present study showed that a high prevalence was detected in younger age groups and that the prevalence significantly decreased with age in Bhutan. The decrease in the *H. pylori* infection rate with age might be due to the overuse of antibiotics in Bhutan. Antibiotics are frequently used in any infectious diseases in Bhutan (Dr. Lotay, personal communication). Even under these conditions, the prevalence of *H. pylori* infection was relatively high in all age groups.

Several clinical tests have been developed to diagnose *H. pylori* infection. However, there is still no established "gold standard" for the diagnosis of *H. pylori*, and thus the combination of two or more tests should be applied to determine the accurate prevalence of infection. In this study, we combined 5 different tests and considered *H. pylori*-positivity to be at least one positive test among the five tests. In this study, the serological test showed the highest positive rate compared with the other 4 tests. Although the serological test is widely used in epidemiological studies and not affected by local changes in the stomach that could lead to false-negatives, as in the other tests, this test cannot distinguish between current and past infections because *H. pylori* IgGs persist even after the disappearance of this bacterium<sup>[9,14]</sup>. Therefore,

Table 2 Prevalence of *Helicobacter pylori* infection in each diagnosis *n* (%)

| Diseases           | <i>n</i> | Positive   |
|--------------------|----------|------------|
| Gastritis          | 307      | 219 (71.3) |
| Gastric ulcer      | 25       | 23 (92.0)  |
| Duodenal ulcer     | 22       | 20 (90.9)  |
| Gastric cancer     | 5        | 3 (60.0)   |
| Duodenal erosion   | 5        | 3 (60.0)   |
| Duodenal tumor     | 1        | 1 (100.0)  |
| Reflux esophagitis | 7        | 4 (57.1)   |

results that are positive in the serological test and negative in the endoscopic tests may indicate a past infection. Culture from biopsy specimens has the potential of leading to a high sensitivity, given that only one bacterium can multiply and provide billions of bacteria. However, both strict transport conditions and careful handling in the laboratory are necessary<sup>[14]</sup>. Histopathological positivity depends on the density of *H. pylori* biopsy sites; thus, these tests can occasionally show false negative results<sup>[14]</sup>. In addition, the histological diagnosis of *H. pylori* infection is very much dependent on the expertise of the pathologists. The rapid urease test, such as the CLO test, can be useful as a rapid diagnostic method. However, these results can also be affected by the bacterial load<sup>[14]</sup>. A high proportion of the elderly population develops gastric atrophy and intestinal metaplasia, which can lead to a hostile environment for *H. pylori* and thus fewer bacteria and potentially a negative result. A detailed study in histological scoring is necessary for further study. Moreover, endoscopic tests, including the CLO test, culture and histological examination, can be affected in bleeding patients with peptic ulcers<sup>[10]</sup>. However, although the reason is not clear, all peptic ulcer cases in our study were not in the active bleeding phase, indicating that we do not need to consider any effects of bleeding.

The lowest infection rate was found in Thimphu, which is the capital city of Bhutan. The prevalence of *H. pylori* infection in Thimphu was significantly lower than that of the rural cities of Punakha and Wangdue. In Thimphu, the sanitary conditions are better than those of Punakha and Wangdue, which supports the possibility that sanitary conditions may be important factors for *H. pylori* infection. In fact, the prevalence of *H. pylori* infection was very low in individuals less than 10 years old (*i.e.*, approximately 5%) and increased with age in Japan<sup>[15]</sup>. Overall, it was higher among individuals born before 1950 and lower in those born thereafter. There was a rapid change in the sanitary conditions and standard of living in Japan after World War II, and clean public water systems were introduced in Japan in the 1950s. Therefore, sanitary conditions, such as a full equipment rate of water and sewage, are considered to be important factors for *H. pylori* infection<sup>[8]</sup>.

The prevalence of *H. pylori* in patients with peptic ulcers was significantly higher than that in patients with gastritis, which is consistent with previous reports<sup>[16-18]</sup>. This observation suggests that *H. pylori* infection is a risk factor for the development of peptic ulcers and gastric cancer, even in Bhutan. In addition, we found 5 gastric

cancer patients among the 372 volunteers. The ASR of gastric cancer in Bhutan was reported to be 24.2/100000 based on the small number in the registry (114 cases) in 2008<sup>[4]</sup>. This observation supports the high incidence of gastric cancer in Bhutan.

In conclusion, the high incidence of gastric cancer in Bhutan may be attributed to the high prevalence of *H. pylori* infection. Therefore, the eradication therapy of *H. pylori* can contribute to a decrease in *H. pylori*-related diseases, such as peptic ulcers and gastric cancer. However, we should be cautious regarding eradication therapy in Bhutan. Even when eradication therapy for *H. pylori* has succeeded, the infection frequently recurs in patients in developing countries, where there is a high prevalence of *H. pylori* infection<sup>[19]</sup>. Such repeat infections are either due to a recurrence of the original infection or reinfection with a new strain. Improving the sanitary conditions to decrease the prevalence of *H. pylori* in Bhutan is important.

## ACKNOWLEDGMENTS

We thank Kudo Y, Yano K and Chaithongrat S for their technical assistance.

## COMMENTS

### Background

Bhutan is a small landlocked country in South Asia and the prevalence of *Helicobacter pylori* (*H. pylori*) infection in Bhutan has not been investigated.

### Research frontiers

The prevalence of *H. pylori* in Bhutan was 73.4% and the high incidence of gastric cancer and peptic ulcer disease in Bhutan may be attributed to the high prevalence of *H. pylori* infection.

### Innovations and breakthroughs

This is the first study exploring the extremely high prevalence of *H. pylori* infection in Bhutan. Many tests for *H. pylori* detection such as serological test rapid urease test, culture, histology and immunohistochemistry were performed and analyzed.

### Applications

The study results suggest that high incidence of gastric cancer and peptic ulcer disease in Bhutan may be attributed to the high prevalence of *H. pylori* infection. Therefore, *H. pylori* eradication therapy can contribute to reduce these *H. pylori*-related diseases.

### Peer review

This is a study in which authors analyzed the prevalence of *H. pylori* infection in different cities, age group and each disease. The results are very interesting and suggest that *H. pylori* eradication and improve of sanitation should be considered to reduce *H. pylori* infection and related diseases in Bhutan.

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## Prognostic value of preoperative mean corpuscular volume in esophageal squamous cell carcinoma

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Received: December 17, 2012 Revised: March 1, 2013

Accepted: March 21, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To evaluate whether preoperative mean corpuscular volume (MCV) is a prognostic indicator in patients with resectable esophageal squamous cell carcinoma (ESCC).

**METHODS:** A total of 298 consecutive, prospectively enrolled patients with histologically diagnosed ESCC who underwent surgery with curative intent from 2001 to 2011 were retrospectively evaluated. Patients were excluded if they had previous malignant disease, distant metastasis at the time of primary treatment, a history of neoadjuvant treatment, had undergone non-radical resection, or had died of a non-tumor-associated

cause. Survival status was verified in September 2011. Pathological staging was performed based on the 2010 American Joint Committee on Cancer criteria. Preoperative MCV was obtained from blood counts performed routinely within 7 d prior to surgery. Receiver operating characteristic (ROC) curve analysis was used to determine a cutoff for preoperative MCV.

**RESULTS:** The 298 patients consisted of 230 males and 68 females, with a median follow-up of 30.1 mo. ROC analysis showed an optimal cutoff for preoperative MCV of 95.6 fl. Fifty-nine patients (19.8%) had high (> 95.6 fl) and 239 (80.2%) had low ( $\leq$  95.6 fl) preoperative MCV. Preoperative MCV was significantly associated with gender ( $P = 0.003$ ), body mass index ( $P = 0.017$ ), and preoperative red blood cell count ( $P < 0.001$ ). The predicted 1-, 3- and 5-year overall survival (OS) rates were 72%, 60% and 52%, respectively. Median OS was significantly longer in patients with low than with high preoperative MCV (27.5 mo vs 19.4 mo,  $P < 0.001$ ). Multivariate analysis showed that advanced pT ( $P = 0.018$ ) and pN ( $P < 0.001$ ) stages, upper thoracic location ( $P = 0.010$ ), lower preoperative albumin concentration ( $P = 0.002$ ), and high preoperative MCV ( $P = 0.001$ ) were negative prognostic factors in patients with ESCC. Preoperative MCV also stratified OS in patients with T3, N1-N3, G2-G3 and stage III tumors.

**CONCLUSION:** Preoperative MCV is a prognostic factor in patients with ESCC.

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**Key words:** Preoperative markers; Mean corpuscular volume; Prognosis; Resectable; Esophageal neoplasms

**Core tip:** Elevated mean corpuscular volume (MCV) has been shown to predict the risk of esophageal squamous cell carcinoma (ESCC). We hypothesized that pretreatment MCV could predict prognosis. In analyzing 298 patients with ESCC, we found that the optimal cut-off

for preoperative MCV was 95.6 fl. Multivariate analysis showed that high (> 95.6 fl) preoperative MCV was a negative prognostic factor, along with advanced stage, upper thoracic location and lower preoperative albumin, in patients with ESCC. Median overall survival was significantly longer in patients with low ( $\leq$  95.6 fl) than high preoperative MCV (27.5 mo *vs* 19.5 mo,  $P < 0.001$ ).

Zheng YZ, Dai SQ, Li W, Cao X, Li Y, Zhang LJ, Fu JH, Wang JY. Prognostic value of preoperative mean corpuscular volume in esophageal squamous cell carcinoma. *World J Gastroenterol* 2013; 19(18): 2811-2817 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2811.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2811>

## INTRODUCTION

Elevated mean corpuscular volume (MCV) has long been recognized as a biomarker for alcoholic and folate deficient patients<sup>[1-3]</sup>. Although the nature of the relationship between them remains unclear, recent reports suggested that alcohol-induced folate deficiency can lead to macrocytosis<sup>[4]</sup>. In addition, MCV was found to be higher in Asian heavy drinkers with inactive aldehyde dehydrogenase-2 (ALDH2)<sup>[5,6]</sup> and to be a marker for alcohol abuse with inactive heterozygous ALDH2<sup>[7,8]</sup>, suggesting that acetaldehyde is an important contributor to macrocytosis.

Alcohol abuse, and acetaldehyde and folate deficiency, all indicative of poor physical condition, were found to increase susceptibility to esophageal carcinoma<sup>[3,9-12]</sup>, as was macrocytosis<sup>[7,13]</sup>. In addition, patients with more advanced malignancies frequently present with more severe hematological anomalies<sup>[14,15]</sup>. These findings led us to hypothesize that pretreatment MCV may predict the prognosis of patients with esophageal carcinoma. We therefore analyzed the association between preoperative MCV and different clinicopathological parameters, as well as the prognostic significance of preoperative MCV in patients with esophageal squamous cell carcinoma (ESCC).

## MATERIALS AND METHODS

### Patients selection

This study was a retrospective analysis of a prospectively collected database (2001-2011) of 298 consecutive patients with histologically diagnosed ESCC who underwent surgery with curative intent at the Cancer Center of Sun Yat-Sen University, Guangzhou, China. Patients with previous malignancy, distant metastasis, neoadjuvant treatment, non-radical resection (R1/R2), or non-tumor-associated death were excluded. Tumors were pathologically staged using the American Joint Committee on Cancer (2010) staging system. Patients were followed-up in the outpatient clinic every 3-6 mo during the first 3 years and every 12 mo thereafter. Demography and clinical details were extracted from the database (Table 1). Survival status

was verified in September 2011 using the best available methods. The study protocol was approved by the medical ethics committee of the Cancer Center of Sun Yat-Sen University, which waived the requirement for informed consent due to the retrospective nature of the study.

### Preoperative MCV

Preoperative MCV was determined from preoperative blood counts, performed routinely within 7 d prior to surgery, using a Beckman Counter blood analyzer (version STKS, Beckman Counter Inc., Fullerton, CA, United States). The cut-off for preoperative MCV was defined by receiver operating characteristic (ROC) curve analysis, with the point maximizing the area under the curve being selected.

### Statistical analysis

All statistical analysis were performed using the SPSS 19.0 software package (SPSS, Inc., Chicago, IL, United States). The ROC curve was generated and analyzed using MedCalc statistical software package 11.0.1 (MedCalc Software bvba, Mariakerke, Belgium). Correlations between preoperative MCV and clinicopathological characteristics were assessed using the Pearson's  $\chi^2$  test. Overall survival (OS) was defined as the interval from the date of surgery to the date of death, or last follow-up. Multivariate Cox regression analysis was performed for all parameters found to be significant by the univariate analysis. Survival was analyzed using the Kaplan-Meier method, and differences between curves were assessed by the Log-Rank test. Statistical significance was defined as a  $P$  value < 0.05.

## RESULTS

### Patient baseline characteristics and preoperative MCV

The 298 patients consisted of 230 males and 68 females, with a median preoperative MCV of 91.0 fl (range: 61.4-112.4 fl). ROC curve analysis showed that the optimal cut-off point maximizing (0.588) was 95.6 fl ( $P = 0.0123$ ), with a sensitivity of 0.867 and a specificity of 0.324. Using this cut-off, 59 patients (19.8%) had high (> 95.6 fl) and 239 (80.2%) had low ( $\leq$  95.6 fl) preoperative MCV. The correlations between preoperative MCV and clinicopathologic parameters are summarized in Table 1. Preoperative MCV was significantly associated with gender ( $P = 0.003$ ), body mass index (BMI) ( $P = 0.017$ ), and preoperative red blood cell (RBC) count ( $P < 0.001$ ; Figure 1).

### Survival and preoperative MCV

Over a median follow-up of 30.1 mo, 102 of the 298 patients (34.2%) died of cancer-related causes, whereas the other 196 (65.8%) survived. The median survival time was 25.8 mo (range: 1.6-116.1 mo), and the predicted 1-, 3- and 5-year OS rates after primary surgery were 72%, 60%, and 52% respectively. Median OS was significantly longer in patients with low than high preoperative MCV (27.5 mo *vs* 19.4 mo,  $P < 0.001$ ; Figure 2).

To determine factors independently prognostic of pa-

**Table 1** Clinicopathological parameters and preoperative mean corpuscular volume *n* (%)

| Characteristics                                       | Case numbers      | Preoperative MCV |           | <i>P</i> value<br>Pearson's $\chi^2$ test |
|---|-------------------|------------------|-----------|---|
|   |                   | Low              | High      |   |
| Age, yr (mean $\pm$ SE)                               | 58.2 $\pm$ 9.2    |                  |           |   |
| $\leq$ 65   | 231               | 184 (79.7)       | 47 (20.3) |   |
| > 65  | 67                | 55 (82.1)        | 12 (17.9) | 0.660                                     |
| Gender  |                   |                  |           |   |
| Male  | 230               | 176 (76.5)       | 54 (23.5) |   |
| Female  | 68                | 63 (92.6)        | 5 (7.4)   | 0.003                                     |
| BMI, kg/m <sup>2</sup> (mean $\pm$ SE)                | 22.3 $\pm$ 3.2    |                  |           |   |
| $\leq$ 20   | 65                | 46 (70.8)        | 19 (29.2) |   |
| > 20 and $\leq$ 25                                    | 180               | 144 (80.0)       | 36 (20.0) |   |
| > 25  | 53                | 49 (92.5)        | 4 (7.5)   | 0.017                                     |
| Smoking index   | 440.1 $\pm$ 483.1 |                  |           |   |
| $\leq$ 400  | 171               | 141 (82.5)       | 30 (17.5) |   |
| > 400   | 127               | 98 (77.2)        | 29 (22.8) | 0.257                                     |
| Preoperative RBC, $\times 10^{12}$ /L (mean $\pm$ SE) | 4.5 $\pm$ 0.6     |                  |           |   |
| $\leq 4.0^1$  | 56                | 31 (55.4)        | 25 (44.6) |   |
| > 4.0   | 242               | 208 (86.0)       | 34 (14.0) | < 0.001                                   |
| Preoperative albumin, g/L (mean $\pm$ SE)             | 42.9 $\pm$ 4.6    |                  |           |   |
| $\leq 43^2$   | 149               | 115 (77.2)       | 34 (22.8) |   |
| > 43  | 149               | 124 (83.2)       | 25 (16.8) | 0.191                                     |
| pT status, UICC <sup>7th</sup> (mean $\pm$ SE)        |                   |                  |           |   |
| T1  | 33                | 31 (93.9)        | 2 (6.1)   |   |
| T2  | 52                | 44 (84.6)        | 8 (15.4)  |   |
| T3  | 213               | 164 (77.0)       | 49 (23.0) | 0.051                                     |
| N0  | 138               | 116 (84.1)       | 22 (15.9) |   |
| N1  | 89                | 67 (75.3)        | 22 (24.7) |   |
| N2  | 51                | 42 (82.4)        | 9 (17.6)  |   |
| N3  | 20                | 14 (70.0)        | 6 (30.0)  | 0.250                                     |
| Histologic grade                                      |                   |                  |           |   |
| G1  | 94                | 71 (75.5)        | 23 (24.5) |   |
| G2  | 156               | 127 (81.4)       | 29 (18.6) |   |
| G3  | 48                | 41 (85.4)        | 7 (14.6)  | 0.324                                     |
| pTNM stage (UICC <sup>7th</sup> )                     |                   |                  |           |   |
| Stage I   | 37                | 32 (86.5)        | 5 (13.5)  |   |
| Stage II  | 120               | 100 (83.3)       | 20 (16.7) |   |
| Stage III   | 141               | 107 (75.9)       | 34 (24.1) | 0.191                                     |
| Tumor location  |                   |                  |           |   |
| Upper   | 48                | 37 (77.1)        | 11 (22.9) |   |
| Middle  | 150               | 125 (83.3)       | 25 (16.7) |   |
| Lower   | 100               | 77 (77.0)        | 23 (23.0) | 0.393                                     |

<sup>1</sup>Normal limit of red blood cell count; <sup>2</sup>Mean value of preoperative hemoglobin. MCV: Mean corpuscular volume; Low: Low preoperative MCV ( $\leq 95.6$  fl); High: High preoperative MCV ( $> 95.6$  fl); BMI: Body mass index; RBC: Red blood cell; UICC: Union for International Cancer Control.

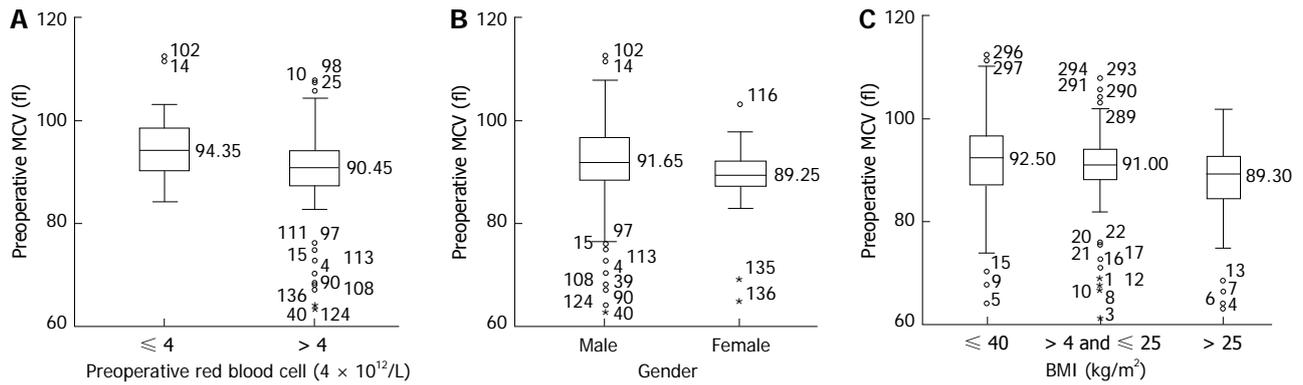
tient survival, we analyzed OS using a Cox proportional hazards model. All parameters found to be potentially significant in univariate analysis were included in a multivariate analysis. We found that pT status ( $P = 0.018$ ), pN status ( $P < 0.001$ ), tumor location ( $P = 0.010$ ), preoperative albumin concentration ( $P = 0.002$ ), and preoperative MCV ( $P = 0.001$ ) were significantly prognostic of survival in this patient cohort (Table 2). When we analyzed the effect of preoperative MCV on OS in patients classified by clinicopathological factors, preoperative MCV was predictive of OS in patients with T3 ( $P < 0.001$ ), N1-N3 ( $P < 0.001$ ), G2-G3 ( $P < 0.001$ ), and stage III ( $P = 0.001$ ) tumors (Figure 2 and Table 3).

## DISCUSSION

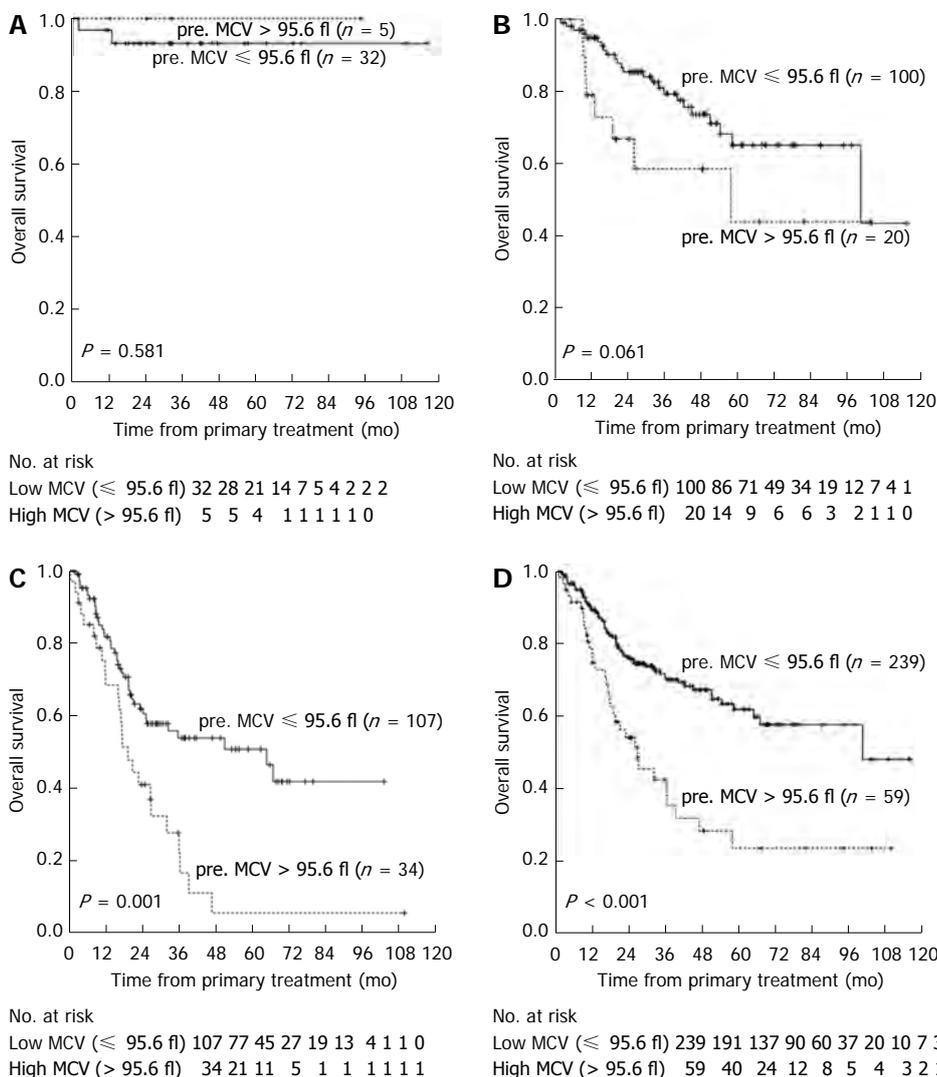
Hematologic parameters have been reported to correlate significantly with prognosis in patients with advanced

malignant disease<sup>[15-18]</sup>. MCV is considered a sensitive indicator of alcohol abuse and folate deficiency<sup>[1,2,4,6,19]</sup>. Recently, MCV was found to be a biomarker for alcohol abuse accompanied by inactive heterozygous ALDH2, and also allowed for the prediction of ESCC risk<sup>[8]</sup>. To our knowledge, however, no previous study has assessed the relationship between MCV and the prognosis of patients with ESCC.

Using ROC curve analysis, we found that a cut-off of 95.6 fl was a statistically significant predictor of OS. Moreover, high ( $> 95.6$  fl) MCV was significantly correlated with male gender, lower BMI, and RBC  $\leq 4 \times 10^{12}$ /L. Folate deficiency has been shown to inhibit red cell maturation, as well as increasing erythrocyte fragility, resulting in increased hemolysis and lower RBC count, which consequently results in macrocytosis<sup>[20]</sup>. Lower BMI may accompany poor nutritional status, which was associated with elevated MCV<sup>[13,21]</sup>. The significant correlation



**Figure 1** Box plot of preoperative mean corpuscular volume stratified by preoperative red blood cell count (A), gender (B) and body mass index (C). MCV: Mean corpuscular volume; BMI: Body mass index.



**Figure 2** Kaplan-Meier estimates of the probability of overall survival according to preoperative mean corpuscular volume in stage I cohort (A), stage II cohort (B), stage III cohort (C), and all cohorts (D). pre. MCV: Preoperative mean corpuscular volume.

between high MCV and male gender may be related to the association between macrocytosis and alcohol abuse, since overdrinking is much more frequent in males than in females<sup>[6-8]</sup>. Furthermore, MCV tended to be associated with pT status ( $P = 0.051$ ), consistent with findings showing that preoperative MCV may provide a complementary advantage in assessing tumor invasiveness<sup>[17]</sup>.

Although TNM stage is the best predictor of survival in cancer patients, OS may differ widely in patients with the same TNM stage tumors who receive the same treatment, suggesting that other, as yet undetermined factors may affect prognosis. Since preoperative hematologic parameters have been predictive of patient prognosis<sup>[15,22-25]</sup>, we performed univariate and multivariate analyses of

**Table 2** Univariate and multivariate Cox regression analysis for overall survival

| Factors  | Univariate analysis |                      | Multivariate analysis |                      |
|--|---------------------|----------------------|-----------------------|----------------------|
|  | HR (95%CI)          | P value <sup>1</sup> | HR (95%CI)            | P value <sup>1</sup> |
| Age, yr  | 1.007 (0.985-1.029) | 0.527                |                       |                      |
| Gender (male <i>vs</i> female)   | 0.744 (0.464-1.196) | 0.222                |                       |                      |
| Smoking index ( $\leq 400$ <i>vs</i> $> 400$ )                                     | 1.391 (0.940-2.060) | 0.099                | 1.302 (0.874-1.940)   | 0.194                |
| BMI, kg/m <sup>2</sup> ( $\leq 20$ <i>vs</i> $> 20$ ; $\leq 25$ <i>vs</i> $> 25$ ) | 0.878 (0.638-1.207) | 0.422                |                       |                      |
| Preoperative MCV, fl ( $\leq 95.6$ <i>vs</i> $> 95.6$ )                            | 2.495 (1.644-3.787) | $< 0.001$            | 2.108 (1.372-3.241)   | 0.001                |
| Preoperative RBC, $\times 10^{12}/L$ ( $\leq 4$ <i>vs</i> $> 4$ )                  | 0.685 (0.433-1.082) | 0.105                | 0.835 (0.507-1.350)   | 0.462                |
| Preoperative albumin, g/L  | 0.954 (0.919-0.990) | 0.012                | 0.938 (0.900-0.977)   | 0.002                |
| pT status (pT1 <i>vs</i> pT2 and pT3)  | 1.641 (1.147-2.348) | 0.007                | 1.589 (1.084-2.327)   | 0.018                |
| pN status (pN0 <i>vs</i> pN1, pN2 and pN3)   | 1.954 (1.603-2.382) | $< 0.001$            | 1.957 (1.602-2.392)   | $< 0.001$            |
| Histologic grade (G1 <i>vs</i> G2 and G3)  | 0.961 (0.714-1.293) | 0.791                |                       |                      |
| Tumor location<br>(upper thoracic <i>vs</i> middle thoracic and lower thoracic)    | 0.798 (0.605-1.051) | 0.109                | 0.692 (0.522-0.916)   | 0.01                 |

<sup>1</sup>Cox proportional hazards model. HR: Hazard ratio; BMI: Body mass index; MCV: Mean corpuscular volume; RBC: Red blood cell.

**Table 3** Comparison of prognosis in specified cohort stratified by preoperative mean corpuscular volume

| Variable         | Case numbers | Overall survival (mo)<br>(mean $\pm$ SE) | P value<br>Log-Rank test |
|------------------|--------------|--|--------------------------|
| All cohort       |              |  | $< 0.001$                |
| Low              | 239          | 33.7 $\pm$ 24.7                          |                          |
| High             | 59           | 25.8 $\pm$ 24.2                          |                          |
| pT status        |              |  |                          |
| T1-T2            |              |  | 0.075                    |
| Low              | 75           | 39.1 $\pm$ 27.1                          |                          |
| High             | 10           | 27.6 $\pm$ 22.7                          |                          |
| T3               |              |  | $< 0.001$                |
| Low              | 164          | 31.2 $\pm$ 23.2                          |                          |
| High             | 49           | 25.4 $\pm$ 24.7                          |                          |
| pN status        |              |  |                          |
| N0               |              |  | 0.464                    |
| Low              | 116          | 40.0 $\pm$ 26.0                          |                          |
| High             | 22           | 35.5 $\pm$ 28.7                          |                          |
| N1-N3            |              |  | $< 0.001$                |
| Low              | 123          | 27.9 $\pm$ 22.0                          |                          |
| High             | 37           | 20.0 $\pm$ 19.2                          |                          |
| Histologic grade |              |  |                          |
| G1               |              |  | 0.211                    |
| Low              | 71           | 32.9 $\pm$ 27.6                          |                          |
| High             | 23           | 27.4 $\pm$ 24.3                          |                          |
| G2-G3            |              |  | $< 0.001$                |
| Low              | 168          | 34.1 $\pm$ 23.4                          |                          |
| High             | 36           | 24.8 $\pm$ 24.4                          |                          |
| pTNM stage       |              |  |                          |
| Stage I          |              |  | 0.581                    |
| Low              | 32           | 37.9 $\pm$ 27.1                          |                          |
| High             | 5            | 37.6 $\pm$ 32.6                          |                          |
| Stage II         |              |  | 0.061                    |
| Low              | 100          | 39.6 $\pm$ 25.4                          |                          |
| High             | 20           | 31.1 $\pm$ 27.7                          |                          |
| Stage III        |              |  | 0.001                    |
| Low              | 107          | 27.0 $\pm$ 21.6                          |                          |
| High             | 34           | 20.9 $\pm$ 19.8                          |                          |

Low: Low preoperative mean corpuscular volume ( $\leq 95.6$  fl); High: High preoperative mean corpuscular volume ( $> 95.6$  fl).

factors predictive of OS in patients with ESCC. We found that pathological stage, tumor location, preoperative albumin concentration, and preoperative MCV were prognostic factors in our patient cohort.

We also found that OS was significantly shorter in patients with upper-thoracic cancer than those with middle and lower-thoracic esophageal cancer. A study of 605 patients with ESCC also found that median OS was significantly shorter in patients with upper thoracic cancer than in those with middle and lower thoracic tumors (45.9 mo *vs* 82.2 and 93.8 mo;  $P < 0.001$ )<sup>[26]</sup>. Due to their anatomical location, carcinomas of the upper thoracic esophagus often result in early invasion of adjacent structures and extensive lymph node metastasis<sup>[27]</sup>. The prognostic significance of preoperative albumin concentration may be due to it being a sensitive indicator of nutrition, liver function, and metabolic response to disease; thus patients with lower albumin concentrations may present with poorer physical status, decreasing both their response and tolerance to treatment<sup>[28,29]</sup>. Similar findings were reported in patients with adenocarcinoma of the gastric cardia<sup>[30]</sup>.

Although we found that preoperative MCV was prognostic in patients with ESCC, there is no evidence that MCV has a direct effect on tumor progression or patient prognosis. MCV, however, is a marker of internal folate concentration. Folate acts to transfer one-carbon moieties, thus playing a central role in DNA synthesis, replication, repair, and methylation<sup>[31]</sup>. Folate deficiency leads to aberrant DNA methylation, which has been reported to be a predictor of clinical outcome in patients with esophageal cancer<sup>[32]</sup>. A recent study of 125 ESCC patients who underwent surgical resection showed that median OS was significantly longer in patients with high than with low/moderate folate intake (4.59 years *vs* 3.06 years;  $P = 0.007$ )<sup>[33]</sup>. Similar results were reported in patients with advanced gastric cancer who were treated with chemotherapy<sup>[34]</sup>.

Another factor linking MCV with prognosis in ESCC is macrocytosis, which may be an indicator of malnutrition, a negative prognostic factor in various human cancers<sup>[21,35,36]</sup>. In addition, crystal osmotic pressure was shown to be a major regulator of red cell volume in internal environments<sup>[37]</sup>. Dysphagia, a frequently observed symptom in patients with advanced esophageal cancer,

restricts intake, thus reducing serum concentrations of electrolytes, glucose, and amino acids. This, in turn, may decrease crystal osmotic pressure, leading to red cell dilation. Our finding, that preoperative MCV was related to pT stage and BMI, was consistent with results suggesting that increased MCV was associated with tumor invasiveness and nutritional status<sup>[3,17]</sup>. Thus, taken together, these results suggest that preoperative MCV may be a marker reflecting internal folate concentration, nutritional status, and tumor invasiveness, thus comprehensively predicting prognosis in patients with ESCC. MCV assays are also convenient and inexpensive to perform, allowing for wide clinical application and suggesting that they may be crucial in preoperative assessment.

To further evaluate the prognostic significance of preoperative MCV, we performed subgroup analysis in patients with ESCC. We found that MCV resulted in the stratification of OS in patients with T3, N1-N3, G2-G3, and stage III tumors, but not in patients with T1-T2, N0, G1, or stage I / II tumors. These findings, however, may be due to the small sample size of these subgroups. Moreover, the relatively good prognosis in patients with T1/2, N0, G1, and stage I / II tumors may mask the significance of preoperative MCV.

This study has limitations and potential biases. Due to its retrospective nature, records of alcohol consumption by patients were incomplete and folic acid concentrations were not tested in most patients. Furthermore, we could not determine whether preoperative MCV was a better predictor of OS than conventional prognostic factors. Finally, our small sample size may reflect a selection bias to some extent.

In conclusion, in patients with resectable ESCC, OS was significantly longer in patients with low ( $\leq 95.6$  fl) than high ( $> 95.6$  fl) preoperative MCV. Additional studies, however, are required to validate our results.

## COMMENTS

### Background

Surgical resection remains the treatment of choice for patients with localized esophageal carcinoma. Routine preoperative blood tests of red blood cells, white blood cells, and platelet counts can help estimate surgical risk. Significant hematologic variations frequently observed in patients with advanced malignant diseases may predict prognosis.

### Research frontiers

Elevated mean corpuscular volume (MCV) has long been recognized as a biomarker for alcohol abuse and folate deficiency. In addition, MCV was reported to be higher in Asian heavy drinkers with inactive aldehyde dehydrogenase-2 (ALDH2), and was found to be a marker of alcohol abuse in individuals with inactive heterozygous ALDH2, suggesting that acetaldehyde may be an important contributor to macrocytosis. A recent study showed that macrocytosis was a risk factor for esophageal carcinoma.

### Innovations and breakthroughs

The authors observed a correlation between macrocytosis and prognosis in patients with esophageal carcinoma. Overall survival was significantly shorter in patients with elevated MCV than those with lower MCV. Utilizing receiver operating characteristic curve analysis, the authors determined an optimal cut-off point for MCV, which was both reasonable and objective.

### Applications

These results suggest that preoperative MCV may be used to predict prognosis in patients with esophageal cancer. Routine blood tests should be performed

shortly before surgery in these patients, and those with elevated MCV, especially greater than 95.6 fl, should be carefully evaluated to assess the risks and feasibility of surgery.

### Terminology

MCV, representing the mean volume of a single red blood cell, is determined by indirect calculation. Clinically, this parameter is often used in the differential diagnosis of various type of anemia.

### Peer review

This is an article on an unusual topic. The value of MCV has been known up to now as risk factor for esophageal carcinoma, but it is not known as prognostic factor.

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**P- Reviewers** Mann O, Kopacova M **S- Editor** Zhai HH  
**L- Editor** Rutherford A **E- Editor** Li JY



## Increased CD163 expression is associated with acute-on-chronic hepatitis B liver failure

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Supported by Grants from Key Project of Chinese Ministry of Science and Technology, No. 2012ZX10002007 and No. 2013ZX10002001; National Natural Science Foundation of China, No. 81171579 and No. 81201287; and Natural Science Foundation of Shandong Province, No. ZR2010HM070 and No. ZR2010HQ040

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Received: November 13, 2012 Revised: March 14, 2013

Accepted: March 23, 2013

Published online: May 14, 2013

### Abstract

**AIM:** To assess CD163 expression in plasma and peripheral blood and analyze its association with disease in acute-on-chronic hepatitis B liver failure (ACHBLF) patients.

**METHODS:** A retrospective study was conducted from January 1, 2011 to January 1, 2012. Forty patients with ACHBLF (mean age  $44.48 \pm 12.28$  years, range 18-69 years), 40 patients with chronic hepatitis B (CHB) (mean age  $39.45 \pm 12.22$  years, range 21-57 years) and 20 age- and sex-matched healthy controls (mean age  $38.35 \pm 11.97$  years, range 28-60 years) were included in this study. Flow cytometry was used to analyze the frequency of CD163+ peripheral blood mononuclear cells (PBMCs) and surface protein expression of CD163. Real-time transcription-polymerase chain re-

action was performed to assess relative CD163 mRNA levels in PBMCs. Plasma soluble CD163 (sCD163) levels were measured by enzyme-linked immunosorbent assay. Clinical variables were also recorded. Comparisons between groups were analyzed by Kruskal-Wallis *H* test and Mann-Whitney *U* test. Statistical analyses were performed using SPSS 15.0 software and a *P* value < 0.05 was considered statistically significant.

**RESULTS:** Flow cytometry showed that the population of CD163+ PBMCs was significantly greater in ACHBLF patients than in CHB patients and healthy controls ( $47.9645\% \pm 17.1542\%$ ,  $32.0975\% \pm 11.0215\%$  vs  $17.9460\% \pm 6.3618\%$ ,  $P < 0.0001$ ). However, there were no significant differences in mean fluorescence intensity of CD163+ PBMCs within the three groups ( $27.4975 \pm 11.3731$ ,  $25.8140 \pm 10.0649$  vs  $20.5050 \pm 6.2437$ ,  $P = 0.0514$ ). CD163 mRNA expression in ACHBLF patients was significantly increased compared with CHB patients and healthy controls ( $1.41 \times 10^{-2} \pm 2.18 \times 10^{-2}$ ,  $5.10 \times 10^{-3} \pm 3.61 \times 10^{-3}$  vs  $37.0 \times 10^{-4} \pm 3.55 \times 10^{-4}$ ,  $P = 0.02$ ). Plasma sCD163 levels in patients with ACHBLF were significantly increased compared with CHB patients and healthy controls ( $4706.2175 \pm 1681.1096$  ng/mL,  $1089.7160 \pm 736.8395$  ng/mL vs  $435.9562 \pm 440.8329$  ng/mL,  $P < 0.0001$ ). In ACHBLF patients, plasma sCD163 levels were significantly positively associated with model for end-stage liver disease scores ( $r = 0.5075$ ,  $P = 0.008$ ), hepatitis B virus-DNA ( $r = 0.6827$ ,  $P < 0.0001$ ), and negatively associated with prothrombin activity ( $r = -0.3348$ ,  $P = 0.0347$ ), but had no correlation with total bilirubin ( $r = 0.2551$ ,  $P = 0.1122$ ). Furthermore, sCD163 was obviously elevated in non-surviving patients compared with surviving patients with ACHBLF ( $5344.9080 \pm 1589.5199$  ng/mL vs  $3641.7333 \pm 1264.5228$  ng/mL,  $P = 0.0321$ ).

**CONCLUSION:** CD163 and sCD163 may be related to disease severity and prognosis in ACHBLF patients.

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**Key words:** Acute-on-chronic hepatitis B liver failure; Model for end-stage liver disease; CD163; Soluble CD163; Real-time transcription-polymerase chain reaction

**Core tip:** This study included three groups, acute-on-chronic hepatitis B liver failure (ACHBLF) patients, chronic hepatitis B (CHB) patients and healthy controls. Flow cytometry was used to analyze the frequency of CD163+ peripheral blood mononuclear cells (PBMCs) and surface protein expression of CD163. Real-time transcription-polymerase chain reaction was performed to assess relative CD163 mRNA levels in PBMCs. The population of CD163+ PBMCs was significantly larger in ACHBLF patients than in CHB patients and healthy controls. CD163 mRNA expression in ACHBLF patients was significantly increased compared with healthy controls. Plasma soluble CD163 (sCD163) levels were markedly increased and correlated with disease severity and prognosis in ACHBLF patients. CD163 and sCD163 may be useful biomarkers for ACHBLF.

Ye H, Wang LY, Zhao J, Wang K. Increased CD163 expression is associated with acute-on-chronic hepatitis B liver failure. *World J Gastroenterol* 2013; 19(18): 2818-2825 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2818.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2818>

## INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem worldwide. It is thought to be one of the main causes of liver-related chronic and acute diseases<sup>[1]</sup>. With severe acute exacerbation of the disease, some chronic hepatitis B (CHB) patients may progress to liver failure. We define this progression as acute-on-chronic hepatitis B liver failure (ACHBLF), and constitutes about 70% of all acute-on-chronic liver failure in areas with a high incidence of hepatitis B<sup>[2]</sup>. ACHBLF has an extremely poor prognosis due to a lack of understanding of its pathogenesis. Currently, clinical observations have shown that ACHBLF may be related to strong immune responses. Therefore, it is important to fully understand the course of the immune pathogenesis of ACHBLF. If we can identify efficient markers which predict disease progression, this may be of great help in the treatment of ACHBLF<sup>[3]</sup>.

Innate immune cells such as macrophages can be activated by acute and chronic inflammation and produce various cytokines. These immunological cytokines induce and cause liver tissue injury. Activated macrophages play important roles in the production of these immune cytokines. Monocytes and macrophages can be activated into M1 and M2 subpopulations in response to different environmental signals which are mainly derived from inflammatory diseases. Those of the M2 subpopulation inhibit the inflammatory state<sup>[4-6]</sup>.

CD163 is a member of a scavenger receptor family and is expressed mainly on activated macrophages.

It is a specific M2 macrophage marker. Soluble CD163 (sCD163) emerges from the shedding of CD163 from the cell surface in the plasma<sup>[6-9]</sup>. It is now evident that sCD163 and CD163 are very useful as biomarkers of macrophage activation in various inflammatory diseases. It is strongly indicated that CD163 and sCD163 may be involved in the pathogenesis of liver failure. However, the exact expression of CD163 and sCD163 in ACHBLF patients has not been fully elucidated<sup>[10,11]</sup>.

This study aimed to evaluate peripheral blood CD163 and plasma sCD163 expression in macrophages from patients with ACHBLF.

## MATERIALS AND METHODS

### Patients

Forty patients with ACHBLF, 40 patients with CHB and 20 healthy controls from Qilu Hospital of Shandong University, were included in this retrospectively study.

Blood samples collected from January 2011 to January 2012 at the Department of Hepatology, Qilu Hospital of Shandong University, were separated into plasma and stored at -70 °C until use.

Patients with CHB had more than twice the normal alanine aminotransferase level. All groups were matched for sex and age. ACHBLF patients had a history of CHB, with plasma total bilirubin (TBIL)  $\geq 85$   $\mu\text{mol/L}$ , prothrombin activity (PTA)  $< 40\%$ , and complications such as hepatic encephalopathy (no less than grade II), ascites or hepato-renal syndrome. According to the Asian Pacific Association for the Study of the Liver guideline, all ACHBLF patients received inpatient treatment. We excluded patients who underwent liver transplantation, had hepatocellular carcinoma or other metastatic liver tumors or who had received immunotherapy or anti-viral treatment within 6 mo. Patients with a history of alcohol abuse, intravenous drug abuse, pregnancy, concomitant chronic hepatitis C, human immune deficiency virus infection or autoimmune hepatitis were also excluded. Hepatitis B surface antigen in healthy controls ( $n = 20$ ) (age-, sex- and race-matched) was negative. Experiments and procedures were conducted with the guidance of the Helsinki Declaration of 1975<sup>[12]</sup>. The study was approved by the local Ethical Committee of Qilu Hospital of Shandong University. Prior to the collection of blood, informed consent was obtained from each patient. The characteristics of the enrolled subjects are summarized in Table 1.

### RNA extraction and real-time reverse-transcriptase polymerase chain reaction

Six milliliters of peripheral venous blood were collected from each subject and Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) was used for isolation of PBMCs. Total RNA in PBMCs was extracted using Trizol (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. Two micrograms of total RNA were converted into cDNAs using the RevertAid™ First

**Table 1** Baseline characteristics of the subjects enrolled in the present study

| Patients                             | ACHBLF          | CHB           | Healthy control |
|--------------------------------------|-----------------|---------------|-----------------|
| Cases                                | 40              | 40            | 20              |
| Age, yr                              | 44.48 ± 12.28   | 39.45 ± 12.22 | 38.35 ± 11.97   |
| Sex (male/female)                    | 23/17           | 26/14         | 12/8            |
| HBeAg (+)/HBeAg (-)                  | 19/21           | 28/12         | NA              |
| PTA, %                               | 45.36 ± 25.14   | 98.70 ± 17.56 | 108.00 ± 13.56  |
| TBIL, μmol/L                         | 322.08 ± 166.00 | 33.39 ± 66.35 | 18.65 ± 6.55    |
| HBV DNA, log <sub>10</sub> copies/mL | 322.08 ± 166.00 | 3.20 ± 1.44   | NA              |
| Encephalopathy grade III/IV          | 12.12%          | 0%            | 0%              |
| Ascites                              | 30.30%          | 0%            | 0%              |
| Mortality                            | 62.5%           | 0%            | 0%              |

ACHBLF: Acute-on-chronic hepatitis B liver failure; CHB: Chronic hepatitis B; HBeAg: Hepatitis B e antigen; PTA: Prothrombin activity; TBIL: Total bilirubin; HBV: Hepatitis B virus; NA: Not available.

Strand cDNA Synthesis Kit (Fermentas, Lithuania) and real-time polymerase chain reaction (RT-PCR) was carried out on a Lightcycler (Roche Diagnostics, Germany). RT-PCR amplification mixtures (20 μL) contained 75 ng template cDNA, 10 μL 2 9 SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Takara, Japan) and 200 nmol/L forward and reverse primer. The real-time PCR reaction was performed as follows: the initial step was 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. β-actin was used as the endogenous control for the normalization of RNA quantity and quality differences in all samples.

The primers were as follows: CD163 forward 5'-TCTGGCTTGACAGCGTTTC-3'; CD163 reverse 5'-TGTGTTTGTTCCTGGATT-3'; β-actin forward 5'-CGGGAAATCGTGCGTGACATT-3'; β-actin reverse 5'-GGAGTTGAAGGTAGTTTCGTGG-3' (Fermentas, Lithuania). Gene specific amplifications were demonstrated with melting curve data and gel-migration analyses.

### Flow cytometric analysis

Freshly drawn peripheral whole blood samples of 200 μL were stained with isotype-matched control antibody or a relevant antibody (CD14, CD163) for 30 min at room temperature in the dark. Anti-human CD163 PE, anti-human CD14 APC and isotype-matched control antibody were purchased from eBioscience (San Diego, CA, United States). Following incubation, erythrocytes were lysed with RBC lysis buffer (Whole Blood Lysing Kit, R and D Systems, United States). Finally, the cells were washed three times, resuspended in 500 μL of PBS containing 0.5% formaldehyde, and analyzed using a FACS Calibur (BD Bioscience, PharMingen). The samples were analyzed with a Becton-Dickinson FACS Calibur machine. Separate gates were established for the macrophages. The amount of CD163 in peripheral blood was assessed using flow cytometry (Coulter counter)<sup>[13-15]</sup> (Figure 1).

### Determination of plasma levels of sCD163

SCD163 was determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R and D, Minneapolis, MN, United States). All

samples were analyzed in duplicate following the manufacturer's protocol. The sensitivity of the assay was 0.1 ng/mL, and the intra-assay and inter-assay coefficients of variation were less than 3% and 6%, respectively.

### Clinical and laboratory parameters

Measurement of liver function tests: TBIL and hematological tests including PTA were performed using standard methods in a clinical setting. Hepatitis B markers were tested using a commercially available radioimmunoassay (Abbott). The level of HBV DNA was quantified using a DNA assay (sensitivity > 500 copies/mL). Model for end-stage liver disease (MELD) scores were calculated according to the Malinchoc formula:  $r = 9.57 \times \log_e [\text{creatinine (mg/dL)}] + 3.78 \times \log_e [\text{bilirubin (mg/dL)}] + 11.2 \times \log_e (\text{INR}) + 6.43 \times (\text{etiology: } 0 \text{ if cholestatic or alcoholic, } 1 \text{ otherwise})$ <sup>[16,17]</sup>.

### Statistical analysis

Statistical analysis were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, United States). Comparisons between groups were analyzed by Kruskal-Wallis *H* test and Mann-Whitney *U* test. The Spearman rank correlation test was used for correlation analysis. All statistical analysis were two-sided, and a *P* value < 0.05 was considered statistically significant.

## RESULTS

### Frequency of circulating CD163+ PBMCs and mean fluorescence intensity

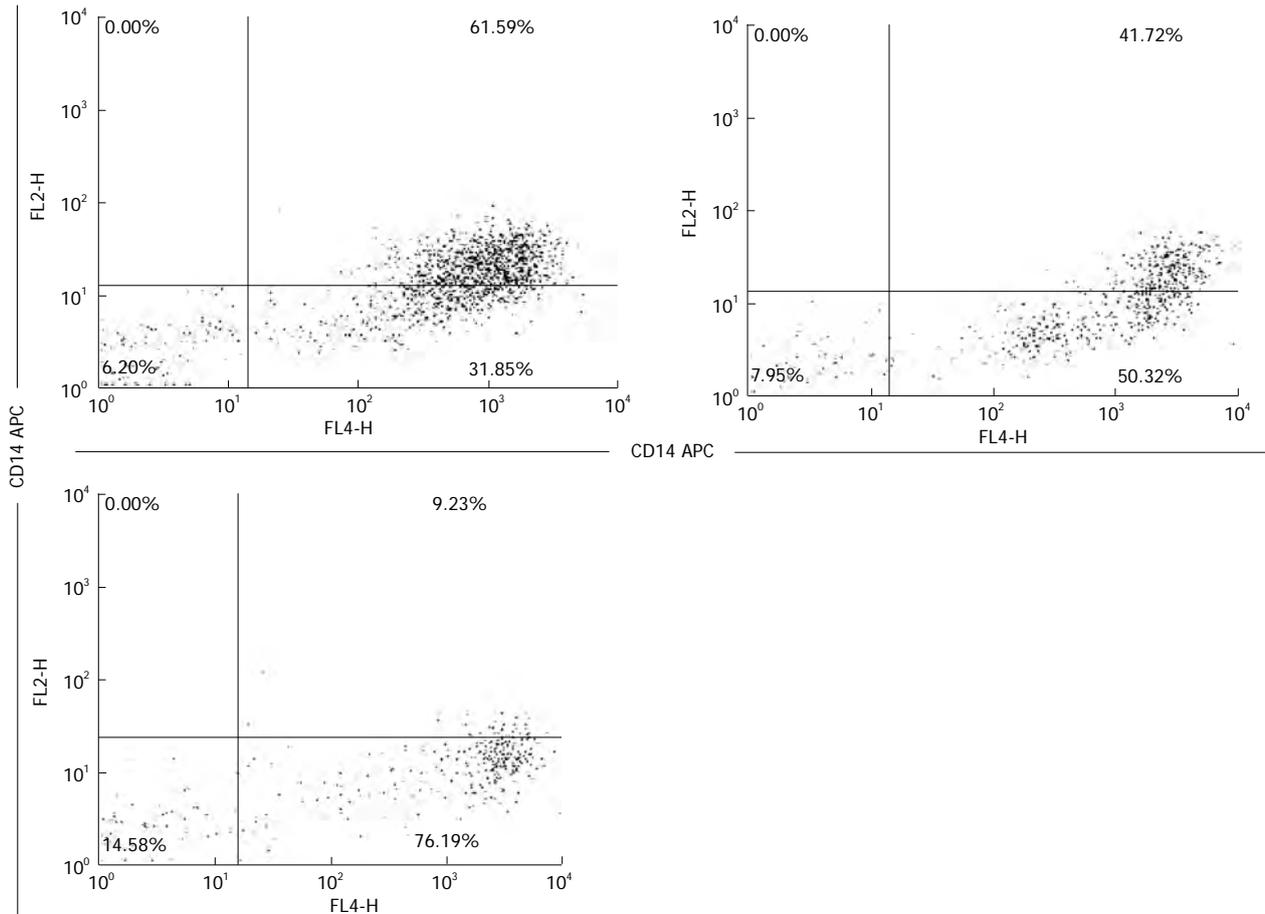
We determined the frequency of CD163+ PBMCs using flow cytometry and found that in ACHBLF patients, the frequency was markedly higher than that in the healthy control group and CHB patients, respectively (Figure 2A). There was a significant difference in the frequency of CD163+ PBMCs within the three groups (47.9645% ± 17.1542%, 32.0975% ± 11.0215% *vs* 17.9460% ± 6.3618%, *P* < 0.0001). We also evaluated the average mean fluorescence intensity (MFI) of CD163+ PBMCs using flow cytometric analysis. However, there were no significant differences in MFI in CD163 + PBMCs within the three groups (27.4975 ± 11.3731, 25.8140 ± 10.0649 *vs* 20.5050 ± 6.2437, *P* = 0.0514) (Figure 2B).

### Plasma levels of sCD163 in ACHBLF patients

We evaluated the plasma levels of sCD163 by ELISA. The results showed that the levels of sCD163 in ACHBLF patients were markedly higher than those in CHB patients and the healthy control group. Significant differences in plasma sCD163 were found within ACHBLF patients, CHB patients and healthy controls (4706.2175 ± 1681.1096 ng/mL, 1089.7160 ± 736.8395 ng/mL *vs* 435.9562 ± 440.8329 ng/mL, *P* < 0.0001) (Figure 2C).

### Increased mRNA expression of CD163 in PBMCs from ACHBLF patients

We also determined the mRNA level of CD163 in PBMCs



**Figure 1** Percentage of CD163+ peripheral blood mononuclear cells. The representative results of CD163+ peripheral blood mononuclear cells are shown. ACH-BLF: Acute-on-chronic hepatitis B liver failure; CHB: Chronic hepatitis B.

using RT-PCR. No significant differences in the mRNA level of CD163 were found within the three groups ( $1.41 \times 10^{-2} \pm 2.18 \times 10^{-2}$ ,  $5.10 \times 10^{-3} \pm 3.61 \times 10^{-3}$  vs  $37.0 \times 10^{-4} \pm 3.55 \times 10^{-4}$ ,  $P = 0.02$ ). The mRNA levels of CD163 in ACHBLF patients and CHB patients were significantly higher than those in healthy controls. No significant difference in the mRNA level of CD163 was observed in ACH-BLF and CHB patients ( $P > 0.05$ ) (Figure 2D).

#### **Increased plasma sCD163 levels in patients with ACHBLF**

To determine whether the increase in plasma levels of sCD163 correlated with liver injury, we analyzed the plasma levels of sCD163 in ACHBLF patients with clinical and laboratory parameters which indicated liver function or DNA replication in ACHBLF. The results showed that plasma levels of sCD163 were positively correlated with MELD score ( $r = 0.5075$ ,  $P = 0.008$ ), plasma HBV-DNA levels ( $r = 0.6827$ ,  $P < 0.0001$ ) and negatively correlated with PTA ( $r = -0.3348$ ,  $P = 0.0347$ ), but had no correlation with TBIL ( $r = 0.2551$ ,  $P = 0.1122$ ) (Figure 3A-D).

#### **Plasma levels of sCD163 influence disease progression in ACHBLF patients**

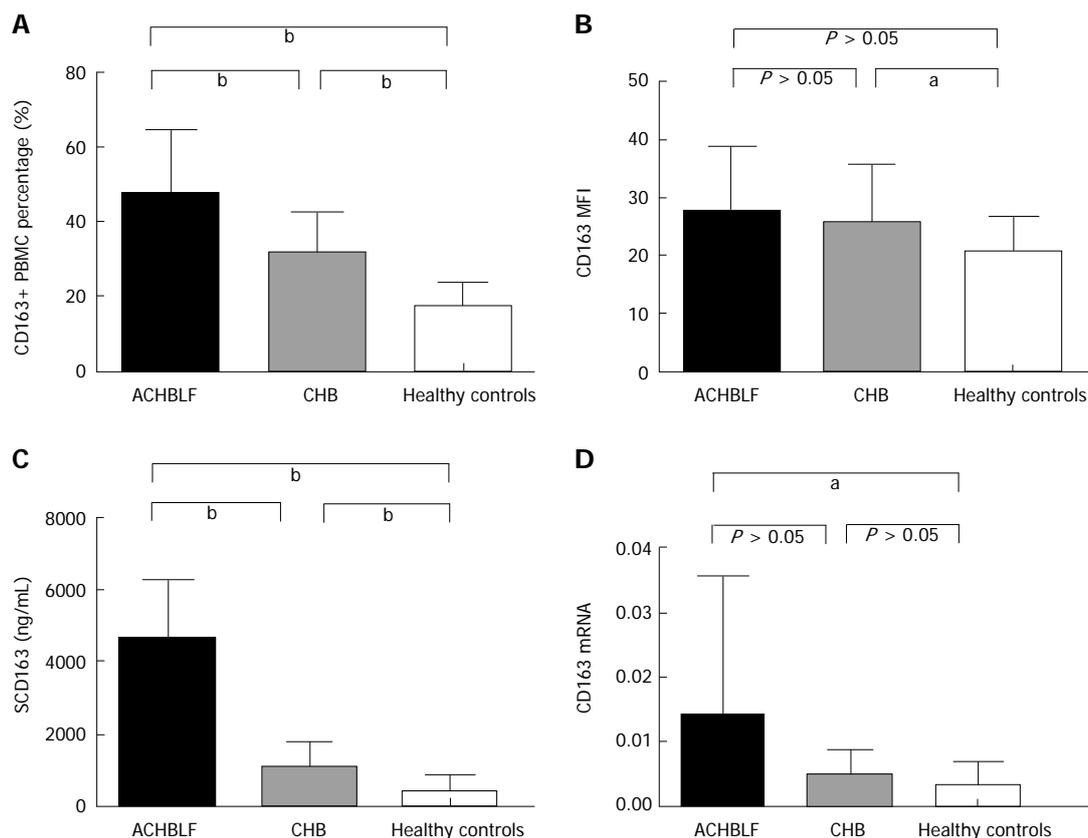
ACHBLF patients were grouped into survivors and non-

survivors after 3 mo of follow up. The plasma levels of sCD163 were increased in non-survivors compared with survivors ( $5344.9080 \pm 1589.5199$  ng/mL vs  $3641.7333 \pm 1264.5228$  ng/mL,  $P = 0.0321$ ) (Figure 3E).

## **DISCUSSION**

Although sCD163 is thought to play an important role in the pathogenesis of liver failure, this is the first study on the frequency of CD163+ PBMCs and CD163 mRNA level in ACHBLF patients<sup>[18,19]</sup>. Increased plasma sCD163 levels were correlated with disease severity in ACHBLF patients. These results strongly suggest that the role of CD163 in the immune response might affect the prognosis of ACHBLF.

CD163 is a scavenger receptor, and its expression has been proved to be associated with inhibition of inflammation. Furthermore, during the innate immune response, CD163 plays an important role in the host defense against inflammation<sup>[5,20,21]</sup>. The soluble form of CD163 is also a biomarker associated with inflammation<sup>[18]</sup>. In the present study, we first found that the frequency of CD163+ PBMCs and CD163 mRNA expression in PBMCs were dramatically increased in ACHBLF patients compared with healthy controls. These results could be interpreted together with previous reports to suggest that CD163 might



**Figure 2** Acute-on-chronic hepatitis B liver failure ( $n = 40$ ), chronic hepatitis B ( $n = 40$ ) and healthy controls ( $n = 20$ ). A: The frequency of CD163+ peripheral blood mononuclear cells (PBMCs) in acute-on-chronic hepatitis B liver failure (ACHBLF) patients was significantly higher than that in chronic hepatitis B (CHB) patients and healthy controls ( $P < 0.01$ ); B: There were no significant differences in mean fluorescence intensity (MFI) of CD163+ PBMCs within the three groups ( $P > 0.05$ ); C: The plasma levels of soluble CD163 (sCD163) in ACHBLF patients were significantly higher than those in CHB patients and healthy controls ( $P < 0.01$ ); D: The mRNA levels of CD163 in ACHBLF patients and CHB patients were significantly higher than those in healthy controls ( $P < 0.01$ ). Significant differences were calculated using the Kruskal-Wallis  $H$  test and Mann-Whitney  $U$  test ( $^aP < 0.05$ ,  $^bP < 0.01$  between the two groups).

have a key position in the immunolesion of ACHBLF.

There were no significant differences in MFI of CD163+ PBMCs within the three groups. This result is very interesting. From previous literature, which mainly included randomly selected patients, CD163 was inversely correlated with sCD163 plasma levels<sup>[22]</sup>. Lipopolysaccharide and other pro-inflammatory cytokines induce shedding of CD163 from the surface of isolated monocytes. During infection, the expression of monocyte surface CD163 decreases. In contrast, some studies have shown that with chronic and acute inflammation, the expression of surface CD163 increased. Through CD163 shedding, decreased surface CD163 was followed by recovery and induction of surface CD163 to higher levels<sup>[23-25]</sup>. We speculate that surface CD163 expression might vary according to different inflammatory states in patients and different stages of inflammation. More research is needed to confirm this.

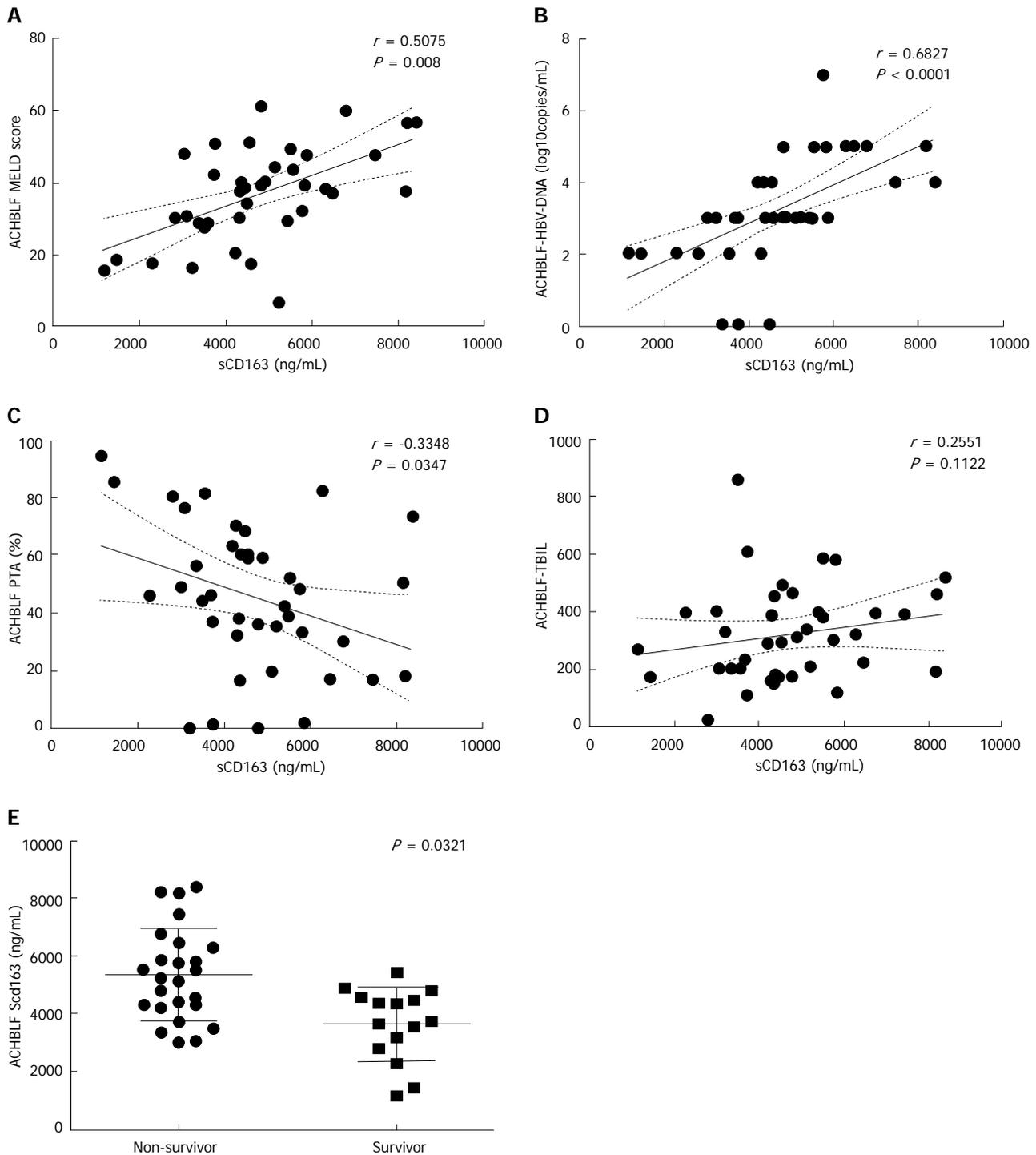
In this study, we observed that peripheral mRNA levels of CD163 in ACHBLF patients were high and there was a significant difference between the three groups, however, mRNA levels of CD163 in ACHBLF patients were not significantly different to those in CHB patients. During inflammation, we speculate that plasma sCD163

levels might increase dramatically, but mRNA levels increase slowly<sup>[26-28]</sup>.

In the present study, we found that plasma sCD163 levels were significantly increased in patients with ACHBLF compared to CHB patients and healthy controls. Plasma sCD163 levels were positively associated with HBV DNA levels, MELD scores and were negatively correlated with PTA. In addition, there were no associations with TBIL. It was demonstrated that plasma sCD163 correlated with the severity of ACHBLF<sup>[29-31]</sup>.

The MELD scoring system is widely accepted and is used to predict prognosis in patients with liver failure. We chose the MELD scoring system to evaluate the prognosis of ACHBLF patients over a three month period. The results strongly indicated the potential role of plasma sCD163 levels in forecasting the prognosis of ACHBLF patients. Furthermore, plasma sCD163 levels were obviously elevated in ACHBLF patients who did not survive compared with surviving ACHBLF patients<sup>[16,17]</sup>.

We found significant associations between plasma sCD163 levels and HBV DNA in ACHBLF patients. These results indicate that HBV may contribute to the aggravated macrophage immune response besides activation of HBV<sup>[32,33]</sup>. HBV might play a key role in promot-



**Figure 3** Linear correlation of plasma CD163 levels with disease severity markers in acute-on-chronic hepatitis B liver failure patients. A-C: Plasma CD163 was positively correlated with model of end stage liver disease (MELD) score, hepatitis B virus (HBV)-DNA and negatively correlated with prothrombin activity (PTA); D: There was no correlation between plasma CD163 levels and total bilirubin (TBIL); E: In acute-on-chronic hepatitis B liver failure (ACHBLF) patients, non-survivors had elevated plasma CD163 levels compared with survivors.

ing disease progression during host immune responses caused by the infection in ACHBLF patients. The exact role of HBV in CD163 immune response requires further clarification<sup>[34-36]</sup>.

The present study has limitations, and it would be very interesting to determine the dynamic and histologic expression of CD163 in a future study.

In conclusion, our study demonstrated three major findings. First, the population of CD163+ PBMCs was significantly greater in ACHBLF patients than in CHB patients and healthy controls. Second, CD163 mRNA expression in ACHBLF patients was significantly increased compared with CHB patients and healthy controls. Third, plasma sCD163 levels were markedly increased and cor-

related with disease severity and prognosis in ACHBLF patients. Our findings indicate that CD163 and sCD163 may serve as useful biomarkers and new therapeutic targets for ACHBLF.

## COMMENTS

### Background

Acute-on-chronic hepatitis B liver failure (ACHBLF) constitutes about 70% of all acute-on-chronic liver failure (ACLF) in areas with a high incidence of hepatitis B. ACHBLF has an extremely poor prognosis due to a lack of understanding of its pathogenesis.

### Research frontiers

ACHBLF has an extremely poor prognosis due to a lack of understanding of its pathogenesis. It is important to fully understand the course of the immune pathogenesis of ACHBLF. It may be very helpful to identify a new biomarker which indicates the prognosis of ACHBLF.

### Innovations and breakthroughs

This is the first study on the frequency of CD163+ peripheral blood mononuclear cells and CD163 mRNA level in ACHBLF patients. Authors found that increased plasma soluble CD163 (sCD163) levels were correlated with disease severity in ACHBLF patients. These findings strongly suggest that the role of CD163 and sCD163 in the immune response might affect the prognosis of ACHBLF.

### Applications

Their findings indicate that CD163 and sCD163 may serve as useful biomarkers and new therapeutic targets for ACHBLF. These biomarkers should be determined as early as possible in ACHBLF patients.

### Terminology

ACLF was first used in 1995 to describe a rapid deterioration of liver function due to acute insult to the liver in patients with ongoing or chronic liver disease. Diagnosis of ACLF may be based on the following: acute hepatic insult manifesting as jaundice (serum bilirubin  $\geq 85$   $\mu\text{mol/L}$  and coagulopathy international normalized ratio  $> 1.5$  or prothrombin activity  $< 40\%$ ) complicated within 4 wk by ascites and/or encephalopathy in a patient with previously diagnosed or undiagnosed chronic liver disease. ACHBLF is ACLF with acute or chronic hepatitis B.

### Peer review

Overall, this paper is of a very good quality. Study design is appropriate and clear, and helps to answer a clinical relevant question. Data analysis is sufficient. Manuscript is well structured, language is of sufficient quality.

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P- Reviewer He JY S- Editor Gou SX L- Editor A  
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## Massive hepatic necrosis with toxic liver syndrome following portal vein ligation

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Received: November 16, 2012 Revised: January 23, 2013

Accepted: February 2, 2013

Published online: May 14, 2013

### Abstract

Right portal vein ligation (PVL) is a safe and widespread procedure to induce contralateral liver hypertrophy for the treatment of bilobar colorectal liver metastases. We report a case of a 60-year-old man treated by both right PVL and ligation of the glissonian branches of segment 4 for colorectal liver metastases surrounding the right and median hepatic veins. After surgery, the patient developed massive hepatic necrosis with secondary pulmonary and renal insufficiency requiring transfer to the intensive care unit. This so-called toxic liver syndrome finally regressed after hemofiltration and positive oxygen therapy. Diagnosis of acute congestion of the ligated lobe was suspected. The mechanism suspected was an increase in arterial inflow secondary to portal vein

ligation concomitant with a decrease in venous outflow due to liver metastases encircling the right and median hepatic vein. This is the first documented case of toxic liver syndrome in a non-cirrhotic patient with favorable issue, and a rare complication of PVL.

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**Key words:** Colorectal liver metastases; Portal vein ligation; Liver failure; Toxic liver syndrome; Hemofiltration

Dupré A, Gagnière J, Tixier L, Ines DD, Perbet S, Pezet D, Buc E. Massive hepatic necrosis with toxic liver syndrome following portal vein ligation. *World J Gastroenterol* 2013; 19(18): 2826-2829 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2826.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2826>

### INTRODUCTION

Portal vein embolization (PVE) was first used by Makuuchi *et al*<sup>[1]</sup> to induce liver hypertrophy before major hepatectomy in biliary cancers. The technique was then applied to hepatocellular carcinoma and colorectal liver metastases (CRLM) to decrease postoperative morbidity and mortality<sup>[2,3]</sup>. PVL and portal vein embolization (PVE) are generally safe with few side effects in non-cirrhotic patients<sup>[4]</sup>. Suppression of the right portal flow causes atrophy of the right lobe, and induces the production of various growth factors and proinflammatory cytokines that prepare hepatocytes for growth (for review, see<sup>[5]</sup>). Whereas arterial ischemia can induce massive liver necrosis, the suppression of portal flow leads to progressive apoptosis with minor consequences for liver function<sup>[6]</sup>, owing mainly to a compensatory increase in arterial blood flow in the deprived lobe, a phenomenon called “hepatic arterial buffer response (HABR)”<sup>[7]</sup>. Here we report a case of massive hepatic necrosis of both the right lobe

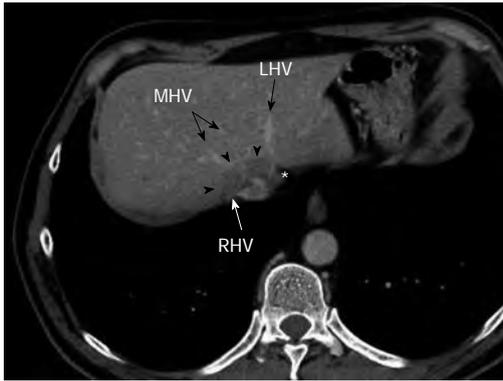


Figure 1 Preoperative multidetector computed tomograph at portal venous phase showing a large metastasis (arrowheads) encircling the confluence of the right hepatic vein and middle hepatic vein, without any thrombosis, and displacing the end of the left hepatic vein (asterisk). RHV: Right hepatic vein; MHV: Middle hepatic vein; LHV: Left hepatic vein.

and segment 4 after PVL in a patient treated for CRLM, with secondary cardiac, pulmonary and renal insufficiency due to toxic liver syndrome.

## CASE REPORT

A 60-year-old man was referred to our tertiary center for treatment of metachronous bilobar colorectal liver metastases. He had no medical history. Two years before he had undergone right colectomy for stage II colon cancer with no adjuvant anticancer therapy. During oncologic follow-up, three CRLM were detected by multidetector computed tomography scan: two in the right lobe, and one large one encircling the right and middle hepatic veins and displacing the left hepatic vein (Figure 1). Neoadjuvant chemotherapy (six courses of FOLFOX with cetuximab) was administered, with control imaging showing objective response according to RECIST criteria<sup>[8]</sup>. Radical resection required right hepatectomy enlarged to segment 4 (right trisectionectomy according to the Brisbane terminology<sup>[9]</sup>). To improve left lobe hypertrophy, we decided to perform a two-stage surgical procedure: first, right PVL with ligation of the glissonian branches of segment 4, and assessment of resectability using intraoperative liver ultrasonography (US) followed by right trisectionectomy. At first stage laparotomy, the liver was normal with no sign of chemotherapy-related injury. CRLM were confined to the right lobe and did not involve the left hepatic vein. A right PVL was performed with ligation of the glissonian branches of segment 4.

The immediate postoperative course was marked by a massive peak of transaminase and moderate liver insufficiency (Figure 2). The patient developed pulmonary and renal insufficiency with oligoanuria necessitating transfer to the intensive care unit (ICU) for non-invasive positive pressure ventilation and hemofiltration. US of the hepatic pedicle did not show any vascular thrombosis. Multidetector computed tomography (MD-CT) performed at postoperative day (POD) 2 showed massive hepatic necrosis of segment 4 and of a large part of the right hemi-

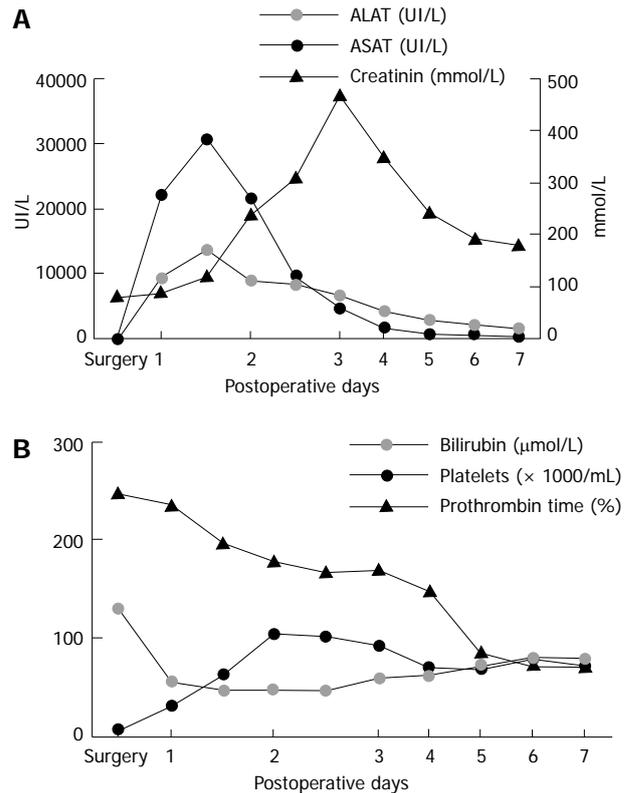


Figure 2 Postoperative blood tests. A: Transaminases increased from postoperative day 1, reached a peak at postoperative day 3, then returned to normal value on postoperative day 7. Serum creatinin started to increase after the peak of transaminases, as observed in the toxic liver syndrome, and decreased after hemofiltration; B: Platelets decreased regularly as from the first postoperative day. Total bilirubin increased from the first postoperative day and reached a plateau around 100 μmol/L. Prothrombin time fell rapidly to 47% at postoperative day 1 and thereafter increased progressively. ASAT: Aspartate amino transferase; ALAT: Alanine amino transferase.

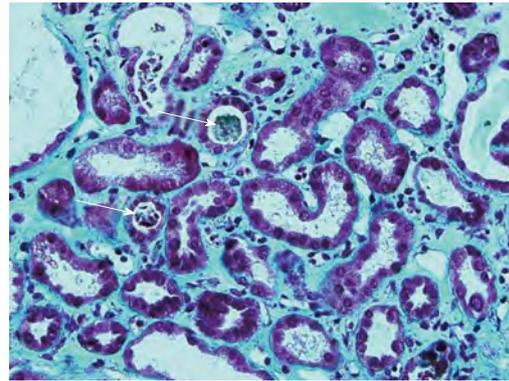
liver, hypertrophy of the left lobe and ascites (Figure 3). Renal biopsy revealed acute tubular necrosis without any particularity (Figure 4). Liver biopsy was not performed because of relative liver insufficiency and ascites. The level of transaminases started to decrease progressively from POD 2, and hemofiltration was stopped after recovery of renal function at POD 12. Finally, the patient was discharged from ICU at POD 15. A liver abscess developed in segment 8 near the necrotic area. Percutaneous drainage showed infected collection without biliary leak, with favorable outcome after appropriate antibiotic treatment. The patient was discharged from hospital after 52 d.

## DISCUSSION

Portal vein occlusion by ligation or embolization is a safe procedure widely used to induce liver hypertrophy of the future remnant liver before major liver resection. The mortality of this procedure is these procedures nil and major morbidity is about 1%<sup>[6]</sup>. In the event of post-procedure symptoms, which occur in more than 50% of patients, transaminase levels peak at a level less than three times baseline 1 to 3 d after embolization and return to baseline in 7-10 d<sup>[10]</sup>. Fever and post-embolization syn-



**Figure 3** Postoperative day 2 non-enhanced multidetector computed tomograph scan. Massive hepatic necrosis of the medial segment of the left lobe (segment 4, white arrowhead) and of a large part of the right lobe (black arrowhead) are shown, with remarkable hypertrophy of the lateral segments of the left lobe (red zone). Note also ascites (asterisk).



**Figure 4** Renal biopsy showing acute tubular necrosis: Tubules dilated with sometimes a low-lying epithelium and pleomorphic nuclei. The brush border of the proximal tubular cells is missing. The lumen of some tubules contains rare tubular cell necrosis (white arrows). There is a diffuse interstitial edema (Masson's trichrome,  $\times 40$ ).

drome, which are frequently observed after trans-arterial chemoembolization, are rare in PVE or PVL. Markers of liver insufficiency (bilirubin, prothombin time) are usually not affected. Hepatic necrosis of the ligated lobe has never been reported as a major complication<sup>[4]</sup>. Despite having different courses within the liver, arterial and portal blood flow drain in a common terminal hepatic veinule<sup>[11]</sup>. Thus, occlusion of a branch of the portal vein induces an ipsilateral HABR that guarantees rapid normalization of overall blood flow in the ligated lobe<sup>[12]</sup>. Flow in the hepatic artery of the ligated lobe increases by more than 3-fold for this compensatory effect and is usually well tolerated as venous outflow can absorb the overflow<sup>[13]</sup>. Hepatic necrosis involves the interruption of hepatic arterial flow with secondary damage due to liver hypoxemia. Interestingly, portal arterialization by arterioportal shunting restores normal oxygen supply within the liver and can prevent liver necrosis<sup>[14]</sup>. Hence, hypoxemia is the main cause of liver necrosis and explains why portal vein obstruction alone cannot induce hepatic necrosis. In contrast, impairment of venous outflow following compression by liver tumors may be very deleterious in this situation. Studies on venous drainage of right lobe grafts in living donor liver transplantation have demonstrated that non reconstruction of the middle hepatic vein can lead to congestion of the anterior sector, with possible severe liver dysfunction<sup>[15,16]</sup>. In such cases, assessment of hepatic tissue oxygenation using near-infrared spectrometry has confirmed congestion with hypoxemia of the anterior sector<sup>[17]</sup>. As a consequence, portal veins can become the draining veins to ensure adequate venous outflow, *via* a mechanism of regurgitation<sup>[18]</sup>. Finally, it can be speculated that compression of the right and median hepatic veins by liver metastases silently reduced the flow in these veins, with partial recovery of liver outflow through the portal system. PVL in this condition could have induced acute congestion with secondary necrosis of the right liver. Congestion was probably worsened by increased hepatic arterial inflow due to hepatic arterial buffer response secondary to PVL.

Acute congestion probably led to massive hepatic ne-

crosis of the right lobe with partial liver insufficiency. In our patient, massive hepatic necrosis caused renal, cardiac and respiratory dysfunction. This so-called “toxic liver syndrome (TLS)” was first described by Ringe *et al*<sup>[19]</sup> in fulminant hepatic failure. Definition is based on complete liver necrosis associated with cardiovascular shock, renal, and possibly respiratory failure requiring vasopressor support, hemodialysis, and mechanical ventilation<sup>[19]</sup>. The pathophysiology is still unclear but seems to involve toxic metabolites released from the necrotic liver, such as cytokines or cardiosuppressive factors, known to play a role in cardiac and pulmonary instability after liver ischemia-reperfusion syndrome<sup>[20,21]</sup>. Furthermore, intra-abdominal hypertension related to an increase in the volume of the intra-abdominal organs and ascites can impair cardiac preload by reduced venous return and pulmonary function and by limitation of abdominal wall expansion<sup>[22]</sup>. TLS is invariably fatal, and all the studies on the topic have been published in the setting of two-stage liver transplantation<sup>[19,23-26]</sup>. To the best of our knowledge, there are no documented cases of reversible TLS. The syndrome usually develops following graft failure or acute rejection in liver transplantation, but can also have traumatic, toxic or postoperative causes<sup>[19]</sup>. Spontaneous evolution of TLS is fatal mainly because of its association with liver failure<sup>[19]</sup>. One approach is wo-stage liver transplantation, which consists in (1) removing the necrotic liver to avoid toxic syndrome and stabilize the patient; and (2) restoring hepatic function with transplantation<sup>[27]</sup>. In our case, the patient did not develop fatal complications, for at least two reasons. First, only a part of the liver was involved by necrosis so that TLS was comparatively moderate. Second, the remnant liver spared by necrosis was neither cirrhotic nor fibrotic and was able to ensure sufficient hepatic function to minimize liver insufficiency, as demonstrated by the rapid hypertrophy visualized on MD-CT. Taken together, these two factors may have limited the consequences of TLS and directly contributed to the patient's favorable clinical evolution.

Portal vein ligation must be avoided when impaired venous return is suspected. There is the risk of massive

hepatic necrosis occurring due to hepatic congestion and secondary toxic liver syndrome, which can be fatal. This is the first documented observation of a spontaneously reversible toxic liver syndrome with minor hepatic failure.

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P- Reviewer Luca V S- Editor Jiang L L- Editor A  
E- Editor Li JY



## Polyarteritis nodosa diagnosed by surgically resected jejunal necrosis following acute abdomen

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Received: January 18, 2013 Revised: March 2, 2013

Accepted: March 23, 2013

Published online: May 14, 2013

### Abstract

The differential diagnosis of acute abdomen is typically extremely broad in range, with vasculitis posing a rare but potentially life-threatening cause of acute abdomen. Here, we report a case of acute abdomen with bowel wall thickening limited to jejunum, accompanied by unexplained renal dysfunction. Later, the patient was diagnosed as having polyarteritis nodosa based on surgically resected jejunal necrosis. Despite aggressive treatment, including the use of steroid pulse therapy and continuous hemodiafiltration, the patient died. Although polyarteritis nodosa is extremely rare in patients with acute abdomen, acute abdomen is relatively common manifestation of that. And it is reported that involvement of small intestine suggests poorer prognosis. Our case highlights the importance of vasculitis as a differential diagnosis of patients with atypical acute abdomen. In this report, we not only

review possible clues that might have led to an earlier diagnosis in this case, but also attempt to draw some lessons for treating similar cases in the future.

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**Key words:** Acute abdomen; Polyarteritis nodosa; Jejunal necrosis

**Core tip:** Our case shows the importance of vasculitis, including polyarteritis nodosa, as a differential diagnosis in case of acute abdomen. Here we provide comprehensive review of gastrointestinal organ involvement in Polyarteritis nodosa, and concluded that gastrointestinal lesions, especially small intestinal lesion, is relatively common manifestation and that suggests high mortality. Then we draw two findings, bowel wall thickening limited to jejunum and unexplained renal dysfunction, as possible clues that might led us to earlier diagnosis in this case. Additionally, we discuss possible relationship between pathophysiology of intestinal ischemia and radiological findings.

Hiraike Y, Kodaira M, Sano M, Terazawa Y, Yamagata S, Terada S, Ohura M, Kuriki K. Polyarteritis nodosa diagnosed by surgically resected jejunal necrosis following acute abdomen. *World J Gastroenterol* 2013; 19(18): 2830-2834 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2830.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2830>

### INTRODUCTION

Polyarteritis nodosa is a systemic vasculitis affecting medium- and small-sized arteries<sup>[1,2]</sup>. The affected sites can include the kidneys, gastrointestinal tract, heart, peripheral and central nervous system, and skin. Accurately diagnosing the disease is difficult because there are no

known markers, making biopsy a mandatory procedure. Here we report the case of a patient with acute abdomen diagnosed as polyarteritis nodosa based on surgically resected jejunal necrosis.

## CASE REPORT

A 70-year-old female with a history of diabetes mellitus, hypertension, and gallbladder stone visited the emergency department at our institution complaining of severe abdominal pain. Upon examination, she appeared slightly distressed; her temperature was 36.6 °C, blood pressure 153/101 mmHg, pulse 100 beats/min, and oxygen saturation 98% on room air. Epigastric tenderness without rebound or guarding tenderness, and pitting edema of all extremities were noted. The laboratory findings were remarkable for elevated white blood cell count (20650/ $\mu\text{L}$ ), C-reactive protein (8.62 mg/dL), and impaired renal function (BUN 70.2 mg/dL, creatinine 2.63 mg/dL). Urinalysis showed 1+ protein and 2+ occult blood. Although her platelet count had fallen to  $8.6 \times 10^4/\mu\text{L}$ , coagulation tests were normal. Serologies for hepatitis B virus, hepatitis C virus, anti-nuclear antibodies, cytoplasmic-Anti-Neutrophil Cytoplasmic Antibodies (ANCA), and perinuclear-ANCA were also negative. Abdominal computed tomography (CT) without contrast showed bowel wall thickening almost limited to jejunum, a stone at the neck of gallbladder, and a moderate amount of ascites surrounding the liver and the Douglas's pouch (Figure 1). Based on these findings, the patient was admitted to our institution with a diagnosis of acute abdomen, and intravenous sulbactam/cefoperazone was started.

Although her abdominal pain improved spontaneously after admission, her response to the antibiotic was minimal and her inflammatory reaction remained high. On day 6 following admission, the patient complained of severe abdominal pain. An emergent CT showed deterioration of the edema in the jejunum and the ascites (Figure 1). Although her antibiotic regimen was switched to imipenem/cilastatin, no improvement was observed. In light of these findings, an emergent laparotomy was carried out with tentative diagnoses including a serious intestinal infection refractory to antibiotics, a thrombosis or occlusion of the mesenteric vasculatures, and an intestinal lymphoma. Laparotomy findings included hemorrhagic ascites measuring more than 2000 mL, and a swollen, dark red jejunum. Twenty centimeters of the jejunum was resected, and an end-to-end anastomosis was conducted. The pathology was consistent with a diagnosis of polyarteritis nodosa (Figure 2). The pathological finding of a post-surgical skin lesion on her forearm was also consistent with the diagnosis.

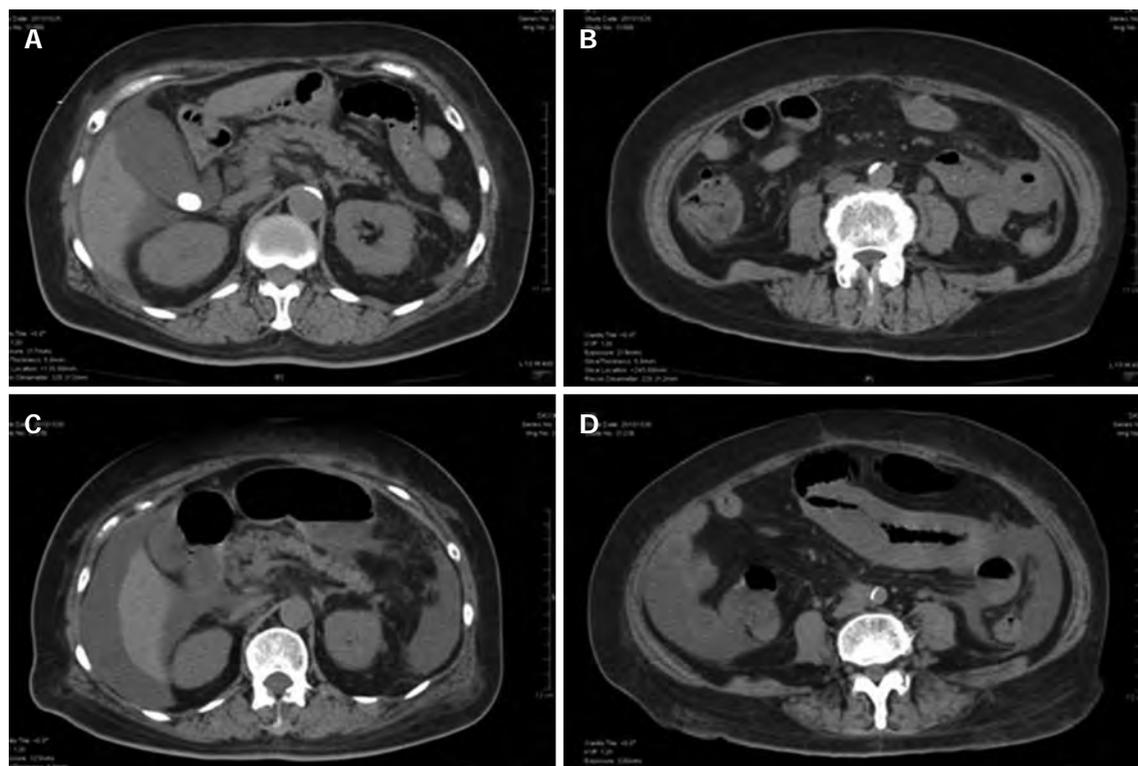
On day 17 following admission, steroid pulse therapy (methylprednisolone 1000 mg/d for 3 d) was prescribed, followed by a maintenance dose of steroids (60 mg/d for 8 d). Although her inflammatory reaction and renal function initially improved, the patient suffered a relapse of disease activity and her general status deteriorated despite

continuous hemodiafiltration and extensive use of antibiotics including ceftriaxone and ceftazidime. On day 32 following admission, an acute onset of left hemiparesis was observed, with normal head CT findings (Figure 3). At midnight of the same day, sudden respiratory arrest occurred. Although cardiopulmonary resuscitation was performed immediately after arrest, the patient died. An autopsy was not performed. The clinical course is summarized in Figure 4.

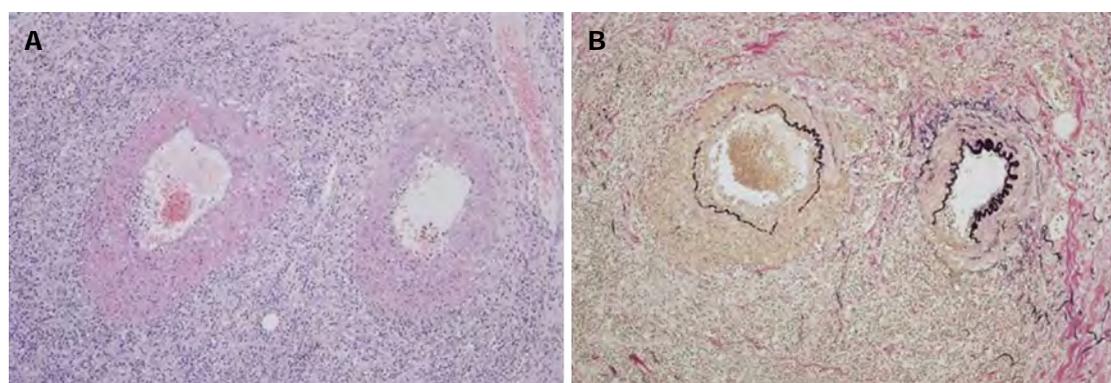
## DISCUSSION

Polyarteritis nodosa is a type of vasculitis affecting medium- and small-sized arteries. Epidemiologic studies of polyarteritis nodosa have generally been difficult to interpret because they have typically included patients with microscopic polyarteritis and other possible diseases as well. Recent studies, however, have estimated the prevalence per one million adults to be approximately 30 in France and Sweden<sup>[3,4]</sup>. Possible symptoms of polyarteritis nodosa include weight loss, livedo reticularis, testicular pain, myalgias or muscle weakness, mono- or polyneuropathy, hypertension, and renal dysfunction<sup>[1]</sup>. Unfortunately, no definitive diagnostic marker has ever been identified, and diagnosis is most often confirmed by biopsy. The prognosis of polyarteritis nodosa depends on the existence of serious organ involvement; in this regard, Guillevin *et al.*<sup>[5]</sup> have established 5 risk factors leading to higher rates of mortality. These include proteinuria > 1 g/d [relative risk (RR) for death was 3.6], renal insufficiency with serum creatinine > 1.58 mg/dL (RR = 1.86), gastrointestinal tract surgery (RR = 2.83), cardiomyopathy (RR = 2.18), and central nervous system involvement (RR = 1.76). When none of the 5 prognostic factors is present, the mortality rate at 5 years is 11.9%. However, when more than 3 factors are present, the mortality rate is as high as 45.95%.

Although polyarteritis nodosa is extremely rare in patients with an acute abdomen, acute abdomen is a relatively common manifestation of polyarteritis nodosa. Levine *et al.*<sup>[6]</sup> reported that 24 of the 54 cases (44%) they reviewed involved gastrointestinal lesions, and 13 required surgical intervention. Of the 24 cases presenting a gastrointestinal lesion, the mortality rate proved higher in the group requiring surgery (3/13, 23%) compared to the group that received more conservative treatment (1/11, 9%). The small intestine was reported to be involved in 3 of the 9 cases with polyarteritis nodosa that required abdominal surgery, 2 of the 3 patients died in such cases, while just 1 of the 6 patients died when the small intestine was spared<sup>[7]</sup>. According to an investigation of autopsy summaries in Japan, the small intestine is the most common site of gastrointestinal lesions and it is involved in 12 of the 15 cases (80%), while the large intestine is involved in 10 cases, liver and pancreas in 9 cases, gallbladder in 8 cases, stomach in 7 cases, tongue and esophagus in 6 cases, mesenterium in 3 cases, and appendix in 2 cases<sup>[8]</sup>. In summary, the small intestine is often involved in polyarteritis nodosa with acute



**Figure 1** Abdominal computed tomography findings. A, B: On day 1 showed ascites and bowel wall thickening; C, D: On day 6, both of the ascites and the bowel wall thickening, especially jejunum, had severely worsened.



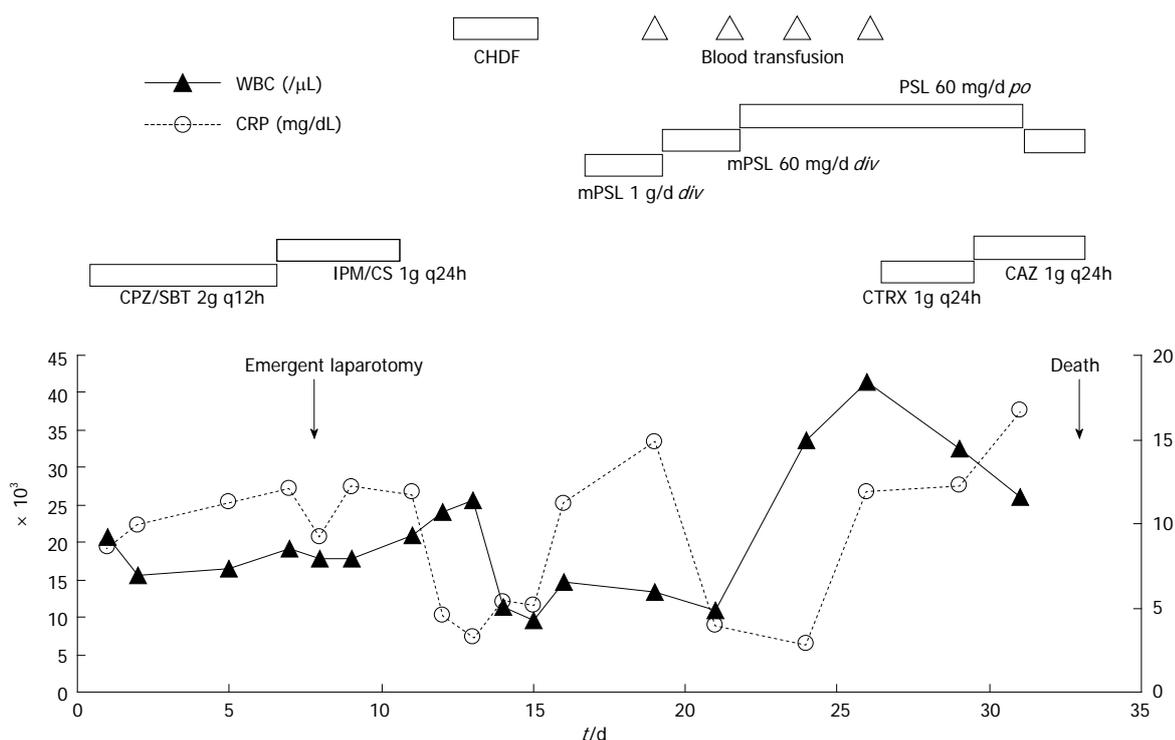
**Figure 2** Pathological specimen from the resected jejunum showing fibrinoid necrosis of the arterial wall and infiltration of inflammatory cells around the arteries. A: Hematoxylin and eosin staining. B: Elastica van Gieson staining. Original magnification  $\times 100$ .



**Figure 3** Head computed tomography, which revealed no clear evidence consistent with left hemiparesis.

abdomen and this situation suggests poor prognosis.

In the case of the 70-year-old female patient described here, we needed 6 d before a laparotomy was carried out and 16 d before a definitive diagnosis of polyarteritis nodosa could be made. Reviewing our case retrospectively, there were some findings inconsistent with a typical intestinal infection. First, the findings were remarkable for the presence of renal dysfunction with proteinuria and microscopic hematuria. Second, her abdominal pain and intestinal edema were so severe and resistant to antibiotics. Additionally, in our case, bowel wall thickening was limited to jejunum. We believe that such situation is very unlikely and there is no typical etiology that could explain the symptoms and findings. If we had considered these find-



**Figure 4 Clinical course of the patient.** Cefoperazone/sulbactam (CPZ/SBT) was started at day 1 and switched to imipenem/cilastatin (IPM/CS) at day 7. Ceftriaxone (CTRX) was started at day 26 and switched to ceftazidime (CAZ) at day 29. Steroid pulse therapy [methylprednisolone (mPSL) 1 g/d for 3 d] was started at day 16, and changed to maintenance dose of mPSL (60 mg/d) at day 19, then that was switched to oral steroid (PSL 60 mg/d) at day 22. According to deterioration of general status, steroid was again administered intravenously since day 31. Continuous hemodiafiltration (CHDF) was performed from day 13 to day 15, and intermittent blood transfusion was done at day 19, 21, 23 and 26. WBC: White blood cell; CRP: C-reactive protein.

ings more carefully, we might have suspected vasculitis, or other connective tissue diseases as a differential diagnosis. Nevertheless, since a biopsy remains indispensable for the diagnosis of polyarteritis nodosa and the patient's possible biopsy site was limited to the intestine at that time, we believe that a correct diagnosis was extremely difficult to ascertain in her situation.

Following diagnosis, we performed steroid pulse therapy (methylprednisolone 1000 mg/d for 3 d), followed by a maintenance dose of steroids (60 mg/d for 8 d). Although her inflammatory reaction and renal function initially improved after beginning pulse therapy, the patient subsequently worsened. Although repetitive steroid pulse therapy or another type of immunosuppressive therapy might have been effective in this case, as has been described in the literature<sup>[9]</sup>, we unfortunately did not have sufficient time to start such a treatment before the patient died.

Intestinal ischemia has generally been diagnosed by CT, and bowel wall thickening is one of the most reported findings in that situation<sup>[10]</sup>. Recently, magnetic resonance imaging (MRI) has begun to be regarded as an alternative modality to detect intestinal ischemia. *In vivo* study with animal model showed that bowel wall thickening was absent from arterial mesenteric ischemia, and bowel wall with reduced thickness resembling sheets of paper is rather observed 2-4 h after the onset, when evaluated by MRI<sup>[11]</sup>. CT findings in our case are possibly late findings after arterial occlusion, findings caused by reperfusion, or

that suggestive of venous infarction. Advances in imaging modality would further clarify the relationship between pathophysiology of intestinal ischemia and radiological findings.

At approximately 12 h before death, a left hemiparesis was observed in our patient, although a head CT revealed no obvious findings. It is difficult to state the cause of the patient's left hemiparesis since she died before a more thorough investigation could be conducted, but it is possible that it stemmed from complications of polyarteritis nodosa, including brain infarction and polyneuropathy<sup>[12-14]</sup>.

In conclusion, we have reported here a case of acute abdomen diagnosed as polyarteritis nodosa based on surgically resected jejunal necrosis. Vasculitis cannot be ruled out as a differential diagnosis in cases of acute abdomen, especially if the patient exhibits atypical findings, such as unexplained renal dysfunction or severe bowel wall thickening limited to small intestine. Additionally, it is worth noting that vasculitis cannot be ruled out even though serum markers, including ANCA, are negative, since there are no definitive diagnostic markers for polyarteritis nodosa.

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P- Reviewer Grassi R S- Editor Wen LL L- Editor A  
E- Editor Zhang DN



## Two case reports of gastroendoscopy-associated *Acinetobacter baumannii* bacteremia

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Supported by A Grant from the Changhua Christian Hospital, partially

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Received: February 6, 2012 Revised: March 11, 2013

Accepted: March 13, 2013

Published online: May 14, 2013

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**Key words:** Endoscopy; *Acinetobacter baumannii*; Bacteremia; Antibiotic prophylaxis

**Core tip:** After a literature review, we suggest that correct gastroendoscopy technique and skill in drainage procedures, as well as antibiotic prophylaxis, are of paramount importance in minimizing the risk of gastroendoscopy-associated bacteremia. Gastroenterologists should give more attention to gastroendoscopy-related infections, and increased clinical alertness may be the best way to reduce the impact from these types of infections.

Chen CH, Wu SS, Huang CC. Two case reports of gastroendoscopy-associated *Acinetobacter baumannii* bacteremia. *World J Gastroenterol* 2013; 19(18): 2835-2840 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i18/2835.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i18.2835>

### Abstract

Two cases of gastroendoscopy-associated *Acinetobacter baumannii* (*A. baumannii*) bacteremia were discovered at the study hospital. The first case was a 66-year-old woman who underwent endoscopic retrograde cholangiopancreatography and endoscopic retrograde papillotomy, and then *A. baumannii* bacteremia occurred. The second case was a 70-year-old female who underwent endoscopic retrograde biliary drainage due to obstruction of intra-hepatic ducts, and bacteremia occurred due to polymicrobes (*Escherichia coli*, *viridans streptococcus*, and *A. baumannii*). After a literature review, we suggest that correct gastroendoscopy technique and skill in drainage procedures, as well as antibiotic prophylaxis, are of paramount importance in minimizing the risk of gastroendoscopy-associated bacteremia.

### INTRODUCTION

Gastroendoscopy is a commonly used procedure for diagnosis and therapy, such as in endoscopic retrograde cholangiopancreatography (ERCP). Infection is one of the most common morbidity complications of gastroendoscopy. Septic complications of ERCP include ascending cholangitis, liver abscess, acute cholecystitis, infected pancreatic pseudocyst, infection following perforation of a viscus, and, less commonly, endocarditis and endovascularitis<sup>[1]</sup>. Bacteria can enter the biliary tract by hematogenous or, more frequently, by a retrograde route, and the most common organisms transmitted by ERCP are *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species<sup>[2]</sup>. Here we report two interesting cases of gastroendoscopy-associated *Acinetobacter baumannii* (*A. baumannii*)(GEaAb) bacteremia.

## CASE REPORT

### Case 1

The patient was a 66-year-old woman who visited a gastroenterologist for complaints of right upper quadrant pain and hunger pain. The initial impression was of a gall bladder stone. An ERCP was performed, and it showed a common bile duct of 16.1 mm in diameter and several filling defects in the gall bladder; therefore, an endoscopic retrograde papillotomy (EPT) was executed over the proximal portion of the bile duct, after which a gallstone was removed. The course of the procedure went smoothly. She again began to feel right quadrant pain and fever the next day, so she was admitted for further evaluation and management. At admission, vital sign measurements were: blood pressure, 100/90 mmHg; temperature, 38 °C; pulse rate, 110 beats/min; and respiratory rate, 20 breaths/min. The patient appeared acutely ill. The abdomen was distended and ovoid. There was radiation pain and tenderness to her back, and abdominal fullness over the right quadrant area (positive Murphy's sign), but no rebounding pain. Admission laboratory results revealed the following: white blood cell count, 12700/mm<sup>3</sup>; blood creatinine level, 0.8 mg/dL; serum amylase, 815 IU/L; serum bilirubin, 0.88 mg/dL; aspartate transaminase, 55 U/L; and alanine transaminase, 140 U/L. Abdominal echography revealed peri-pancreatic fluid accumulation. On the first day of admission, the patient was treated with antibiotics (cefazolin 1 g every 8 h plus gentamicin 60 mg every 12 h) and adequate fluid hydration, after initially remaining nil per os (NPO). A blood culture revealed *A. baumannii* on the 4<sup>th</sup> admission day, and the antibiotic treatment was switched to imipenem-cilastatin 500 mg every 6 h according to the antibiotics susceptibility test. Clinically, the source of *A. baumannii* was from the biliary tract, and it could be related to the previous invasive procedure. Because of persistent fever, an abdominal computed tomography was performed and showed a pancreatic abscess; consequently, an echo-guided aspiration was performed on the 5<sup>th</sup> admission day. The fever gradually subsided and follow-up laboratory data showed improvement. The total duration of parenteral imipenem-cilastatin usage was 21 d, after which antibiotic therapy was switched to oral levofloxacin 500 mg per os daily. The patient was discharged on the 44<sup>th</sup> admission day and was followed in the out-patient department (OPD). She has recovered quite well.

### Case 2

This patient was a 70-year-old female with liver cirrhosis related to hepatitis C virus infection. At initial presentation, laboratory test results were as follows: serum bilirubin, 0.52 mg/dL; aspartate transaminase, 42 U/L; alpha-fetoprotein, < 20 ng/mL; and hepatitis C virus-antibody titer, positive. The abdominal computed tomography scan showed multiple nodular hypervascular tumor stains in both lobes of the liver, especially in the right lobe. Hepatocellular carcinoma was highly suspected. Transarterial

chemo-embolization (TACE) of both sides of the liver was performed, and she was regularly followed in the OPD while she received TACE 6 times over the course of 20 mo. Her follow-up laboratory tests showed a serum bilirubin of 8.6 mg/dL, an aspartate transaminase of 72 U/L, and an alkaline phosphatase of 371 U/L. An abdominal echography exam revealed focal dilated intra-hepatic ducts. Hence, an endoscopic retrograde biliary drainage (ERBD) procedure was performed accordingly, at which time a stent (11 Fr) was inserted into the intra-hepatic ducts through the common bile duct. She began to feel right quadrant pain and fever three days later, and she was admitted under the impression of cholangitis. At admission, vital signs included a blood pressure of 100/90 mmHg, a temperature of 38 °C, a pulse rate of 110 beats/min, and a respiratory rate of 20 breaths/min. Murphy's sign was positive. Admission laboratory results revealed the following: white blood cell, 5600/mm<sup>3</sup>; serum total bilirubin, 27.65 mg/dL; serum direct bilirubin, 19.2 mg/dL; aspartate transaminase, 60 U/L; and alanine transaminase, 140 U/L. The abdominal echography showed left intra-hepatic duct dilatation. On the first day of admission, the patient was treated with antibiotics (cefazolin 1 g every 8 h plus gentamicin 60 mg every 12 h) and adequate fluid hydration after initially being NPO. Echo-guided percutaneous transhepatic cholangial drainage was performed. Blood culture revealed polymicrobes (*Escherichia coli*, *viridans streptococcus*, and *A. baumannii*) on the 4<sup>th</sup> admission day, and treatment was changed to imipenem-cilastatin 500 mg every 6 h according to the antibiotics susceptibility test. The fever gradually subsided. Clinically, the source of *A. baumannii* was from the biliary tract, and it could be related to the previous invasive procedure. Follow-up laboratory data did not, however, seem much improved. This patient expired due to severe hepatic failure with multiple organ failure.

### Evidence-based literature review and epidemiological study

After noting those two GEaAb cases in our institute, we conducted an evidence-based literature review (Table 1)<sup>[3-7]</sup>. Norfleet's study showed that 6% of patients who received upper gastrointestinal endoscopic examinations developed bacteremia, but only one of 447 patients acquired *Acinetobacter* bacteremia<sup>[4]</sup>. Maulaz's study described that the bacteremia incidence in cirrhotic patients who received variceal ligation was 2.5%, and only one *Acinetobacter hwoffii* infection was disclosed<sup>[5]</sup>. Only two case reports described post-endoscopic retrograde cholangiopancreatography-associated *Acinetobacter* infection in the United States National Library of Medicine National Institutes of Health<sup>[6,7]</sup>. Additionally, we performed a retrospective cross-sectional epidemiological study in our institute to identify GEaAb bacteremia cases and elucidate the possible sources of infection for a further five years from the year of identifying these two patients. During this period of five years, we disclosed 45 *A. baumannii* bacteremia cases. Most of them resulted from hospital-

Table 1 Evidence-based literature review for gastroendoscopy-associated *Acinetobacter* bacteremia

| Ref.   | Country       | Evaluation  | Risk factors  | Microbiology  | Treatment  | Outcome  |
|--|---------------|---|---|---|--|--|
| Norfleet <i>et al</i> <sup>[3]</sup> , 1981            | United States | 447 patients have been evaluated, of which 6% had bacteremia after upper gastrointestinal endoscopy                                   | Upper gastrointestinal endoscopy  | One case with <i>Acinetobacter</i> sp infection   | NM   | NM   |
| Maulaz <i>et al</i> <sup>[4]</sup> , 2003              | Brazil        | The bacteremia incidence in cirrhotic patients submitted to variceal ligation was 2.5%, showing no difference from the control groups | Endoscopic variceal ligation or esophagogastroduodenoscopy only   | One case with <i>Acinetobacter baumannii</i> infection  | NM   | One case with <i>Acinetobacter baumannii</i> infection is survived |
| Oh <i>et al</i> <sup>[5]</sup> , 2007                  | South Korea   | A total of 364 patients who underwent PTC were included in the study  | Cholangitis and bacteremia were associated with percutaneous transhepatic biliary drainage and tract dilation, catheter migration and blockage with tract maturation, and bile duct injury with PTC | NM  | NM   | NM   |
| Lai <i>et al</i> <sup>[6]</sup> , 2008                 | Taiwan        | Case report   | Endoscopic procedure  | Initial, polymicrobes ( <i>Acinetobacter baumannii</i> , <i>Klebsiella pneumoniae</i> and <i>Enterococcus Faecalis</i> ), then became <i>Acinetobacter</i> genomic species T3TU at day 14 | Ceftazidime and ampicillin-sulbactam, then intravenous gentamicin and ciprofloxacin (parenteral antibiotics for 4 wk) then followed by oral ciprofloxacin and trimethoprim-sulfamethoxazole (for another 13 d), antibiotics used for 61 d in total | Survived   |
| de la Tabla Ducasse <i>et al</i> <sup>[7]</sup> , 2008 | Spain         | Case report   | Post-endoscopic retrograde cholangiopancreatography   | <i>Acinetobacter ursingii</i> infection   | Cefotaxime   | Survived   |

NM: Not mentioned; PTC: Percutaneous transhepatic cholangioscopy.

acquired pulmonary infection. We focused on biliary tract infections and gastroendoscopy-associated *A. baumannii*, but neither a case of biliary tract infection nor a case of GEaAb were disclosed among hospital-acquired infection patients. So, we excluded those hospital-acquired *A. baumannii* patients, and the results were 19 patients with documented non-hospital-acquired *A. baumannii* bacteremia. We focused on these 19 patients (the demographics and clinical presentations are listed in Table 2). We also performed a pulse-field gel electrophoresis (PFGE) analysis according to Seifert's method<sup>[8]</sup>, and the results are shown in Figure 1. Patients 1, 11 and 14 appeared to have had similar fingerprints according to a dendrogram and PFGE (Figure 1). In the analysis of 5 biliary sepsis cases, risk factors included one patient with common bile duct stone, one with diabetes mellitus, one with cholangiocarcinoma, one with liver cirrhosis, and one with hepatocellular carcinoma. In addition, 3 patients had received invasive diagnostic or therapeutic procedures: one had an EPT, one underwent percutaneous transhepatic cholangial drainage, and one had ERBD (Table 2). Two of 5 biliary sepsis cases developed *A. baumannii* bacteremia. All of those patients received prophylactic antibiotics before the invasive medical procedures.

## DISCUSSION

This is the first serial study and case reports of GEaAb bacteremia in Taiwan. In our study, this infection was seen in one patient after an EPT, and in one patient who underwent ERBD. Both diagnostic and therapeutic gastroendoscopy can lead to bacteremia, and gastroendoscopy-associated infection rates up to 27% have been associated with therapeutic procedures<sup>[9-11]</sup>.

## Mechanisms of gastroendoscopy-associated infections

Concerning the mechanisms of gastroendoscopy-associated infections, bacteria can enter the biliary tract by a hematogenous or, more frequently, a retrograde route. Estimates of

Table 2 Demographics and clinical presentations of 19 non-hospital-acquired *Acinetobacter baumannii* bacteremia patients

| No. | Age (yr) | Sex | Chief complaint (d)         | Previous admission | Initial diagnosis of infection | Underlying disease                        | Fever/shock | Route of entry <sup>1</sup> | Treatment (d)  | Outcome |
|-----|----------|-----|-----------------------------|--------------------|--------------------------------|---|-------------|-----------------------------|--|---------|
| P1  | 67       | F   | RUQ pain (1)                | 0                  | Pancreatic abscess             | CBD stone                                 | Y/N         | Biliary tract <sup>1</sup>  | Cefazoline+gentamicin (5), imipenem-clastatin (12), levofloxacin (8) | S       |
| P2  | 76       | F   | Conscious disturbance (3)   | 3                  | Liver abscess                  | DM  | Y/N         | Biliary tract               | Cefmetazole (3), imipenem-clastatin (7), levofloxacin (7)            | S       |
| P3  | 79       | M   | SOB (7)                     | 3                  | Pneumonia                      | Esophageal cancer                         | Y/N         | Respiratory tract           | Co-trimoxazole (4)   | S       |
| P4  | 40       | F   | SOB (3)                     | 2                  | Sepsis                         | Breast cancer                             | Y/N         | Primary <sup>2</sup>        | Cefazoline+gentamicin (5), levofloxacin (7)                          | S       |
| P5  | 74       | M   | Deafness (4)                | 0                  | Sepsis, sudden deafness        | Nil                                       | Y/N         | Primary                     | Cefazoline + gentamicin (1)  | S       |
| P6  | 24       | F   | Fever, right flank pain (1) | 2                  | Acute pyelonephritis           | Pelvic cancer                             | Y/N         | Urinary tract               | ampicillin-sulbactam (7), co-trimoxazole (3)                         | S       |
| P7  | 76       | F   | Chest pain (1)              | 0                  | Sepsis                         | AMI                                       | N/Y         | Primary                     | Cefmetazole (4), imipenem-clastatin (7)                              | S       |
| P8  | 80       | F   | SOB (1)                     | 1                  | Urinary tract infection        | Right renal stone                         | Y/N         | Urinary tract               | Cefazoline (5), amikacin (7)   | S       |
| P9  | 82       | M   | Hematuria (1)               | 1                  | Urinary tract infection        | Old CVA                                   | Y/N         | Urinary tract               | Nil  | S       |
| P10 | 1        | M   | Fever (1)                   | 0                  | Neonatal infection             | Nil                                       | Y/N         | Primary                     | Ampicillin (5)   | S       |
| P11 | 33       | F   | SOB (3)                     | 1                  | Sepsis                         | Cholangiocarcinoma                        | Y/N         | Primary <sup>3</sup>        | Cefmetazole (1), imipenem-clastatin (7)                              | S       |
| P12 | 64       | M   | Abdominal pain (3)          | 2                  | Cholangitis                    | Liver cirrhosis, uremia, AF               | N/Y         | Biliary tract               | Cefazoline (5), gentamicin (14)                                      | E       |
| P13 | 73       | F   | Epigastric pain (1)         | 0                  | Urosepsis                      | DM  | Y/N         | Urinary tract               | Imipenem-clastatin (14)  | S       |
| P14 | 70       | M   | RUQ pain (1)                | 2                  | Cholangitis                    | HCC, HCV                                  | N/N         | Biliary tract <sup>4</sup>  | Cefamet (5), imipenem-clastatin (9)                                  | E       |
| P15 | 79       | M   | Loss of consciousness (1)   | 1                  | Sepsis                         | DM  | N/N         | Primary                     | Cefazoline (7)   | S       |
| P16 | 79       | F   | Fever (1)                   | 4                  | Lung abscess                   | DKA                                       | Y/Y         | Respiratory tract           | Cefuroxime (2)   | E       |
| P17 | 80       | M   | Dysuria (7)                 | 1                  | Urinary tract infection        | DM, urethral stricture                    | Y/N         | Urinary tract               | imipenem-clastatin (14)  | S       |
| P18 | 51       | F   | Right limb weakness (2)     | 4                  | Pneumonia                      | Chf                                       | N/N         | Respiratory tract           | Cefazoline (7), co-trimoxazole (7)                                   | S       |
| P19 | 80       | F   | Hematuria (3)               | 1                  | Urinary tract infection        | Left hydronephrosis, right urethral stone | N/N         | Urinary tract               | Cefazoline (3), urotactin (11)                                       | S       |

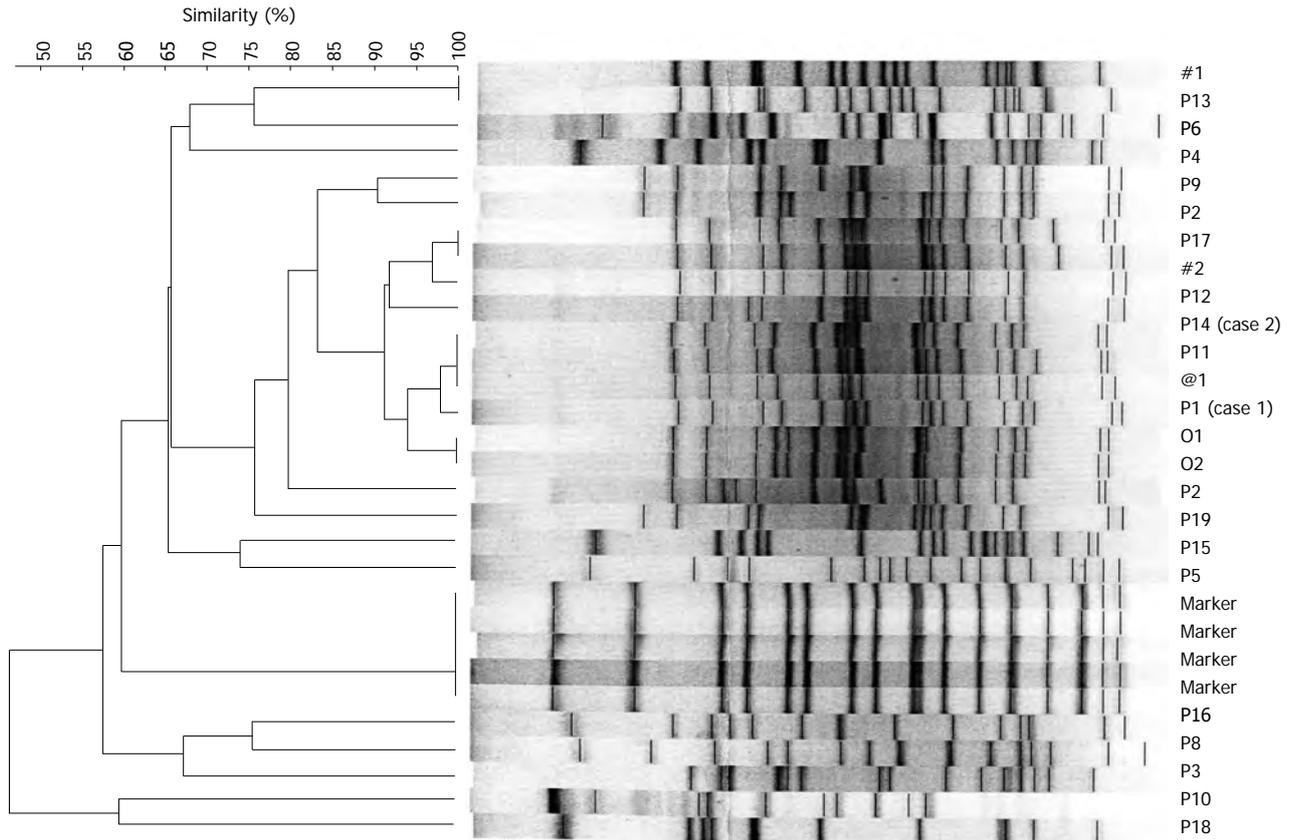
<sup>1</sup>This patient is case one, and she had received the endoscopic papillotomy one day before the episode of *Acinetobacter baumannii* (A. *baumannii*) bacteremia; <sup>2</sup>This patient had suspected hepatocellular carcinoma; <sup>3</sup>This patient had received the percutaneous transhepatic cholangial drainage three days before the episode of A. *baumannii* bacteremia, and the route of entry of A. *baumannii* could result from biliary tract. He was categorized to primary bacteremia due to lack of typical clinical symptoms and signs of biliary sepsis; <sup>4</sup>This patient is case two, and she had received the endoscopic retrograde biliary drainage one day before the episode of A. *baumannii* bacteremia. RUQ pain: Right upper quadrant pain; SOB: Shortness of breath; AMI: Acute myocardial infarction; TIA: Transient ischemic attack; CBD: Common bile duct; DM: Diabetes mellitus; DKA: Diabetic ketoacidosis; CHF: Congestive heart failure; HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; AF: Atrial fibrillation; CVA: Cerebrovascular attack; co-trimoxazole: Sulfamethoxazole-trimethoprim; S: Survived; E: Expired; P: Patient number; F: Female; M: Male; Y: Yes; N: No.

the incidence of clinically significant cholangitis have ranged from 0.4% to more than 10% (mean, 1.4%), depending upon the study population<sup>[12]</sup>. Entrance into the blood stream is presumably through minor trauma by the endoscope<sup>[13]</sup>. Another factor influencing the rate of cholangitis is the use of prophylactic antibiotics<sup>[14]</sup>. Results from these studies were similar to the results in our study.

### Organisms associated with gastroendoscopy-associated infections

The most frequent organisms responsible for cholangitis and biliary sepsis are enteric bacteria, such as *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species<sup>[2]</sup>. *A. baumannii*, which was reported in this study, is rare, so we performed a molecular epidemiological study. Patients 1, 11 and 14 appeared to have had similar fingerprint patterns according to a dendrogram and PFGE (Figure 1), and those 3 patients had experienced invasive gastrointestinal endoscopic procedures. Although we suspect the relationship, we still cannot prove the causal association between the procedure and infection in this study. We lacked direct microbiological evidence as well as estimates of the infection rate in the gastroendoscopy room. Also, there was no significant evidence of endemic *A. baumannii* infection at Changhua County.

In conclusion, we believe that accurate gastroendoscopy techniques and skill in drainage procedures are of paramount importance for minimizing the risk of GEAb bacteremia. Antibiotic prophylaxis is also widely considered to be indicated in selected patients. Gastroenterologists should give more attention to gastroendoscopy-related infections,



**Figure 1** Dendrogram and pulse-field gel electrophoresis patterns of *SgrAI*-digested chromosome DNA of 24 *Acinetobacter baumannii* isolates. #1, #2: Nosocomial *Acinetobacter baumannii* (*A. baumannii*) strain isolated from the same period; @1: Environmental *A. baumannii* strain isolated from the endoscopic room; O1, O2: Outbreak *A. baumannii* strains; P: Clinical *A. baumannii* strains isolated from the numbered patient among 19 non-hospital-acquired *A. baumannii* bacteremia patients.

and increased clinical alertness may be the best way to reduce the impact from these types of infections.

## ACKNOWLEDGMENTS

The authors thank Changhua Christian Hospital for the kind gift of the clinical *A. baumannii* strains. The authors thank the Gastroendoscopy Room of Changhua Christian Hospital for surveillance cooperation. The authors thank Chou CS and Laiu JC of the Central Branch Office, Center for Disease Control, Taichung for the PFGE assistance.

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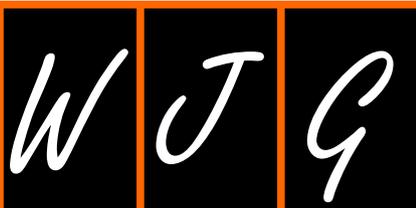
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# World Journal of *Gastroenterology*

*World J Gastroenterol* 2013 May 21; 19(19): 2841-2978



**EDITORIAL**

- 2841** Biliary complications following liver transplantation  
*Kochhar G, Parungao JM, Hanouneh IA, Parsi MA*

**REVIEW**

- 2847** Chemokines, chemokine receptors and the gastrointestinal system  
*Miyazaki H, Takabe K, Yeudall WA*
- 2864** Herbal hepatotoxicity: Challenges and pitfalls of causality assessment methods  
*Teschke R, Frenzel C, Schulze J, Eickhoff A*

**ORIGINAL ARTICLE**

- 2883** Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver  
*Chowdhury S, Chen Y, Yao TW, Ajami K, Wang XM, Popov Y, Schuppan D, Bertolino P, McCaughan GW, Yu DMT, Gorrell MD*
- 2894** Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats  
*Akyazi I, Eraslan E, Gülçubuk A, Ekiz EE, Çıraklı ZL, Haktanir D, Bala DA, Özkurt M, Matur E, Özcan M*
- 2904** Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa  
*Adas G, Adas M, Arikan S, Sarvan AK, Toklu AS, Mert S, Barut G, Kamali S, Koc B, Tatal F*
- 2913** Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma  
*Sun W, Dong WW, Mao LL, Li WM, Cui JT, Xing R, Lu YY*
- 2921** Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation  
*Wang QM, Du JL, Duan ZJ, Guo SB, Sun XY, Liu Z*

**BRIEF ARTICLE**

- 2935** Long-term follow-up study of gastroduodenal lesions after radioembolization of hepatic tumors  
*Rodríguez-Lago I, Carretero C, Herráiz M, Subtil JC, Betés M, Rodríguez-Fraile M, Sola JJ, Bilbao JI, Muñoz-Navas M, Sangro B*

- 2941** Predominant mucosal *IL-8* mRNA expression in non-*cagA* Thais is risk for gastric cancer  
*Yamada S, Kato S, Matsuhisa T, Makonkawkeyoon L, Yoshida M, Chakrabandhu T, Lertprasertsuk N, Suttharat P, Chakrabandhu B, Nishiumi S, Chongraksut W, Azuma T*
- 2950** Current application situation of gastrointestinal endoscopy in China  
*Zhang XL, Lu ZS, Tang P, Kong JY, Yang YS*
- 2956** Intraperitoneal perfusion of cytokine-induced killer cells with local hyperthermia for advanced hepatocellular carcinoma  
*Wang XP, Xu M, Gao HF, Zhao JF, Xu KC*
- 2963** Effect of Danzhijiangtang capsule on monocyte chemoattractant protein-1 mRNA expression in newly diagnosed diabetes subclinical vascular lesions  
*Fang ZH, Liu Y, Bao TT, Ni YQ, Liu J, Shi GB, Wu JP, Yang JP, Zhang H*
- 2969** Theory of mind deficits in patients with esophageal cancer combined with depression  
*Cao Y, Zhao QD, Hu LJ, Sun ZQ, Sun SP, Yun WW, Yuan YG*

## CASE REPORT

- 2974** Successful liver resection in a giant hemangioma with intestinal obstruction after embolization  
*Zhou JX, Huang JW, Wu H, Zeng Y*

**APPENDIX** I-VI Instructions to authors

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**AIMS AND SCOPE** *World Journal of Gastroenterology* (*World J Gastroenterol*, *WJG*, print ISSN 1007-9327, online ISSN 2219-2840, DOI: 10.3748) is a peer-reviewed open access journal. *WJG* was established on October 1, 1995. It is published weekly on the 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, and 28<sup>th</sup> each month. The *WJG* Editorial Board consists of 1352 experts in gastroenterology and hepatology from 64 countries.

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**INDEXING/ABSTRACTING** *World Journal of Gastroenterology* is now indexed in Current Contents<sup>®</sup>/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch<sup>®</sup>), Journal Citation Reports<sup>®</sup>, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports<sup>®</sup>, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16951 (7/74); Current Articles: 677 (1/74); and Eigenfactor<sup>®</sup> Score: 0.06035 (5/74).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 23/F, Lucky Plaza, 315-321 Lockhart Road, Wanchai, Hong Kong, China

Fax: +852-65557188  
Telephone: +852-31779906  
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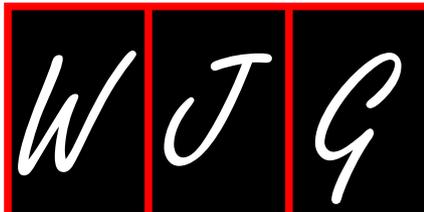
**PUBLICATION DATE**  
May 21, 2013

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## Biliary complications following liver transplantation

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Received: February 17, 2013 Revised: March 23, 2013

Accepted: April 10, 2013

Published online: May 21, 2013

Stricture; Leak; Endoscopic retrograde cholangiopancreatography

**Core tip:** Biliary complications continue to be a major cause of morbidity in liver transplant recipients. In this article, we review the etiology, as well as the main types of biliary complications according to the technique of biliary reconstruction and liver transplant procedure performed. Their management is also discussed with endoscopic techniques emerging as the preferred treatment option, obviating the need for surgery in majority of patients.

Kochhar G, Parungao JM, Hanouneh IA, Parsi MA. Biliary complications following liver transplantation. *World J Gastroenterol* 2013; 19(19): 2841-2846 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2841.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2841>

### Abstract

Biliary tract complications are the most common complications after liver transplantation. These complications are encountered more commonly as a result of increased number of liver transplantations and the prolonged survival of transplant patients. Biliary complications remain a major source of morbidity in liver transplant patients, with an incidence of 5%-32%. Post liver transplantation biliary complications include strictures (anastomotic and non-anastomotic), leaks, stones, sphincter of Oddi dysfunction, and recurrence of primary biliary disease such as primary sclerosing cholangitis and primary biliary cirrhosis. The risk of occurrence of a specific biliary complication is related to the type of biliary reconstruction performed at the time of liver transplantation. In this article we seek to review the major biliary complications and their relation to the type of biliary reconstruction performed at the time of liver transplantation.

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**Key words:** Liver transplantation; Complication; Biliary;

### INTRODUCTION

Since the first experiences with liver transplantation in the 1960s, this procedure has become a standard treatment for end stage liver disease. Limited primarily by the donor liver supply, the number of orthotopic liver transplants (OLT) has continued to increase. In the United States alone, according to the American Liver Foundation, 6500 liver transplantations were performed in 2005. Although, because of constant improvements in surgical techniques, the rate of biliary complications following liver transplantation has been decreasing; they remain a major source of morbidity and mortality<sup>[1,2]</sup>. Post liver transplantation biliary complications include strictures, leaks, stones or debris, and sphincter of Oddi dysfunction (SOD). T-tube biliary reconstruction, Roux-en-Y anastomosis, ischemia, reperfusion injury, hepatic artery thrombosis (HAT), cytomegalovirus infection, and primary sclerosing cholangitis are some of the risk factors that have been implicated in biliary complications.

## TYPES OF BILIARY RECONSTRUCTION

The choice of biliary anastomosis is a major determinant of the risk of biliary complications after OLT<sup>[3,4]</sup>. The two most common forms of biliary reconstruction are choledochocholedochostomy (CC, duct-to-duct anastomosis) and choledochojejunostomy (CJ, connection of the bile duct to a portion of jejunum). The choice of biliary reconstruction is determined by multiple factors, including the underlying liver pathology, the size of donor and recipient bile ducts, prior transplant or previous biliary surgery, and the preference of the performing surgeon. There are no clear-cut guidelines on the optimal type of biliary reconstruction, and considerable variability exists between surgeons.

CC is the most common biliary reconstruction procedure performed during OLT. This type of reconstruction is usually preferred because the procedure is technically easier, it preserves the function of Sphincter of Oddi, and it also allows easy endoscopic access to the biliary system after the surgery<sup>[5]</sup>. Furthermore, the preservation of the sphincter of Oddi, theoretically decreases the risk of ascending cholangitis as it serves as a barrier against the reflux of enteric contents into the biliary tree. CC can be performed either with or without a T-tube. Routine use of a T-tube allows direct measurement of bile output and color in the early post-operative period, maintains easy access for radiological evaluation of the biliary system and allows rapid decompression of the biliary tree if needed. It also may reduce the risk of anastomotic stricture formation. On the other hand, the use of T-tubes has been associated with bile leak and cholangitis at the time of their removal. A recent retrospective study of 180 patients demonstrated an increased rate of overall complications (33% *vs* 15.5%) and an increased rate of cholangitis (10% *vs* 2.2%) in patients with a T-tube compared to those without. In that study patients without a T-tube had an increased survival rate compared to the T-tube population (80.1% *vs* 72.8%), an observation that was attributed to higher complication rates among those with a T-tube<sup>[5]</sup>. This observation is supported by a recent meta-analysis consisting of 1027 patients, in which those without a T tube had a decreased incidence of cholangitis and peritonitis with overall decreased rate of biliary complications. Interestingly, this meta-analysis did not show any significant differences between the T-tube and non T-tube groups in terms of other complications such as anastomotic bile leaks, fistulas, choledochojejunostomy revisions, stenting of the bile duct, hepatic artery thromboses, retransplantation, and mortality due to biliary complications<sup>[6]</sup>.

CJ is another type of biliary reconstruction usually recommended in patients with pre-existing biliary disease such as primary sclerosing cholangitis, or prior biliary surgery, and also when there is a size mismatch between donor and recipient ducts. Compared to CC, CJ takes longer to perform and adversely affects the ability to perform an endoscopic evaluation of the biliary system after the liver transplantation. Potential complications of

CJ include intestinal perforation, stricture, leakage, and bleeding at jejunostomy site.

## DIAGNOSIS OF BILIARY COMPLICATIONS

The presentation of biliary complications varies considerably. Some complications such as bile leaks may occur immediately in the post-operative period, while others may take weeks to develop. The clinical presentation can vary from asymptomatic patient with moderate liver enzyme elevations to a septic patient with fever and hypotension due to ascending cholangitis. Whenever a biliary complication is suspected, work-up usually begins with laboratory evaluation and an abdominal doppler ultrasound. Abdominal ultrasounds are relatively inexpensive, and are easy to perform. An abdominal ultrasound allows for the evaluation of the biliary tree and the corresponding hepatic vasculature. The positive predictive value of abdominal ultrasound is very high, especially in the presence of dilated bile ducts. In the absence of dilated bile ducts, the sensitivity of the ultrasound for detecting biliary obstruction ranges from 38%-68%<sup>[7]</sup>. In the event that the ultrasound does not reveal evidence of bile duct dilatation despite clinical suspicion, the next step can be magnetic resonance cholangiopancreatography (MRCP) or endoscopic retrograde cholangiopancreatography (ERCP), depending on their availability. MRCP has excellent sensitivity (93%-100%) in detecting biliary strictures; and can also offer a road map for the endoscopist in planning the necessary intervention<sup>[8]</sup>. Another advantage of MRCP is that it does not carry the invasive risk involved with ERCP or other interventions such as percutaneous transhepatic cholangiography (PTC). The upside of ERCP and PTC, on the other hand, is that they both offer a potential therapeutic advantage over MRCP. It should be noted, however, that ERCP is associated with a high failure rate in patients with Roux-en-Y reconstruction, except when double balloon enteroscopy is available to assess the biliary tree. PTC is usually reserved in cases where ERCP cannot be performed.

### *Bile leaks after OLT*

Bile leaks, along with strictures, account for the majority of complications post-OLT. Bile leaks occur in 2%-25% of cases after liver transplantation and can be classified in two categories: early bile leaks, which present within 4 wk of OLT; and late bile leaks, which present beyond this time<sup>[9-12]</sup>.

**Etiology:** Early bile leaks usually occur at the anastomotic site or at the T-tube insertion site. They can be caused by ischemia, relative downstream obstruction, sphincter of Oddi hypertension, or as a result of T-tube removal<sup>[13]</sup>. The majority of bile leaks after OLT are associated with either planned or inadvertent T-tube removal, and the leak often occurs at the T-tube insertion site. One factor that has been shown to predict development of bile leak after T-tube removal is the presence of mucosal duct irregularities on cholangiography.

**Presentation:** The presentation varies with extent of the leak. Bile leak should be suspected in any patient who develops abdominal pain, fever or any sign of peritonitis after liver transplant, especially after T-tube removal. Bile leaks not related to T-tube removal typically present within the first 30 d after OLT. Some patients, especially those on corticosteroids, may be asymptomatic, with no signs of pain or fever. In such cases, any unexplained elevations in serum bilirubin, fluctuation in cyclosporine levels, or bilious ascites should raise suspicion for a bile leak.

**Management:** Once the clinical suspicion of a bile leak following T-tube removal is raised, initial management usually involves pain control with analgesics, intravenous fluids, and supportive care. Biliary leaks due to ischemia are difficult to treat since the cause is usually not corrected by endoscopic or radiologic intervention. Leaks due to other causes usually respond to non-operative diversion of biliary flow, such as unclamping of the T tube, endoscopic sphincterotomy with or without endoscopic stenting at the time of ERCP, or placement of a PTC. ERCP with stenting of the bile duct, sphincterotomy, nasobiliary drainage, or a combination of these techniques has been shown to have high rates of success<sup>[9,14]</sup>. Most studies report resolution of symptoms in 85%-100% of the cases<sup>[15]</sup>. Although one study reported better results with nasobiliary drainage; most centers use an internal biliary stent to overcome the difference in pressure in the bile duct and the gut. The stent usually remains in place for several weeks. PTC is commonly used in cases where ERCP cannot be performed, or in patients with Roux-en-Y reconstruction where the biliary orifice cannot be reached with a regular sideview endoscope. In rare cases, surgical intervention may become necessary<sup>[9]</sup>.

### **Biliary strictures**

Post liver transplantation biliary strictures are usually classified as anastomotic or non-anastomotic. The incidence of biliary stricture ranges from 5%-15% after deceased donor liver transplantation and 28%-32% after living donor liver transplantation<sup>[7]</sup>. Strictures are commonly seen as late complications, occurring approximately 5-8 mo after transplantation.

### **Anastomotic strictures**

Anastomotic strictures (AS) at the site of biliary anastomosis are frequent after OLT and can occur in both CC and CJ type of reconstruction. AS are more common after CJ than CC due to the direct bilioenteric connection<sup>[1,12]</sup>.

**Etiology:** The pathogenesis of AS is believed to be from inadequate mucosa-to-mucosa anastomosis, surgical technique, local tissue ischemia, and the fibrotic nature of the healing process<sup>[16]</sup>. Early bile leak is also considered to be a risk factor for developing AS<sup>[9]</sup>. In those with a T-tube,

strictures at the CC anastomosis are often not typically evident until after removal of the T-tube. A slight and transient narrowing of the biliary lumen occurs frequently in biliary anastomosis shortly after the OLT due to post-operative edema. It is uncertain how many of these cases progress to clinically significant strictures.

**Presentation:** Biliary stricture should be suspected in any OLT patient who presents with jaundice, fever, abdominal pain, or even in patients with asymptomatic biochemical cholestasis. Dilatation of the bile ducts proximal to the biliary anastomosis may be observed on imaging studies in some patients but is not a pre-requisite for diagnosis. Histologic findings may be suggestive of biliary obstruction, such as pericholangitis or bile duct proliferation.

**Management:** When a clinically significant AS is found, treatment is warranted. In recent years, ERCP has seen an increase in popularity in the management of AS. Although results differ markedly, studies have demonstrated good response after endoscopic therapy in over 75% of the patients<sup>[17,18]</sup>. Endoscopic treatment is thus regarded as the treatment of choice for AS, especially in the CC group of patients. Stenting of the stricture during ERCP is performed with or without balloon dilatation of the stricture. The initial stent is then exchanged for a larger stent or multiple stents every 3 mo for an average of 1 year to dilate the stricture and prevent clogging and stone formation. In patients with CJ reconstruction, the initial treatment usually involves stenting by percutaneous approach. Some centers have reported that early strictures respond better to therapy than late strictures. If a stricture does not respond to endoscopic or percutaneous therapy, surgery may be indicated. Previous endoscopic or percutaneous treatment has not shown to influence the success rate of surgery in treating such complications.

### **Non anastomotic strictures**

Non anastomotic strictures (NAS), also known as ischemic type strictures, are well known and have been described since the beginning of liver transplantation. They are frequently hilar in location, but can also be diffusely intrahepatic. NAS tend to be longer and multiple on presentation. NAS incidence ranges from 5%-15% with mean time to presentation of 3.3-5.9 mo post-OLT<sup>[19,20]</sup>.

**Etiology:** A few theories have been proposed for the development of NAS. The blood supply to the supraduodenal bile duct is predominantly from vessels which are resected during OLT. The remaining blood supply to the donor bile duct then comes from the hepatic artery and its branches, which are tenuous and highly susceptible to ischemic injury. In patients with NAS, up to 50% have demonstrable HAT<sup>[21]</sup>. Prolonged cold ischemia time has also been shown to be responsible for the development of NAS. Besides ischemia, an immunological cause has also been proposed. This is mainly due to the observa-

tion of an increased incidence of NAS in cases with ABO-incompatible grafts, in patients with autoimmune hepatitis or primary sclerosing cholangitis, in patients suffering from chronic ductopenic rejection, and those with a CC chemokine receptor 5delta32 polymorphism. In many cases, NAS are probably multifactorial in origin with injury resulting from one or more of the above mechanisms<sup>[7,22]</sup>.

**Management:** The presentation of NAS is similar to that of AS. NAS are more difficult to manage than AS, as treatment in each case has to be individualized. It is therefore difficult to make generalized recommendations for management of NAS. In cases with early HAT, aggressive management with either revascularization or early re-transplantation has been recommended. In NAS not associated with HAT, endoscopic or percutaneous therapy are often attempted first. Repeated dilatation with stenting seems to be the most accepted treatment form<sup>[14]</sup>. Treatment success depends upon stricture severity, number, and location. Extra-hepatic strictures generally respond better to therapy. Different studies report variable treatment success rates, ranging from 50%-70%<sup>[10,23]</sup>. If radiological and endoscopic therapies fail, surgery may become necessary. Success rates are higher if surgery is done within 2 years of OLT and if the liver biopsy does not show any significant fibrosis. Re-transplantation may also be considered in patients with treatment failure, or in the presence of secondary biliary cirrhosis, recurrent cholangitis, or progressive cholestasis.

### SOD

Another common occurrence after OLT is a mild increase in the size of donor and recipient common bile ducts. In certain cases, significant dilatation of both recipient and donor bile duct in association with biochemical abnormalities occurs in the absence of cholangiographic evidence of obstruction. In these cases, SOD is suspected. The incidence of SOD is reported to be up to 70%<sup>[11,24]</sup>.

**Etiology:** The pathogenesis of SOD is attributed to denervation of the sphincter during OLT. This leads to an increase in basal pressure, thus causing increased pressures in the choledochal duct<sup>[24]</sup>. Very few studies have directly assessed the pressures in the sphincter of Oddi post-OLT. Two types of SOD have been proposed on the basis of pathogenic mechanisms: stenosis and dyskinesia<sup>[16,25,26]</sup>. Any process that leads to chronic inflammation and fibrosis, can lead to sphincter stenosis. Dyskinesia, on the other hand, is usually seen as a result of functional disturbance of the sphincter.

**Management:** There have been virtually no clinical trials that demonstrate the best treatment option for SOD. In recent years, endoscopic therapy with sphincterotomy with or without stenting has been the most acceptable treatment option for SOD.

### Biliary stones, sludge, and casts

On ERCP, stones, sludge and casts are usually seen as a defect in the contrast column and described as “filling defects”. Intrinsic bile duct obstruction, in the form of biliary stones and sludge, can virtually occur at any time following the OLT. Sludge is described as a thick collection of mucous, calcium bicarbonate and cholesterol crystals, which, when left untreated, can go on to form biliary stones. Sludge and casts usually occur within the first year of transplant, while stones tend to occur later on.

**Etiology:** Theoretically, anything that increases the viscosity of bile or reducing flow can predispose to the formation of sludge, and stones. Bile duct mucosal damage due to obstruction, ischemia, or infection is thought to play a role in the development of casts. Of patients presenting with biliary stones and sludge, most will have an underlying stricture. In addition, medications such as cyclosporine may play a role in bile lithogenicity by inhibiting bile secretion and promoting functional biliary stasis. Bile in transplant patients has been shown to be supersaturated with cholesterol, which is aggravated by T-tube drainage and depletion of the bile acid pool.

**Presentation:** Patients commonly present with abdominal pain, cholestatic liver tests, and uncommonly with cholangitis.

**Management:** A study demonstrated that cholangiography is the only reliable imaging method for sludge; ultrasonography and CT scans are of limited value. If sludge alone is present, then it would be reasonable to first attempt medical treatment with ursodeoxycholic acid. Endoscopic therapy with sphincterotomy, lithotripsy and stone extraction are successful in treating majority of filling defects, especially biliary stones<sup>[23]</sup>.

### Biloma

Bile rupture and spilling of bile within the liver and abdominal cavity may result in the formation of a biloma. Small bilomas, especially ones that communicate with the biliary tree, may resolve on their own. Although bilomas can generally be treated with antibiotics and percutaneous drainage, some may require placement of a biliary stent in the extrahepatic bile duct<sup>[9]</sup>. Surgical drainage of a biloma is viewed as a last resort option.

### Hemobilia

One of the rare complications seen after OLT is hemobilia. It is usually associated with percutaneous liver biopsy or PTC. The commonly described triad of right upper quadrant pain, jaundice, and gastrointestinal bleed is seen only in a minority of patients. Treatment of hemobilia requires both hemostasis and treatment of any associated biliary obstruction by clots. The bleeding stops spontaneously with supportive therapy and correction of coagulopathy in some cases. Embolization of the

bleeding vessel by interventional radiology is required if bleeding is persistent or severe<sup>[27]</sup>. Removal of clots from the biliary tree for relief of obstruction is usually done by ERCP.

### Ductopenia

Ductopenia (also referred to as bile duct paucity and vanishing bile duct syndrome) is a descriptive term for small intrahepatic bile duct loss from any cause. In post liver transplantation patients, acute and chronic rejection and ischemia are the most common culprits. The diagnosis is established by liver biopsy in the appropriate clinical setting. Treatment depends mainly on the underlying etiology of ductopenia.

## CONCLUSION

Biliary complications following liver transplantation are relatively common and continue to be a challenging aspect in the management of such patients. The development of these complications is heavily influenced by the type of anastomosis during surgery. The majority of biliary complications after liver transplantation are now a days being managed endoscopically rather than surgically. ERCP, in particular, has proven to be relatively safe and effective in the management of these complications.

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**P- Reviewers** Ayoub WS, Bogdan D **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Xiong L



## Chemokines, chemokine receptors and the gastrointestinal system

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Author contributions: All the authors generated the ideas and contributed to the writing of this paper.

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Received: September 12, 2012 Revised: March 22, 2013

Accepted: April 27, 2013

Published online: May 21, 2013

### Abstract

The biological properties of tumor cells are known to be regulated by a multitude of cytokines and growth factors, which include epidermal growth factor receptor agonists and members of the transforming growth factor  $\beta$  family. Furthermore, the recent explosion of research in the field of chemokine function as mediators of tumor progression has led to the possibility that these small, immunomodulatory proteins also play key roles in carcinogenesis and may, therefore, be potential targets for novel therapeutic approaches. In this review, we will summarize recently reported findings in chemokine biology with a focus on the gastrointestinal tract.

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**Key words:** Chemokine; Receptor; Signal transduction;

Tumor progression; Targeted therapeutics; Digestive system; Cancer

**Core tip:** The chemokine network makes an attractive target for therapeutic intervention in many tumor types, including those of the gastrointestinal tract. However, we need to define more selective and specific targets, to minimize systemic side effects during treatment.

Miyazaki H, Takabe K, Yeudall WA. Chemokines, chemokine receptors and the gastrointestinal system. *World J Gastroenterol* 2013; 19(19): 2847-2863 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2847.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2847>

### INTRODUCTION

#### *Cancer development and progression in the gastrointestinal tract*

Cancer is a disease in which normal cells acquire genetic and epigenetic abnormalities<sup>[1,2]</sup>, leading to disorientation of conventional processes for the maintenance of normal cell physiology. These aberrant genetic and epigenetic modifications to the normal cell induce abnormal cell motility, proliferation, and survival, eventually enabling these cells to invade into adjacent tissues and even to migrate to regional or distant organs where they may grow continuously as metastatic lesions<sup>[3]</sup>. For many cancer patients, metastasis is generally the major cause of disease-related death<sup>[4,5]</sup>. Therefore, it is indispensable to elucidate the basic molecular mechanisms of tumor development to identify effective therapeutic targets, which can possibly reduce the side-effects of treatment, and define useful molecular markers for early detection and prediction of disease course. Especially, the newly emerged concept of personalized medicine may require in-depth assessment of potential therapeutic molecular target(s) in each individual case through analysis of sig-

naling pathways and subsequent validation of treatment efficacy<sup>[6]</sup>.

Numerous reports have identified molecules that play key roles in development of gastrointestinal cancer. Amongst these, which include growth factors and their receptors<sup>[7-9]</sup>, signaling pathway components<sup>[10,11]</sup>, transcription factors<sup>[12,13]</sup>, matrix remodeling enzymes<sup>[14,15]</sup> and cytokines<sup>[16-18]</sup>, a milestone finding by Müller *et al*<sup>[19]</sup> made chemokines one of the most intensively studied molecular targets to understand the mechanisms of organ-specific metastasis. The chemotactic cytokines or chemokines contribute to the tumor microenvironment by establishing a chemokine gradient, which is important for the process of chemoattraction and subsequent cell motility and infiltration for metastasis<sup>[20,21]</sup>. In this review, we will summarize the current status of chemokine-related studies in digestive tract cancer.

Recent progress has made it clear that the non-tumor cells, such as stromal fibroblasts and inflammatory cells present in the microenvironment, also play critical roles in establishing the metastatic phenotype of tumor cells<sup>[22,23]</sup>. Indeed, a role for inflammation in cancer progression is well-recognized, with many different cancer types having an inflammatory component<sup>[24]</sup>. Tumor cell infiltration is aided by the presence of tumor-associated macrophages (TAMs), dendritic cells and lymphocytes<sup>[25-28]</sup>. While TAMs are able to kill tumor cells, they also play an important role in enhancing tumor development by secreting matrix metalloproteinases (MMPs)<sup>[29,30]</sup>, growth factors [interleukins (ILs), vascular endothelial growth factors (VEGFs)] and pro-angiogenic factors that support tumor cell growth, neovascularization, and tumor cell invasion through the stromal tissues<sup>[31]</sup>.

### Chemokines and signal transduction

**Chemokines and chemokine receptors:** In humans, the chemokine superfamily is comprised of more than 50 small secreted proteins. These molecules function as immune modulators, chemoattractants and as activators of lymphocytes. They are sub-divided into four major groups based on the relative position of conserved cysteine residues near to the N-terminus: CXC-, CC-, C-, and CX<sub>3</sub>C-chemokines<sup>[32-34]</sup>. The CXC-chemokines can be further subdivided according to the presence or absence of a three amino acid motif immediately N-terminal to the first cysteine. Therefore, a glutamic acid-leucine-arginine (ELR) sequence defines the ELR<sup>+</sup> CXC-chemokines, which generally function as activators and chemoattractants for neutrophils. In contrast, lymphocytes constitute the target cell type for most ELR<sup>-</sup> CXC chemokines. The CC-chemokines play both chemoattractant and immunomodulatory roles. CCL5 attracts monocytes, eosinophils and memory T-cells, and is one of the human immunodeficiency virus (HIV)-suppressive factors secreted by CD8<sup>+</sup> T-cells<sup>[35]</sup>. Similarly, CCL3 and the closely related CCL4 are also capable of inhibiting HIV infection of target cells, in addition to their pro-inflammatory and chemoattractant functions<sup>[35]</sup>. Furthermore, CC-chemokines are also reported to be involved

in lymphocyte recirculation and homing to secondary organs (CCL21), as well as T-cell trafficking within the thymus (CCL19)<sup>[36-38]</sup>.

While the major biological functions ascribed to chemokines regulate leukocyte trafficking and recruitment to inflammatory foci, chemokine receptors are also expressed on non-immune cells, and act as key modulators of the biological functions of other cell types. For example, CCL3 is a negative regulator of keratinocyte growth<sup>[39]</sup>, while the CXC-chemokine ligand (CXCL) 5<sup>[40]</sup>, CXCL8<sup>[41,42]</sup> and other ELR<sup>+</sup> chemokines stimulate endothelial cell migration during angiogenesis<sup>[43]</sup>.

To date, 19 chemokine receptors are known to be expressed in mammals<sup>[44]</sup>. Chemokines activate these specific G-protein-coupled receptors (GPCRs) on the surface of target cells. Chemokine receptors consist of an extracellular ligand binding domain, seven transmembrane spans, and an intracellular carboxyl terminus. The transmembrane domain consists of three intracellular domains and three hydrophobic extracellular domains. Variation of the amino-terminus sequence of these receptors defines the specificity for recognition of chemokine ligands, the binding of which results in phosphorylation of Ser/Thr residues in the intracellular domain and induction of f to the N-terminus of the receptor, specific signal transduction will be induced by the release of these heterotrimeric G-proteins (guanine nucleotide-binding proteins -G/G/G) that are bound to the intracellular carboxyl terminus. Each of these heterotrimeric G-proteins consists of several different subtypes<sup>[45]</sup>. Chemokine receptors are subdivided into four types in relation to the class of ligand to which they bind: CXC-chemokine receptors (CXCR), which bind CXC-chemokines; CC-receptors (CCR), for CC-chemokines; and XC-receptors and CX<sub>3</sub>C-receptors (CX<sub>3</sub>CR) for XC- and CX<sub>3</sub>C-chemokines, respectively. Among these receptors and ligands, there is promiscuous binding between several receptors and multiple chemokines (Table 1)<sup>[38]</sup>.

**Mechanisms of signal transduction:** Binding of chemokines to their innate receptors can activate a number of intracellular signaling pathways that lead to cell proliferation and migration. Downstream mediators identified to date include the small GTPase Ras; extracellular signal-regulated kinases (ERK)<sup>[46,47]</sup>; phosphatidylinositol-3-OH kinase (PI-3K)<sup>[47-49]</sup>, and the other small GTPases Rho, Rac and Cdc42<sup>[50,51]</sup>; and some of these are active in pathways that regulate the actin cytoskeleton. For example, it was shown that migration of Jurkat cells in response to CXCL12 through CXCR4 is mediated by both Rac- and Rho-dependent mechanisms. While Rac is activated by G<sub>i</sub> and G<sub>βγ</sub> subunits, activation of RhoA occurs *via* G<sub>α13</sub> and leads to phosphorylation of myosin light chains<sup>[52]</sup>. Furthermore, CXCL8 (IL-8) signaling through CXCR1 has been reported to mediate migration of leukocytes in a β1-integrin-dependent manner which requires downstream activation of p38 and c-Jun N-terminal kinases<sup>[53]</sup>. The quality and the quantity of chemokine signaling is not controlled only by the expression volume of chemokine ligand/receptor

but, also, by proteolytic processing of chemokine ligands. In this regard, MMPs are major modulators of chemokine signaling. For example, MMP9 can activate CXCL8 by processing its amino terminus, but it also abolishes the function of CXCL1 by cleaving it. Likewise, chemotactic activity of CCL7 is decreased by MMP2, and CXCL12 is decreased by multiple MMPs. However, numerous other proteases have also been documented to act upon chemokines and modulate their activity<sup>[54]</sup>. Furthermore, recent observations suggest more complexity in signal transduction mechanisms that are induced by heterodimerization of GPCRs<sup>[55]</sup>. As indicated above, chemokine and chemokine receptor interactions are complex and are connected to multiple combinations of effector proteins and divergent intracellular signaling pathways (Figure 1).

### Effects of chemokines on tumor cell proliferation

Many growth factors and cytokines act to control cell proliferation, either in a positive or a negative manner<sup>[56]</sup>. For example, epidermal growth factor (EGF) and related family members activate the EGF receptor (EGFR) and initiate divergent biochemical cascades that result in transcription of genes involved in cell cycle progression and other processes necessary for growth. In contrast, transforming growth factor-beta negatively regulates epithelial cell growth by inhibiting cell cycle transit. Signal transduction pathways regulated by these and other growth factors frequently become altered during tumorigenesis, resulting in deregulated cell growth. It has now become clear that deregulated function of multiple chemokines also contributes to enhanced tumor cell proliferation.

The ELR<sup>+</sup> CXC-chemokines play important roles in melanoma cell growth<sup>[57,58]</sup>. CXCL1 has also been implicated in non-melanoma skin cancers, including tumors of neural origin and squamous cell carcinomas. Zhou *et al*<sup>[59]</sup> demonstrated that CXCL1 is highly expressed in anaplastic astrocytomas *in vivo*, and reported that this enhanced cell growth, motility, adhesion to extracellular matrix, and invasion *in vitro*, as well as enhanced aggressiveness *in vivo*. Constitutive expression of CXCL1 in squamous cell carcinomas results in formation of an autocrine growth loop through the CXCR2 receptor, while overexpression of CXCL1, CXCL2 and CXCR2 in esophageal cancer also enhances proliferation<sup>[60]</sup>. Furthermore, it was found that CXCL2 activates signal transduction through an ERK dependent pathway<sup>[61]</sup>.

Transcriptional upregulation mediated through nuclear factor  $\kappa$ B (NF $\kappa$ B)-dependent pathways has been reported to be largely responsible for the enhanced levels of CXCL1 in tumor cells. The tumor promoter okadaic acid, which inhibits protein phosphatases and results in hyperphosphorylation of proteins at serine and threonine residues, activates transcription through two response elements in the CXCL1 promoter, utilizing three distinct NF $\kappa$ B subunits (p65, p52 and c-Rel)<sup>[62]</sup>. Upregulation of NF $\kappa$ B by the upstream NF $\kappa$ B-inducing kinase is also an important mechanism by which CXCL1 is upregulated<sup>[63]</sup>. NF $\kappa$ B-dependent induction of CXCL1 is further regulated by poly (ADP-ribose) polymerase-1 (PARP-1). In-

active PARP-1 binds to the CXCL1 promoter and blocks transcription by excluding NF $\kappa$ B. However, activation of PARP-1 causes its promoter binding ability to be lost leading to NF $\kappa$ B upregulation of CXCL1. In melanoma cells, PARP-1 is highly expressed and active<sup>[64]</sup>, and may be a major contributor to melanoma cell proliferation *via* chemokine-dependent mechanisms, together with constitutively expressed NF $\kappa$ B.

### Chemokines in tumor cell migration, invasion and homing

In terms of cancer metastasis, chemokine-dependent mechanisms for targeting to specific secondary sites is now widely recognized after studies showed upregulation of CXCR4 and CCR7 in breast cancer cells and that activation of these receptors could induce actin polymerization, migration and invasion<sup>[19]</sup>. Importantly, ligands for these receptors were shown to be expressed in organs that represent the primary sites for breast cancer metastasis, strongly suggesting that ligand-receptor "homing" functions *in vivo* to target tumor cells to sites of secondary growth. Organ-specific metastasis has been reported for different tumor types, including breast<sup>[65]</sup>, ovary<sup>[46]</sup> and epidermoid carcinomas<sup>[60]</sup>. Further, more than twenty tumor types have been documented to overexpress CXCR4<sup>[66]</sup>. Upregulation of CXCR4 expression in tumor cells through the action of VEGF also appears to be an important mechanism to further enhance invasiveness<sup>[67]</sup>.

CXCR4/CXCL12 overexpression is associated with metastasis to lung, liver, lymph nodes and bone marrow. Rearrangement of the actin cytoskeleton and alteration in cell polarity are fundamental processes required for cell motility, regulated at least in some cases by CXCL12-CXCR4 pathways<sup>[68]</sup>. CXCL12-CXCR4 signaling may also contribute to tumor progression by upregulating protease expression. In prostate cancer cells, various MMPs were shown to be modulated by CXCL12<sup>[69]</sup>. However, these effects were not consistent for all cell lines examined, suggesting that cell-specific factors may influence the response to CXCL12. In glioma cells, CXCL12 induced expression of MMP15 but not gelatinases. RNA interference studies proved that glioma cell invasiveness *in vitro*, and tumor aggressiveness *in vivo*, is due to the upregulation of MMP15 by CXCL12<sup>[70]</sup>. Chemokine-receptor interactions in skin, as a frequent metastatic site of malignant melanoma, may be another example: CCL27 is highly expressed in skin and the CCR10 receptor for this ligand is frequently upregulated in melanoma cells<sup>[71,72]</sup>.

Furthermore, mounting evidence points to cancer-associated stromal fibroblasts playing important roles in modulating tumor cell behaviour<sup>[73,74]</sup>. As p53 mutation or loss has been shown to occur in stromal cells<sup>[75,76]</sup>, this may upregulate CXCL12 and enhance proliferation and motility<sup>[77]</sup>.

### Chemokines and tumor cell survival

The majority of metastatic tumor cells fail to colonize secondary lesions successfully, most likely due to induction of programmed cell death<sup>[78]</sup>. For metastasized lesions to grow, enhancement of growth factors as well as

positive molecular interactions with surrounding cells in the lymph node or other organs are indispensable. For example, EGFR signaling can activate survival pathways regulated by protein kinase B (AKT)<sup>[79]</sup>. However, despite the presence of available growth factors, tumor growth at the secondary lesion may still be uncertain: it may also require other factors and/or pathways in order for cells to survive and proliferate. Studies have also shown that resistance to cell death induced by loss of attachment to the extracellular matrix (anoikis) is an important element at least for certain tumors<sup>[80]</sup>. Interestingly, some chemokine-receptor combinations, such as CXCL5-CXCR2<sup>[48]</sup> and CCL19/21-CCR7<sup>[81]</sup>, can activate PI-3K and AKT, key regulators of cell survival. Thus, interaction of chemokines with their receptors could play roles in multiple and complex biological processes that are important for successful survival in metastasized locations. For example, CCL2 is well known for regulating cell migration and is a key mediator of breast cancer cell migration<sup>[82]</sup>. Also using breast cancer cell lines, Fang *et al.*<sup>[83]</sup> demonstrated enhanced cell migration and survival along with increased phosphorylation of Smad3 and mitogen-activated protein kinases (MAPKs) in response to CCL2 and, moreover, they found that levels of the innate receptor CCR2 were elevated in breast cancers, accompanied with CCL2 expression. These investigators also suggested that MAPK and Smad3 signaling function as an independent/alternative mechanism for cell survival. Furthermore, they showed that CCL2-induced Smad3 signaling through MAPKs regulates expression and activity of Rho GTPase, thereby facilitating breast cancer cell motility and survival. Therefore, beyond well-described canonical chemokine ligand/receptor signaling, new molecules may need to be considered as critical players in chemokine signaling in cancer. The CXCL12-CXCR4 signaling pathway has been shown to be important for the survival of leukemic B cells in chronic lymphocytic leukemia (CLL) through activation of AKT and ERK1/2<sup>[84,85]</sup>. Moreover, O'Hayre *et al.*<sup>[86]</sup> recently identified additional molecular targets and novel phosphoproteins as possible mechanisms for cell survival in CLL. Amongst these is programmed cell death factor 4, found in all CLL cells examined, and also heat shock protein 27, which mediates anti-apoptotic signaling and has previously been linked to chemotherapeutic resistance, which was detected in a subpopulation of CLL patients. If the roles of these cell-survival-related proteins are supported by further future studies, it may be worth considering identifying these and other phosphoproteins whose functions are modified by certain chemokines in specific pathological conditions, such as cancer and/or inflammation in gastrointestinal disease.

### Chemokines and angiogenesis

Development of microvessels is another critical event that enables oxygen delivery and nurtures tumor cell survival at both primary and secondary sites<sup>[87]</sup>. Recently, intensive studies in normal angiogenic development using mouse model systems have revealed the importance of the angiogenic chemokine CXCL12 for the organized

development of vessel branching along with neural development (<https://intramural.nhlbi.nih.gov/labs/labsn/pages/publications.aspx>). Furthermore, Komatsu *et al.*<sup>[88]</sup> reported the importance of the small-G protein R-Ras for the development of abnormal collateral capillary systems which may play critical roles in the survival of localized tumors by supporting nutrient and oxygen delivery<sup>[89]</sup>. Nonetheless, it remains unclear how the tumor cells, which express chemokine receptors, respond to chemokines released from these pathological vessels, which are inherently "leaky". Several different chemokines are known to be pro-angiogenic, notably CXCL5 and CXCL8. Koch *et al.*<sup>[90]</sup> clearly demonstrated that CXCL8 could induce neovascularization in a rabbit corneal pocket assay. Furthermore, they also showed that the angiogenic activity present in conditioned media derived from macrophages or monocytes from rheumatoid synovial tissues was dependent upon CXCL8. Indeed, macrophages have been reported to induce malignant progression in a breast cancer model by initiating the angiogenic switch<sup>[91]</sup>. Additionally, CXCL8-CXCR2 signaling facilitates migration and proliferation of endothelial cells<sup>[92]</sup>, and the AKT pathway is important for GPCR-dependent angiogenesis<sup>[93]</sup> following CXCR1 and CXCR2 activation<sup>[49,81]</sup>. Also, the human herpesvirus-8, an etiological agent of the highly vascular Kaposi's sarcoma, induces expression of CXCL8<sup>[94]</sup>, providing further evidence for chemokine involvement in tumorigenesis<sup>[95]</sup>.

Notably, in contrast to the ELR<sup>+</sup> chemokines discussed above, most ELR<sup>-</sup> chemokines have anti-angiogenic or angiostatic activity<sup>[96]</sup>. Among these, CXCL9, CXCL10 and CXCL11 are inducible by other cytokines, including members of the IL family and interferons. The angiostatic response of these cytokine-inducible chemokines is mediated through the CXCR3 receptor, found on the surface of endothelial cells<sup>[97]</sup>. Specifically, an alternatively spliced variant of the receptor - CXCR3B - has been shown to mediate this activity<sup>[98]</sup>. In addition to inhibiting endothelial cell migration, these chemokines also block proliferation. A further critical review focused on the development of tumor angiogenesis is available<sup>[99]</sup>.

## CHEMOKINES IN DIGESTIVE SYSTEMS/ DEVELOPMENT AND PROGRESSION

Compared to normal cells, many cancer cells overexpress chemokine and chemokine receptors. Ligation of overexpressed chemokine receptors on tumor cells and the specific chemokines released from target organs seem to be critical regulators of metastasis<sup>[19,100]</sup>. As outlined above, chemokines and their receptors play various important roles in the regulation of invasion, metastasis and dissemination of cancer cells. Here we review chemokine/chemokine receptor interactions, specifically in digestive organs.

### Oral cavity

In the head and neck region, which includes oral cavity,

**Table 1** Ligand specificity of chemokine receptors implicated in gastrointestinal disease

| CC-chemokines |                    | CXC-chemokines |                 | CX <sub>3</sub> C-chemokine |                     |
|---------------|--------------------|----------------|-----------------|-----------------------------|---------------------|
| Ligands       | Receptors          | Ligands        | Receptors       | Ligands                     | Receptors           |
| CCL2          | CCR2               | CXCL1          | CXCR2/<br>CXCR1 | CX <sub>3</sub> CL1         | CX <sub>3</sub> CR1 |
| CCL3          | CCR1/CCR5          | CXCL2          | CXCR2           |                             |                     |
| CCL4          | CCR5               | CXCL4L1        | CXCR3           |                             |                     |
| CCL5          | CCR1/CCR3/<br>CCR5 | CXCL5          | CXCR2           |                             |                     |
| CCL7          | CCR1/CCR2/<br>CCR3 | CXCL7          | CXCR2           |                             |                     |
| CCL8          | CCR3/CCR5          | CXCL8          | CXCR1           |                             |                     |
| CCL13         | CCR2/CCR3          | CXCL9          | CXCR3           |                             |                     |
| CCL19         | CCR7               | CXCL10         | CXCR3           |                             |                     |
| CCL20         | CCR6               | CXCL11         | CXCR3           |                             |                     |
| CCL21         | CCR7               | CXCL12         | CXCR4/R7        |                             |                     |
| CCL25         | CCR9               | CXCL14         | Unknown         |                             |                     |
| CCL27         | CCR10              | CXCL17         | Unknown         |                             |                     |

CXCL: CXC chemokine ligand; CXCR: CXC-chemokine receptor; CCR: CC-receptor; CX<sub>3</sub>CR: CX<sub>3</sub>C-receptor.

pharynx, larynx, nasal cavity and paranasal sinuses, the head and neck squamous cell carcinoma (HNSCC) accounts for more than 90% of malignant neoplasms<sup>[101]</sup>. Despite intensive efforts, survival rates have shown limited improvement over the decades. When primary tumor location is taken into account, the outcome can be even worse, with advanced hypopharyngeal tumors having a 4% five-year survival<sup>[102]</sup>. Metastasis of HNSCC is generally *via* the lymphatic system to loco-regional sites. Recently, several different chemokines were shown to be highly expressed in HNSCC derived cell lines and patient tumor samples. For example, in a series of 94 HNSCCs, CXCL1 was found to be overexpressed in around 40% of lesions<sup>[103]</sup>. Measurement of microvessel density (MVD) in HNSCCs revealed a correlation between CXCL1 expression and angiogenic activity, as well as with nodal metastasis and infiltration of leukocytes. CXCL8 has long been recognized to participate in autocrine (and possibly paracrine) regulation of HNSCC proliferation<sup>[60]</sup>. Recent studies using global gene expression profiling of primary and synchronous metastatic HNSCC further support previous reports of CXCL8 upregulation<sup>[104]</sup>. Along with this observation, Chen *et al.*<sup>[105]</sup> reported that hydrogen sulfide produced by *Porphyromonas gingivalis* bacteria in the oral cavity induced expression of CXCL8 in gingival and oral epithelial cells. Potentially, this may provide a link between tumor development and the induction of inflammation in periodontal disease, which is associated with persistent bacterial infection, and similar results to this study have also been reported<sup>[106]</sup>.

In addition to CXCL8, another ELR<sup>+</sup> angiogenic chemokine, CXCL5, is highly expressed in some HNSCCs. Data suggest that CXCL5 enhances tumor development by stimulating proliferation, cell motility and invasion<sup>[107]</sup> as knockdown of CXCL5 by siRNA completely inhibited tumorigenicity in a mouse xenograft model.

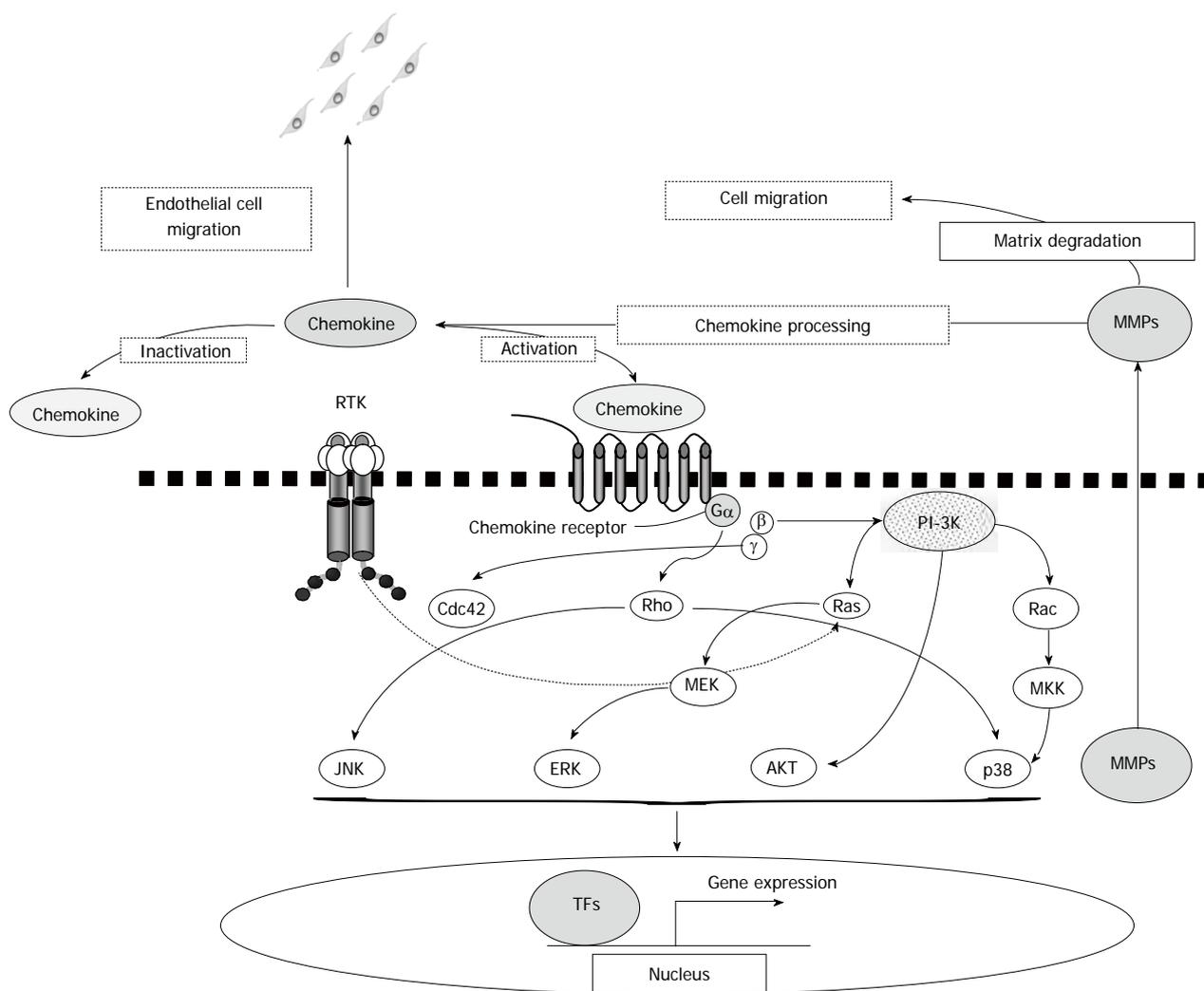
Furthermore, Delilbasi *et al.*<sup>[108]</sup> reported upregulated

CXCR4 expression in squamous carcinomas of the tongue by an immunohistochemical method. Of interest, their experiment exhibited no difference in expression between primary tumors of early and more advanced stage, although invading cells and those which had metastasized to lymph nodes exhibited higher CXCR4 expression, suggesting *in vivo* selection for this phenotype with malignant progression. Clatot *et al.*<sup>[109]</sup> also studied the possible correlation of CXCL12/CXCR4 expression and tumor recurrence and survival in HNSCC patients. They found no meaningful correlation between CXCR4 expression and either recurrence or survival, but a significant difference in CXCL12 expression. Further prospective studies are required to clarify this.

The loss of CCR6 expression in metastatic lesions with concomitant elevation of CCR7 in some HNSCCs was also documented<sup>[110]</sup>. CCL19 and CCL21, ligands for CCR7, induced migration of metastatic cells *in vitro*, whereas primary tumor cells responded to the CCR6 ligand, CCL20. Together, these data suggest that CCR7 upregulation might play a role in targeting tumor cells to sites of secondary growth *in vivo*, by facilitating entry into the lymphatic system and migration to regional lymph nodes. CCR7 signal transduction was also shown to activate cellular invasion and pro-survival pathways by PI-3K and PLC $\gamma$ -dependent, but EGFR-independent, mechanisms<sup>[111]</sup>.

### Esophagus

In the esophagus, several cytokines and chemokines are reported as possible mediators of gastroesophageal reflux, esophagitis, pre-cancerous change (typically Barrett's esophagus) and adenocarcinoma<sup>[112]</sup>. Amongst these, CXCL8 is reported as a molecular marker indicative of response to therapeutic procedures. For example, Oh *et al.*<sup>[113]</sup> compared the expression level of CXCL8 between pre- and post-operation of Nissen fundoplication in reflux esophagitis. They found that CXCL8 expression was significantly reduced postoperatively, as measured by quantitative real-time polymerase chain reaction (qRT-PCR). These authors also reported that CXCL8 expression was higher in patients with reflux compared to those without reflux. Furthermore, they found that patients with the highest CXCL8 expression were those with Barrett's dysplasia and adenocarcinoma. Chemokines and several other cytokines, such as ILs, CXCL8, and VEGFs were found to be upregulated in cancer-related cachexia, although the underlying mechanism is not understood<sup>[114]</sup>. Moreover, chemokine expression in tumors is complicated. Verbeke *et al.*<sup>[115]</sup> screened 51 patients operated on for colon adenocarcinoma, esophageal adenocarcinoma, or esophageal squamous cell carcinoma (SCC) by immunohistochemical staining to examine the expression of CXCL4L1, CXCL8, CXCL10, CXCL12, and VEGF. According to their study, the angiostatic chemokine CXCL4L1 was strongly expressed in colorectal cancer, while there was weaker expression in esophageal cancer. CXCL12 staining was almost negative in esophageal SCC, while stronger staining was observed in adenocarcinoma of the esophagus and colon. VEGF was moderately-to-strong-



**Figure 1 Chemokine-induced signal transduction pathways.** Schematic representation of signaling pathways activated by binding of chemokine ligands to their seven transmembrane G-protein coupled receptors. MMPs: Matrix metalloproteinases; TFs: Transcription factors; RTK: Receptor tyrosine kinase; ERK: Extracellular signal-regulated kinase; MEK: Mitogen-activated protein/extracellular signal-regulated kinase kinase; PI-3K: Phosphatidylinositol-3-OH kinase; MKK: Mitogen-activated protein kinase kinase; JNK: c-Jun N-terminal kinase; AKT: Protein kinase B.

ly positive in all 3 types of cancer, but relatively weaker in esophageal adenocarcinoma. Interestingly, not only was the expression of the angiogenic chemokines CXCL8 and CXCL12 heterogeneous in the samples, but so was the expression of the angiostatic chemokines CXCL10 and CXCL4L1. Based on these results, the roles of chemokines are likely complex in these tumors<sup>[115]</sup>. Recently, *in vitro* and *in vivo* studies using AMD3100, a pharmacological CXCR4 inhibitor, and the human HER2 inhibitor, trastuzumab, have indicated CXCR4 to be a positive regulator of human epidermal growth factor receptor 2 (HER2) expression in esophageal cancer. In this study, the authors also suggested a possible complex relationship between HER2 and CXCR4 in tumor development and metastasis<sup>[116]</sup>. These observations indicate further the involvement of multiple chemokines in molecular events that underpin different stages of cancer development.

### Stomach

In gastric cancer treatment, detection at an early stage of

tumor development is still the most effective curable approach. Amongst different types of gastric cancer, peritoneal dissemination is one of the most incurable conditions and no radical and effective treatment is available to date. Hashimoto *et al*<sup>[117]</sup> showed that the CXCL12/CXCR4 axis is important in peritoneal dissemination of gastric cancer cells. They found that CXCL12 ligation to CXCR4 on the cancer cell surface strongly and rapidly activates AKT-mammalian target of rapamycin signaling and co-activates production of other metastatic mediators, such as MMPs<sup>[117,118]</sup>. Graziosi *et al*<sup>[119]</sup> also suggested possible involvement of the p38 MAPK pathway in the development of peritoneal dissemination. Using an *in vivo* mouse system and administering p38 MAPK inhibitors ML3403 or SB203580, they observed decreased tumorigenesis. Microarray studies using these cancer cells identified several downregulated genes, such as CXCR4, fms-related tyrosine kinase 4, non-receptor spleen tyrosine kinase and collagen  $\alpha 2(IV)$ . Interestingly, p38 MAPK inhibitors induced significant downregulation of multidrug resistance-1 gene expression, a well-

defined marker of resistance to chemotherapy, which possibly induced susceptibility to the cisplatin treatment in this model<sup>[119]</sup>. In a separate study, Zhi *et al.*<sup>[120]</sup> examined the expression of CXCL12 in normal gastric tissue, gastric cancer cell lines, 35 primary gastric carcinomas and corresponding normal gastric tissues. They found that CXCL12 was downregulated in gastric cancer cell lines and patient samples of primary gastric carcinomas compared to normal samples. They provided evidence to show that CXCL12 expression was inversely associated with lymph node metastasis and histological grade. They concluded the possible cause of this downregulation of CXCL12 expression to be hypermethylation of the CXCL12 promoter, as treatment of highly metastatic cancer cell lines with a demethylating agent impaired its invasive phenotype<sup>[120]</sup>. One possible explanation for this reduction in CXCL12 might be to permit CXCR4-expressing gastric cancer cells to sense a CXCL12 gradient from target lymph nodes or other organs, which would otherwise be masked by CXCL12 expressed by the cancer cells. Together, these reports suggest that the peritoneal dissemination may be induced by the dissemination of CXCR4-expressing gastric cancer cells directed into the peritoneal area, which expresses high levels of CXCL12. Therefore, blocking molecular targets such as AKT and/or CXCR4 could be possible treatment strategies to prevent the activation of signaling events induced by CXCR4 ligation mediated by CXCL12 on the cancer cell surface. Moreover, preventing peritoneal dissemination itself might be possible by developing specific inhibitors that prevent binding between CXCR4 and its ligand. In this regard, Manu *et al.*<sup>[121]</sup> investigated plumbagin, which is a CXCR4 expression inhibitor, and it was widely effective in downregulating CXCR4 expression in cancer cell lines irrespective of the tissue of origin. The suggested mechanism of CXCR4 downregulation by plumbagin is through inhibition of NF $\kappa$ B. However, regulation of CXCR4 expression in cancer cells may not be so straightforward as initially expected. Bao *et al.*<sup>[122]</sup> found that HER2, which is frequently overexpressed in gastric cancer, interacts with CD44 and induces CXCR4 expression by blocking expression of microRNA-139. Additionally, EGF signaling may play an important synergistic role in concert with the CXCL12/CXCR4 axis in gastric cancer metastasis, as Yasumoto *et al.*<sup>[123]</sup> found that the EGFR ligands amphiregulin and heparin-binding EGF (HB-EGF)-like growth factor, as well as CXCL12, are highly expressed in malignant ascites. Their work showed that HB-EGF and CXCL12 together enhanced tumor necrosis factor  $\alpha$ -converting enzyme-dependent amphiregulin shedding from human gastric carcinoma (NUGC4) cells, which can promote proliferation of NUGC4 cells in animal models. These experiments strongly imply the possibility that several cancer signaling pathways besides the CXCL12/CXCR4 axis are important for development of gastric cancer metastasis.

When they screened 40 gastric cancer patient samples, Zhao *et al.*<sup>[124]</sup> found that CXCR4 mRNA levels were significantly higher in cases with lymph node metastasis than

those without; they also found that the CXCR4 protein level was correlated with poorly differentiated lesions, more advanced tumor stage and lymph node metastasis. Further, they reported higher CXCL12 mRNA in lymph nodes in patients with metastatic gastric cancer. Ingold *et al.*<sup>[125]</sup> used qRT-PCR to screen CXCL12/CXCR4 mRNA levels in 37 gastric carcinomas, and as well as screening protein levels in 347 gastric carcinomas and 61 matching lymph node metastases using tissue microarrays. They concluded that tumors expressing both CXCL12 in tumor cells and CXCR4 in adjacent microvessels showed a strong correlation with local tumor development and Union for International Cancer Control stages.

Another interesting study recently reported by Xu *et al.*<sup>[126]</sup> describes the possible activation of lymphangiogenesis pathways in gastric cancer by CXCL1 secretion. A technique was established in an animal model for recovery of lymphatic endothelial cells (LECs) from afferent lymph vessels of sentinel lymph nodes, and the gene expression profile between normal LECs and LECs with lymph node metastasis was compared using microarray analysis. They found that CXCL1 stimulated LEC migration and tube formation through FAK-ERK1/2-RhoA activation and reorganization of F-actin. Importantly, it is well known that CXCR2 expression, which is a CXCL1 receptor, is positively correlated with tumor, node, and metastasis (TNM) stage and lymphatic vessel density<sup>[127-130]</sup>. Thus, this study may strongly imply a role for CXCL1 in metastasis mediated through effects on LECs. CXCL8 has also been well studied in gastric cancer development. Using the gastric cancer cell line SCG-7901, Ju *et al.*<sup>[131]</sup> found that CXCL8 can enhance several tumor parameters, such as adhesion to endothelial cells, migration, and invasion. The expression of MMP9, intercellular adhesion molecule (ICAM)-1 and E-cadherin was upregulated in a dose-dependent manner. However, CXCL8 did not affect the proliferation of SCG-7901 cells under these conditions. Several groups have studied polymorphism of the CXCL8 251 allele. Wang *et al.*<sup>[132]</sup> reported the CXCL8 251 allele AA genotype as a risk factor for gastric cancer in Asian groups but not in Caucasian or Mexicans. Furthermore, statistical analysis revealed that the TA and AA polymorphism is significantly associated with the diffuse type of gastric cancer, and the AA genotype was found to be a risk factor for gastric cardia cancer. Song *et al.*<sup>[133]</sup> reported screening results of this allele in *Helicobacter pylori* (*H. pylori*)-infected Korean populations. They found a significant correlation between MMP9 and disease progression in the AA and AT genotype. Angiopoietin-1, which plays an important role in vascular development, showed upregulation, but not VEGF expression or disease progression in the AA genotype. They predicted that the CXCL8 251 AA genotype may be associated with angiogenesis in gastric carcinogenesis in the *H. pylori*-infected population. Vinagre *et al.*<sup>[134]</sup> reported that interaction between CXCL8 251 (AA and AT) allele mutation carriers and the infection of *H. pylori* strain [phosphoinositide PI4, 5P(2) binding protein (s1m1)

cytotoxin-associated gene A product (*cagA*) positive] may have higher risk for development of gastric adenocarcinoma. In addition, Schneider *et al.*<sup>[135]</sup> pointed out the complexity and difficulty in selecting *in vitro* epithelial study models to understand the molecular mechanisms of *H. pylori* infection. Moreover, a meta-analysis carried out by Liu *et al.*<sup>[135]</sup> indicated that the variable results reported by many different studies can be affected by differences of histological type, tumor location, *H. pylori* infection, ethnicity and geographic location<sup>[136]</sup>. Another CXC chemokine, CXCL5, was also reported recently to be a potential molecular marker for cancer development, especially for late stage gastric cancer<sup>[137]</sup>. However, it is not yet confirmed if this observation is a consequence of secondary change as a result of gastric cancer progression, or whether it is one of the primary molecular events leading to tumor development. As CXCL5 is linked to tumor development in other organs<sup>[77,107,138]</sup>, further confirmation is needed through the use of *in vitro* and *in vivo* model systems in the future. Yanagie *et al.*<sup>[139]</sup> performed a comparative analysis of differential gene expression related to chemokines/chemokine receptors and cytokines in established gastric cancer cell lines using a cDNA microarray approach. They found that CC-chemokines CCL2, 5, 21 and CXC-chemokines CXCL1, 7, 8, 12, 14 and chemokine receptor CCR6 were upregulated, while CCL3 and CCL25 were downregulated. These chemokines and their receptors may be potential candidates for cancer diagnosis and/or treatment.

Collectively, in gastric cancer development and metastasis, multiple chemokines likely play important roles. Further studies are required to elucidate the many functional roles of these chemokines, especially in synergistic regulation of the signaling pathways that control development of gastric cancers. Another detailed review on the role of chemokines in esophageal and gastric cancer is available<sup>[140]</sup>.

### Liver

The liver is one of the major metastatic targets of colon cancer and this attributes directly to patient mortality. Many molecules have been proposed as being responsible for the development of hepatic metastases, and accumulated data suggest there are several important signaling pathways involved in the development of both primary and metastatic liver cancer. Among them, the CXCL12-CXCR4, CX3CL1-CX<sub>3</sub>CR1, and the CCL20-CCR6 axes have received much attention<sup>[141]</sup>.

CXCR4 has been found to be a prognostic marker in various types of cancer as it plays an important role in normal stem cell homing. Cancer stem cells also express CXCR4, which implies that this axis may control the trafficking and metastasis of these cells to organs that express CXCL12, and the liver is one of these<sup>[142]</sup>. Recently, Li *et al.*<sup>[143]</sup> reported that the expression of CXCR4 was higher in portal vein tumor thrombus tissue than hepatocellular carcinoma (HCC), and lentivirus-mediated siRNA knockdown of CXCR4 was shown to impair the potential invasiveness of tumor thrombus cells significantly. By

screening tissues from 42 HCC patients, including tumor and adjacent regions, and comparing to tissues from cancer-free individuals, Liu *et al.*<sup>[128]</sup> found that CXCR2 mRNA was significantly higher in HCC than in adjacent or normal liver tissues. Interestingly, TNM staging was not correlated to the level of CXCR2 mRNA but the protein level was relevant to staging, as protein was markedly higher in lesions classified as Stage III/IV. CXCR2 mRNA and protein levels were correlated with intrahepatic metastasis, portal cancer embolus, and low differentiation. Other studies recently reported a possible therapeutic use of CCL2 for prevention of HCC metastasis. In a model of HCC, coupling adenovirus-based CCL2 and the thymidine kinase/ganciclovir expression was shown to prevent intrahepatic metastasis *in vivo*. This combination was also shown to induce an innate immune response involving monocytes/macrophages and NK cells, leading to prolongation of anti-metastatic effects<sup>[144-146]</sup>. CCL2 was also identified by Chen *et al.*<sup>[147]</sup> as a potential target for development of future HCC treatment. Using the recombinant foot-and-mouth disease virus capsid protein VP1 (rVP1), they were able to induce apoptosis of HCC cell lines through deactivation of the AKT pathway and stimulation of caspase cascades *via* Bax. Furthermore, rVP1 downregulated the expression of CCL2 in an AKT-dependent manner, which can support the survival and migration of HCC tumor cell lines<sup>[147]</sup>. CXCR7, a CXCL12 receptor, has also been reported to be upregulated in HCC<sup>[148]</sup>. In another study, shRNA knockdown of CXCR7 expression was found to inhibit many facets of tumor development, such as cell invasion, adhesion, VEGF secretion, endothelial tube formation and tumor growth, although it did not affect metastasis *in vivo*<sup>[149]</sup>. CXCR4, an alternative receptor to CXCR7 for CXCL12, is known to play important roles in liver metastasis. CXCL12 is expressed by endothelial cells and likely by Kupffer cells lining the liver sinusoids. The binding of CXCL12 to CXCR4 activates Rho, Rac and Cdc42, enabling tumor cell extravasation without affecting cell adhesion<sup>[150]</sup>.

In addition to direct roles in cancer development, chemokines make important contributions to chronic inflammatory diseases such as chronic hepatitis, which can be a possible precancerous condition. In chronic hepatitis, regulation of lymphocyte motility is controlled by several independent biochemical pathways. However, these signaling events are not well elucidated despite well-documented observations of lymphocyte recruitment to tissues *via* endothelium. Holt *et al.*<sup>[151]</sup> suggested that activated human liver myofibroblasts (aLMF) affect the migration and accumulation of lymphocytes within the inflamed liver. Also, when cultured *in vitro*, aLMF from inflamed human livers and hepatic stellate cells from non-inflamed livers secrete a distinct profile of cytokines and chemokines. The aLMF-conditioned media had chemotactic activity for lymphocytes, which was partially inhibited by pertussis toxin, implying a requirement for GPCR signaling. Additionally, contribution of GPCR-independent lymphocyte chemotaxis by IL-6, hepatocyte growth factor, and VEGF was also reported<sup>[151]</sup>.

## Pancreas

As a multitude of studies expand our knowledge of pancreatic disease, the important role of chemokines in the development of pancreatic cancer is becoming evident. CCL20 is well known for its expression in various human cancers<sup>[152-155]</sup>. In pancreatic adenocarcinoma (PAC) patients, CCL20 mRNA and protein expression was found to be significantly associated with advanced T-stage<sup>[154]</sup>. It is interesting that CCR6, a canonical CCL20 receptor, is upregulated in chronic pancreatitis, pancreatic cystadenoma and pancreatic carcinoma compared to controls<sup>[154]</sup>. It has also been reported that, in metastatic pancreatic carcinoma, expression of the angiogenic chemokines CXCL5 and CXCL8 is highly elevated compared to pancreatic cystadenoma or chronic pancreatitis<sup>[138]</sup>. This observation suggests a potential contribution of these chemokines to the development of metastatic pancreatic cancer. Furthermore, another report suggests that CXCL5 may be a possible prognostic biomarker for pancreatic cancer. Li *et al.*<sup>[156]</sup> reported that overexpression of CXCL5 is correlated with poorer tumor differentiation, advanced clinical stage, and shorter patient survival. These authors also performed xenograft assays in nude mice, in which they used shRNA downregulation of CXCL5 or antibody-mediated neutralization of CXCR2 and showed attenuation of pancreatic tumor cell growth. They also demonstrated that CXCL5 derived angiogenesis development was ERK, AKT and signal transducer and activator of transcription mediated signaling pathways<sup>[156]</sup>. Moreover, in a clinical study that analyzed 52 PACs and 52 pancreatic neuroendocrine tumors, Hussain *et al.*<sup>[157]</sup> found that expression of CXCL8 and its receptors CXCR1/2 were significantly upregulated compared to normal pancreatic tissues, a finding confirmed by immunohistochemistry and qRT-PCR. This may suggest the existence of autocrine and/or paracrine loops that contribute to the development of these tumors. As in other organs, many reports have suggested the importance of the CXCL12-CXCR4 axis in the development of pancreatic cancers. Cui *et al.*<sup>[158]</sup> compared expression of CXCL12 and CXCR4 between tumor and surrounding tissues. In tumor tissues, CXCL12 expression was significantly lower than that found in paracancerous tissues, normal pancreas, or lymph nodes. In contrast, CXCR4 expression in cancerous tissues was significantly higher than that in normal tissue. Furthermore, expression patterns of the CXCL12/CXCR4 and clinicopathological status showed a strong correlation, including lymph node metastasis. Additionally, CXCL12 expression was significantly associated with MVD but not with microlymphatic vessel density, while CXCR4 expression showed the opposite relationship<sup>[158]</sup>. These observations imply a significant role for CXCL12/CXCR4 signaling in the development and progression of metastatic pancreatic cancer. Also, in another study, samples from 249 PAC patients were screened by immunohistochemistry and tissue microarray for expression of innate CXCL12 receptors CXCR4 and CXCR7. Expression of CXCR7 was found to be associated with tumor grade, inversely associated

with tumor size, and possibly associated with tumor progression and differentiation<sup>[159]</sup>. Interestingly, though, no significant correlation was found between CXCR4 expression and clinicopathological parameters, which may seem to be inconsistent with previous reports<sup>[158]</sup>. However, this may be a manifestation of different mechanisms of pathogenesis. Together with these observations, data from many studies suggest CXCL12-CXCR4 signaling may be a rational therapeutic target to prevent the development and metastasis of pancreatic tumors<sup>[160,161]</sup>. For example, the activation of this axis can be attenuated by suppressing NF $\kappa$ B activity<sup>[162]</sup>. In pancreatic cancer, CX<sub>3</sub>CL1 and its cognate receptor CX<sub>3</sub>CR1, is another possible ligand-receptor combination associated with the pathogenesis of pancreatic cancer. Marchesi *et al.*<sup>[163,164]</sup> reported involvement of CX<sub>3</sub>CR1 in perineural invasion and dissemination of neoplastic cells along intra- and extra-pancreatic nerves. Use of CX<sub>3</sub>CR1 as a possible therapeutic candidate is discussed in detail elsewhere<sup>[165]</sup>. Pancreatic cancer is one of the most aggressive and intractable malignancies amongst all cancers<sup>[166]</sup>. Therefore, finding molecular markers to facilitate accurate diagnosis at early stages of disease is an urgent need. From the evidence available, several chemokine-receptor pairs may be good candidates. A combination of several markers, together with these chemokines, may be promising diagnostic tools, such as CXCL17-ICAM2<sup>[167]</sup> and a classical molecular marker for pancreatic cancer, carbohydrate antigen 19-9, together with CXCL7<sup>[168]</sup>.

## Small and large intestine

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the colon and small intestine. It is now considered that chemokine-mediated chronic inflammation is a direct cause of colitis-associated cancer (CAC). However, the underlying mechanism of CAC is complex and not well elucidated. CC-chemokines and their receptors have long been recognized as key players in tumor promotion and progression during the course of chronic colitis. In several reports, expression of CC-chemokines, including CCL2, is highly upregulated in the colonic mucosa of IBD patients, as well as in the azoxymethane and dextran sulfate sodium experimental colitis model system<sup>[169]</sup>. Observations in the D6 receptor knockout mouse provide another important example of the role of chemokines in IBD. D6 is a silent receptor known to bind to a wide array of pro-inflammatory chemokines promiscuously, including CCL2, 5, 8, 7, and 13 in humans. Compared to wild type mice, D6 knockout mice were found to be more susceptible to chemically-induced colitis and failed to recover from the colitis. This may be due to the lack of the D6 receptor, which usually sequesters overexpressed chemokines, failing to stop the development of symptoms<sup>[170]</sup>, and D6 plays a similar role in preventing liver injury<sup>[171]</sup>. Consistent with a suppressor role for D6, lymphatic vessels expressing D6 were demonstrated in epithelium and connective tissue of both small and large intestine<sup>[172]</sup>. Using a CCR2 knockout mouse or a CCL2 antagonist, Popivanova *et al.*<sup>[173]</sup> showed that

CCL2 is a crucial mediator of colon cancer development, as mice lacking CCL2 had reduced intracolonic macrophage infiltration and COX-2 expression, attenuated neovascularization, and reduced numbers and size of colon tumors.

B-lymphocytes collected from patients with Crohn's disease (CD) express toll-like receptor 2 (TLR2) at the cell surface and secrete high levels of CXCL8, and the clinical disease course is well correlated with CXCL8 expression<sup>[174]</sup>. In contrast, ulcerative colitis (UC) patients also express TLR2, but do not secrete CXCL8 in large amounts. However, CXCL8 is inducible in UC by stimulating TLR2 and its clinical activity correlates inversely with levels of circulating TLR2-positive B cells, converse to CD<sup>[174]</sup>. In a model of experimental colitis, it was shown that infection with *Bacillus polyfermenticus* (*B. polyfermenticus*) affects the biological responses of human intestinal microvascular endothelial cells, including cell migration, tube formation, and permeability through an NF $\kappa$ B/CXCL8 signaling axis<sup>[175]</sup>. Interestingly, this study also reported that *B. polyfermenticus* can accelerate the healing process of colitis by stimulating mucosal angiogenesis. Lopez *et al.*<sup>[176]</sup> found that *Lactobacillus rhamnosus* GG (LGG), a probiotic, can downregulate the flagellin-induced expression of CXCL8. Although the biological mechanisms of LGG in the maintenance of intestinal homeostasis are largely unknown, it is proposed that LGG may modulate induction of CXCL8 by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in the intestinal epithelium<sup>[176,177]</sup>. In colorectal adenocarcinoma cells HCT-116 and HCT-8, immunohistochemistry revealed that diverse stimuli can upregulate CXCL8 expression<sup>[115]</sup>, with upregulation of CXCL4L1 and synergistic CXCL8 and CXCL10 induction in carcinoma cells by IL-1 and TNF- $\alpha$  or immunoreactive fibronectin. In addition, full-length and N-terminally truncated (more active) CXCL8 was identified in HT-29 colorectal adenocarcinoma cells, as well as strong expression of CXCL4L1 and CXCL12 in patient samples<sup>[115]</sup>. Moreover, ERK2 and PI-3K/AKT have been identified as candidate pathways for induction of CXCL8 expression in HCT-15 colon cancer cells and MKN-45 gastric adenocarcinoma cells<sup>[178]</sup>.

Arijs *et al.*<sup>[179]</sup> reported that, in inflamed colonic IBD mucosa, many leukocyte/endothelial cell adhesion molecules (CAMs) and chemokines/chemokine receptors are upregulated, while E-cadherin gene expression was downregulated. Microarray analysis revealed that infliximab (an anti-TNF- $\alpha$  antibody) restores colonic gene expression of endothelial CAMs and most chemokines/chemokine receptors to normal levels of expression, with only CCL20 and CXCL1/2 expression remaining elevated after treatment. In addition to the previously identified 47 integrin-MADCAM1 axis, this study revealed a number of interesting targets for anti-adhesion therapy, including PECAM1, CXCL8, and CCL20, suggesting that anti-TNF- $\alpha$  therapy may work, at least in part, by downregulating certain CAMs<sup>[179]</sup>. Upregulation of CXCL1, CXCR1 and CXCR2 was reported by Oladipo *et al.*<sup>[180]</sup> in tumor epithelium compared to normal adjacent

tissue collected from patients with stage II and III CRC. In their analysis, no overall association between CXCL1, CXCR1 or CXCR2 expression and prognostic endpoints was found, although survival analysis demonstrated an inverse association between CXCL1 and recurrence-free survival in stage III patients. Interestingly, CXCL8 positivity in the tumor infiltrate correlated with earlier disease stage and improved relapse-free survival in multivariate Cox regression analysis<sup>[180]</sup>. Schroepf *et al.*<sup>[181]</sup> screened samples collected from 501 German patients with IBD (336 CD, 165 UC) including 258 children and 243 adults as well as 231 controls. They found CXCR3 pathway-related genes to be significantly overexpressed in inflamed colonic tissue of pediatric CD and UC patients. CXCL9, 10, and 11 are 3 innate ligands for CXCR3, and this study found a correlation between polymorphism in CXCL9 and pediatric CD, while carriers of the hetero- and homozygous genotype variants of CXCL11 rs6817952 were at increased risk for UC in all age groups. Thus, blockade of CXCR3 could be a possible therapeutic avenue in the future<sup>[181]</sup>. In a study using HT-29 colon cancer cells, Lee *et al.*<sup>[182]</sup> reported multiple regulatory roles of IL-17 on chemokine expression. Their results indicated a positive effect of IL-17 on chemokines that recruit neutrophils (CXCL8 and CXCL1) and Th17 cells (CCL20). Contrary to this, IL-17 represses expression of CXCL10, CXCL11, and CCL5, three chemokines that selectively recruit Th1 lymphocytes.

Collectively, these findings suggest that synergistic targeting of critical proteins such as chemokines and their receptors may lead to improved treatment outcomes for inflammatory bowel disease and cancer. Table 2 is attached as a summary of published studies related to chemokine/chemokine receptors and gastrointestinal diseases according to their description in this review.

## CHEMOKINE NETWORK AS A THERAPEUTIC TARGET

With the recent advances in understanding of the many and varied roles of chemokines and their receptors in tumor development and progression, together with the advent of targeted molecular therapies, excellent opportunities exist to develop novel approaches to treat cancer. This would appear to be of particular relevance for gastrointestinal malignancies, where radical improvements in clinical outcome have so far been elusive.

## CONCLUSION

It is clear that chemokine networks play critical roles in inflammatory diseases and cancer progression, and tumor cells may influence their own proliferation as well as affecting stromal and immune system cells, and *vice versa*. Therefore, the chemokine network makes an attractive target for therapeutic intervention in many tumor types, including those of the gastrointestinal tract. However, we need to define more selective and specific targets, to minimize systemic side effects during treatment.

**Table 2 Chemokines/chemokine receptors in this table appear sequentially according to their description in this review**

| Organ         | Chemokines and receptors | Possible role/observed phenomenon   |   |
|---------------|--------------------------|---|---|
| Oral cavity   | CXCL1                    | Angiogenic activity <sup>[103]</sup>  |   |
|               | CXCL8                    | Proliferation, metastasis, tumor development and the induction of inflammation in periodontal disease <sup>[60,104-106]</sup>   |   |
|               | CXCL5                    | Proliferation, cell motility and invasion <sup>[107]</sup>  |   |
|               | CXCR4                    | Enhancement of invasiveness <sup>[108]</sup>  |   |
|               | CXCL12                   | Upregulation in metastasis <sup>[109]</sup>   |   |
|               | CCR6, CCR7               | Involvement in metastatic activity <sup>[110]</sup>   |   |
|               | CCR7                     | Enhancement of invasion <sup>[111]</sup>  |   |
| Esophagus     | CCL20                    | Upregulated with bacterial infection in OSCC cell lines <sup>[152]</sup>  |   |
|               | CXCL8                    | Possible index of inflammation, upregulation in cancer-related cachexia <sup>[113,114]</sup>  |   |
|               | CXCR4                    | Positive regulator of HER <sup>[116]</sup>  |   |
| Stomach       | CXCL12, CXCR4            | Metastasis through activation of AKT-mTOR pathway and MMPs, upregulation in lymph node metastasis, strong correlation with tumor development <sup>[117,118,124]</sup>   |   |
|               | CXCR4                    | Enhancement of metastasis through p38 signaling pathway <sup>[119]</sup>  |   |
|               | CXCL12                   | Acquisition of invasive/metastatic phenotype, enhancement of proliferation when coexpressed with other molecules <sup>[120,123]</sup>   |   |
|               | CXCL1                    | Activation of lymphangiogenesis by stimulating LECs <sup>[126]</sup>  |   |
|               | CXCR2                    | Strong correlation with TNM staging and lymphatic vessel density <sup>[127-130]</sup>   |   |
|               | CXCL8                    | Enhancement of tumor development factors, and a possible risk factor as mutant, association with angiogenesis, development of gastric adenocarcinoma <sup>[132-134,183]</sup>   |   |
|               | CXCL5                    | Marker for late stage gastric cancer <sup>[137]</sup>   |   |
|               | Other candidates         | CC-chemokines (CCL2, 3, 5, 21, 25)/CXC-chemokines (1, 7, 8, 12, 14)/CCR6 <sup>[139]</sup>   |   |
|               | Liver                    | CXCR4   | Metastasis, upregulation in PVTT <sup>[142-143]</sup>   |
|               |                          | CXCR2   | Upregulation in HCC, especially in late stage <sup>[128]</sup>  |
| CCL2          |                          | Application to prevent metastasis, application to prevent HCC by deactivating AKT pathway <sup>[144-147]</sup>  |   |
| CXCR7         |                          | Upregulation in HCC, functional in tumor development and angiogenesis but not in metastasis <sup>[148-149]</sup>  |   |
| CXCL12, CXCR4 |                          | Enhancement of tumor cell extravasation through upregulation of Rho/Rac/Cdc42 <sup>[150]</sup>  |   |
| CCR6          |                          | Upregulation in metastasis <sup>[153]</sup>   |   |
| CCL20         |                          | Enhanced expression in HCC <sup>[155]</sup>   |   |
| D6 receptor   |                          | Prevention of liver injury <sup>[171]</sup>   |   |
| Pancreas      |                          | CCL20   | Associated with tumor staging <sup>[154]</sup>  |
|               |                          | CCR6  | Upregulated in chronic pancreatitis, pancreatic cystadenoma and pancreatic carcinoma <sup>[154]</sup> |
|               | CXCL5, CXCL8             | Upregulation in metastatic pancreatic carcinoma <sup>[140]</sup>  |   |
|               | CXCL5                    | Correlated with poorer tumor differentiation, advanced clinical stage, and shorter patient survival, and ERK, AKT and STAT mediated angiogenesis <sup>[156]</sup>   |   |
|               | CXCL8, CXCR1/2           | Upregulation in adenocarcinomas and neuroendocrine tumors <sup>[157]</sup>  |   |
|               | CXCL12, CXCR4            | Downregulation of CXCL12 and upregulation of CXCR4 in tumors. CXCL12 correlated with MVD but not with MLVD, while CXCR4 showed opposite pattern <sup>[158]</sup>  |   |
|               | CXCR4, CXCR7             | CXCR7 associated with tumor grade, inversely associated with tumor size, and possibly associated with tumor progression and differentiation but not with CXCR4 <sup>[159]</sup>   |   |
|               | CXsCL1, CX3CR1           | Perineural invasion and dissemination of neoplastic cells along intra- and extra-pancreatic nerves <sup>[163,164]</sup>   |   |
|               | CXCL17 (+ ICAM2)         | Diagnostic molecular marker <sup>[167]</sup>  |   |
|               | CXCL7 (+ CA19-9)         | Diagnostic molecular marker <sup>[168]</sup>  |   |
| Colon         | CXCL4L1                  | Upregulation in colorectal cancer <sup>[115]</sup>  |   |
|               | CCL2                     | upregulation in mucosa of IBD <sup>[169]</sup>  |   |
|               | D6 receptor              | Plays role of sequestering several chemokines (in mouse colitis model experiment), plays suppressive role in the development and growth of vascular tumors <sup>[170,172]</sup>   |   |
|               | CCL2, CCR2               | important mediator in colon tumor development <sup>[173]</sup>  |   |
|               | CXCL8                    | Upregulation along with the development of Crohn's disease, affecting biological responses of human intestinal microvascular endothelial cells in colitis model, positively correlated with earlier disease stage and improved relapse-free survival <sup>[164,175,180]</sup> |   |
|               | CXCL10, CXCL41           | Synergistic upregulation with CXCL8 by diverse stimuli, induction by ERK2 and PI-3K/AKT pathway <i>via</i> PAR2 <sup>[175,178]</sup>  |   |
|               | CCL20, CXCL1/2, CXCL8    | Remains high even after the treatment with anti-TNF antibody <sup>[179]</sup>   |   |
|               | CXCL1, CXCR1/2           | Upregulation in stage II and III CRC, upregulation in stage II and III CRC <sup>[180]</sup>   |   |
|               | CXCR3 pathway            | CXCL9-pediatric Crohn's disease, CXCL11-UC in all age groups <sup>[181]</sup>   |   |
|               | Other chemokines         | IL-17 affects CXCL8, CXCL1, CCL20, CXCL10, CXCL11 and CCR5 in colon cancer cells <sup>[182]</sup>   |   |

CXCL: CXC chemokine ligand; CXCR: CXC-chemokine receptor; CCR: CC-receptor; CXsCR: CXsC-receptor; CA19-9: Carbohydrate antigen 19-9; OSCC: Oral squamous cell carcinoma; HER: Human epidermal growth factor receptor; AKT: Protein kinase B; mTOR: Mammalian target of rapamycin; MMPs: Matrix metalloproteinases; LECs: Lymphatic endothelial cells; TNM: Tumor, node, and metastasis; PVTT: Portal vein tumor thrombus; HCC: Hepatocellular carcinoma; STAT: Signal transducer and activator of transcription; ERK: Extracellular signal-regulated kinase; MVD: Microvessel density; MLVD: Microlymphatic vessel density; IBD: Inflammatory bowel disease; PI-3K: Phosphatidylinositol-3-OH kinase; PAR2: Protease-activated receptor-2; CRC: Colorectal cancer; IL: Interleukin; TNF- $\alpha$ : Tumor necrosis factor- $\alpha$ .

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P- Reviewer Fernandez TD S- Editor Zhai HH  
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## Herbal hepatotoxicity: Challenges and pitfalls of causality assessment methods

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Received: February 21, 2013 Revised: April 11, 2013

Accepted: April 17, 2013

Published online: May 21, 2013

toxicity cases, compared to numerous other causality assessment methods, which are inferior on various grounds. Among these disputed methods are the Maria and Victorino scale, an insufficiently qualified, shortened version of the CIOMS scale, as well as various liver un-specific methods such as the *ad hoc* causality approach, the Naranjo scale, the World Health Organization (WHO) method, and the Karch and Lasagna method. An expert panel is required for the Drug Induced Liver Injury Network method, the WHO method, and other approaches based on expert opinion, which provide retrospective analyses with a long delay and thereby prevent a timely assessment of the illness in question by the physician. In conclusion, HILI causality assessment is challenging and is best achieved by the liver specific CIOMS scale, avoiding pitfalls commonly observed with other approaches.

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**Key words:** Herbal hepatotoxicity; Herb induced liver injury; Herbs; Drug hepatotoxicity; Drug induced liver injury; Causality assessment

### Abstract

The diagnosis of herbal hepatotoxicity or herb induced liver injury (HILI) represents a particular clinical and regulatory challenge with major pitfalls for the causality evaluation. At the day HILI is suspected in a patient, physicians should start assessing the quality of the used herbal product, optimizing the clinical data for completeness, and applying the Council for International Organizations of Medical Sciences (CIOMS) scale for initial causality assessment. This scale is structured, quantitative, liver specific, and validated for hepatotoxicity cases. Its items provide individual scores, which together yield causality levels of highly probable, probable, possible, unlikely, and excluded. After completion by additional information including raw data, this scale with all items should be reported to regulatory agencies and manufacturers for further evaluation. The CIOMS scale is preferred as tool for assessing causality in hepa-

**Core tip:** This review focuses on diagnostic causality assessment algorithms that have been used so far in herb induced liver injury (HILI) cases. Detailed information of the various methods with their strengths and weaknesses is provided including their challenges and pitfalls that emerged during the assessing course. For the physician caring for a patient with suspected HILI, the Council for International Organizations of Medical Sciences (CIOMS) scale is the preferred tool for assessing causality compared to numerous other causality assessment methods, which are inferior on various grounds. CIOMS based assessment should start at the day HILI is suspected to ensure completeness of clinical data.

Teschke R, Frenzel C, Schulze J, Eickhoff A. Herbal hepatotoxicity: Challenges and pitfalls of causality assessment methods. *World J Gastroenterol* 2013; 19(19): 2864-2882 Available from:

URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2864.htm>  
 DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2864>

## INTRODUCTION

A total of 60 herbs, herbal drugs, and herbal dietary supplements have been reported to cause herb induced liver injury (HILI), though convincing causality assessment rarely was provided<sup>[1]</sup>. Presented as a tabular compilation, these 60 different herbal products were based on a recent analysis of 185 case reports, spontaneous reports, review articles, and comments. The consideration of possible hepatotoxicity in various reports has been discussed by the National Institutes of Health (NIH) in their recently released LiverTox database, covering a selected group of herbal and dietary supplement (HDS) products<sup>[2,3]</sup>. Among these are: Aloe vera, Black cohosh (BC), Cascara, Chaparral, Chinese and other Asian herbal medicines (*Ba Jiao Lian*, Chi R Yun, Ephedra, *Jin Bu Huan*, Sho Saiko To and Dai Saiko To, *Shou Wu Pian*), Comfrey, Fenugreek, Germander, Ginkgo, Ginseng, Glucosamine, Greater Celandine, Green Tea, Hoodia, Horse Chestnut, Hyssop, Kava, Margosa Oil, Milk Thistle, Noni, Pennyroyal, St John's Wort, Saw Palmetto, Senna, Skullcap, Usnic acid, Valerian, and Yohimbine<sup>[2,3]</sup>. However, causality confirmation was surprisingly rare for individual cases of suspected herbal hepatotoxicity, which often were published as narrative and anecdotal reports without valid and transparent data collection<sup>[1-3]</sup> that require stringent efforts for causality attribution<sup>[4]</sup>.

The focus of this review is on causality assessment methods for herbal hepatotoxicity with particular reference to liver specific evaluation methods. This approach gives insight into challenges and pitfalls of these methods with surprising clinical and regulatory issues. Valid causality assessment of assumed HILI cases is required for further case evaluations, otherwise speculations and fruitless discussions will emerge.

## DATA BASIS FOR CAUSALITY ASSESSMENT

### Herbal product essentials

Herbal product quality aspects are of primary concern, the respective evaluation should start at the day HILI is suspected. The products are destined for human use and must meet the highest possible quality based on specific standards (Table 1)<sup>[4-7]</sup>. Despite fulfilment of quality standards, batch and product variability is common<sup>[4,8-10]</sup>. Therefore, additional specific production quality standards have been described, for instance, as a proposal for a Kava Quality Standardization Code<sup>[8]</sup>. It details standardization of overall herbal quality and specifically addresses chemical, agricultural, manufacturing, nutritional, regulatory, and legislation standardizations. In addition, labelling and consumer leaflet of herbal drugs and herbal dietary supplements should mandatorily provide

a clear definition and identification of the plant family, subfamily, species, subspecies, and variety as classical botanical description for any herb used as an ingredient of a herbal product (Table 1)<sup>[4,8]</sup>.

As an example, several hundred kava varieties exist<sup>[8-11]</sup>, but specific information on kava variety identification was missing in all spontaneous reports and case report publications of suspected hepatotoxicity. This leaves open which kava variety had to be incriminated<sup>[9-17]</sup>. On the other hand, the regulatory recommendation for kava drugs was to use its peeled rhizome<sup>[8,11,15]</sup>. In various HILI cases, it remained unclear, whether unpeeled rhizomes, peeled and unpeeled roots, and/or stem peelings were also used<sup>[8,11,16,17]</sup>. This again hampered any evaluation of the causative agent of kava hepatotoxicity<sup>[16,17]</sup>. For both the United States Food and Drug Administration and the Australian Therapeutic Goods Administration, peeled kava rhizomes were recommended for kava supplements<sup>[18,19]</sup>.

Another point of interest focuses on solvents and solubilizers without regulatory advice<sup>[8,11,15,16]</sup>, as well as on adulterants, impurities, contaminants, or misidentified herbs<sup>[4,7,8,11]</sup>. These key issues of herbal product quality are rarely addressed in publications related to herbal hepatotoxicity<sup>[1,4,8-17,20-33]</sup>.

### Clinical data requirements

Other concerns focus on incomplete clinical evaluation. Beginning at the day HILI is suspected, the physician has to gather all necessary information for an accurate diagnosis and the exclusion of alternative causes under relevant clinical aspects (Tables 1 and 2)<sup>[1,4,13,14,17,20-26,34-59]</sup>. Hepatotoxicity requires strict criteria, best defined by alanine aminotransferase (ALT) and/or alkaline phosphatase (ALP) values<sup>[4]</sup>. Its increases are expressed in multiples of the upper limit of their normal range as  $N^{[60-62]}$ . For ALT, hepatotoxicity has been defined from  $> 2N^{[60,62]}$ ,  $> 3N^{[63]}$  or  $> 5N^{[64]}$ , while ALP values of  $> 2N$  are commonly considered diagnostic<sup>[60,62]</sup>. Restricting ALT increases to  $> 5N$  will eliminate false positive cases and substantiate causality at a higher level of probability<sup>[64]</sup>. Considering patients with  $ALT > 2N$  will include numerous cases with nonspecific increases, with higher requirements for thorough assessment and more stringent exclusion of causes unrelated to the herb(s) under discussion. Also for low threshold  $N$  values, the rate of alternative diagnoses must be higher<sup>[13,14,24-26,35-39]</sup>, and missing a hepatotoxicity definition results in false high case numbers due to overdiagnosing and overreporting<sup>[17,23-26,38,39]</sup>. Special care is required for reporting of confounding variables<sup>[4,13,14,18,24,39]</sup>. For clinicians, a checklist with all clinical details is available for most alternative diagnoses (Table 2)<sup>[62]</sup>.

### Checklist

For a pragmatic approach to assess causality, special attention by the physician is of utmost importance. Only this physician can arrange collection and assessment of all data, thereby providing good data quality. To achieve this, a checklist with all important product and clinical items (Tables 1 and 2) and a valid causality assessment

**Table 1 Essential steps of herbal hepatotoxicity assessments**

| Quality specifications  |
|---|
| Herbal product quality  |
| Good agricultural practices   |
| Good manufacturing practices  |
| Definition of plant family, subfamily, species, subspecies, and variety   |
| Definition of plant part  |
| Definition of solvents and solubilizers   |
| Lack of impurities, adulterants, and misidentifications   |
| Minimum of batch and product variability  |
| Lack of variety to variety variability  |
| Clinical assessment quality   |
| Brand name with details of ingredients, plant parts, batch number, and expiration date  |
| Identification as herbal drug or herbal supplement  |
| Herb as an ingredient of a polyherbal product or an undetermined herbal product   |
| Manufacturer with address   |
| Indication of herbal use with dates of symptoms leading to herbal treatment   |
| Daily dose with details of the application form   |
| Exact date of herb start and herb end   |
| Accurate dates of emerging new symptoms after herb start in chronological order   |
| Accurate date of initially increased liver values   |
| Timeframes of challenge, latency period, and dechallenge  |
| Verification or exclusion of a temporal association   |
| Provided temporal association is verified, evaluation of a causal relationship  |
| Gender, age, body weight, height, body mass index   |
| Ethnicity, profession   |
| Past medical history regarding general diseases and specifically liver diseases   |
| ALT value initially including normal range  |
| ALT values during dechallenge at least on days 8 and 30, as well as later on  |
| ALT values during dechallenge to exclude a second peak  |
| ALT normalization with exact date and actual value  |
| ALP value initially including normal range  |
| ALP values during dechallenge up to 180 d, as well as later on  |
| ALP values during dechallenge to exclude a second peak  |
| ALP normalization with exact date and actual value  |
| AST value initially including normal range  |
| Laboratory criteria for definition of hepatotoxicity and its pattern  |
| Definition of risk factors such as age and alcohol  |
| Alcohol and drug use  |
| Statement regarding actual treatment including steroids or ursodesoxycholic acid  |
| Assessment of preexisting and coexisting liver unrelated diseases   |
| Assessment of preexisting and coexisting liver diseases   |
| Consideration of the several hundreds of other possible liver diseases  |
| Providing details to exclude alternative diagnoses  |
| Assessment and exclusion of hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis E virus, cytomegalovirus, Epstein-Barr virus, HSV, VZV |
| Liver and biliary tract imaging including color Doppler sonography of liver vessels   |
| Specific evaluation of alcoholic, cardiac, autoimmune, and genetic liver diseases   |
| Individual quantitative score of each alternative diagnosis   |
| Comedicated synthetic drugs, herbal drugs, herbal and other dietary supplements   |
| Definition of and search for accidental, unintended reexposure  |
| Assessing of unintended reexposure  |
| Search for evidence of prior known hepatotoxicity of the suspected herb   |
| Assessing of known hepatotoxicity caused by the herb  |
| Qualified data acquisition and documentation of complete data   |
| Transparent presentation of all data  |
| Causality assessment quality  |
| Prospective assessment by the physician suspecting herb induced liver injury  |
| Structured and quantitative method  |
| Liver specific causality assessment method validated for hepatotoxicity   |
| Use of the CIOMS scale  |
| Gathering of all data required for the CIOMS scale item by item   |
| Presentation of individual CIOMS items and of scores to regulatory agency   |
| Gathering all clinical data and presentation to regulatory agency   |
| Excluding all alternative causes and presentation to regulatory agency  |
| Regulatory case assessment by skilled hepatologist with clinical experience   |
| Regulatory assessment with assistance of external experts   |
| Transparent presentation of regulatory verified causality assessment results  |

Required quality specifications of herbal products refer to herbs, herbal drugs, and herbal supplements including herbal mixtures. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; CIOMS: Council for International Organizations of Medical Sciences; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

**Table 2** Check list for herb induced liver injury diagnosis

| Items to be assessed   | Information obtained     |                          |                          |
|--|--------------------------|--------------------------|--------------------------|
|  | Yes                      | No                       | Partial                  |
| Brand name with batch number and expiration date   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Indication of herbal use   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Dates of symptoms leading to herbal treatment  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Daily dose   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Application form of herbal product   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Exact date of herb start   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Exact date of herb end   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Accurate dates of emerging new symptoms after herb start in chronological order  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Accurate date of initially increased liver values  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Time frame of challenge  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Time frame of latency period   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Time frame of dechallenge  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Verification of temporal association   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Exclusion of temporal association  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Gender, age, body weight, height, BMI  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Ethnicity, profession  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Past medical history and actual assessment regarding preexisting general diseases  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Past medical history and actual assessment regarding preexisting liver diseases  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Risk factors such as age and alcohol   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Quantification of alcohol and drug use   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Comedicated synthetic drugs, herbal drugs, herbal and other dietary supplements with all details of product, daily dose, exact dates of start and end of use, indication | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALT value initially including exact date and normal range  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALT values during dechallenge at least on days 8 and 30, and later on, with exact dates  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALT values during dechallenge to exclude a second peak, with exact dates   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALT normalization with exact date and actual value   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALP value initially including exact date and normal range  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALP values during dechallenge up to 180 d, and later on, with exact dates  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALP values during dechallenge to exclude a second peak, with exact dates   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ALP normalization with exact date and actual value   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| AST value initially including normal range   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Laboratory criteria for definition of hepatotoxicity   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Laboratory criteria for injury pattern   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Liver and biliary tract imaging including hepatobiliary sonography, CT, MRT, MRC   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Color Doppler sonography of liver vessels  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Unintended reexposure  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Known hepatotoxicity caused by the herb  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Consideration and exclusion of other possible causes   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Hepatitis A  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Anti-HAV-IgM   |                          |                          |                          |
| Hepatitis B  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| HBsAg, anti-HBc-IgM, HBV-DNA   |                          |                          |                          |
| Hepatitis C  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Anti-HCV, HCV-RNA  |                          |                          |                          |
| Hepatitis E  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Anti-HEV-IgM, anti-HEV-IgG, HEV-RNA  |                          |                          |                          |
| CMV  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| CMV-PCR, titer change for anti-CMV-IgM and anti-CMV-IgG  |                          |                          |                          |
| EBV  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| EBV-PCR, titer change for anti-EBV-IgM and anti-EBV-IgG  |                          |                          |                          |
| HSV  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| HSV-PCR, titer change for anti-HSV-IgM and anti-HSV-IgG  |                          |                          |                          |
| VZV  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| VZV-PCR, titer change for anti-VZV-IgM and anti-VZV-IgG  |                          |                          |                          |
| Other virus infections   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Specific serology of Adenovirus, Coxsackie-B-virus, Echovirus, Measles virus, Rubella virus, Flavivirus, Arenavirus, Filovirus, Parvovirus, HIV, and others              |                          |                          |                          |
| Other infectious diseases  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Specific assessment of bacteria, fungi, parasites, worms, and others   |                          |                          |                          |
| AIH type I   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Gamma globulins, ANA, SMA, AAA, SLA/LP, anti-LSP, anti-ASGPR   |                          |                          |                          |
| AIH type II  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Gamma globulins, anti-LKM-1 (CYP 2D6), anti-LKM-2 (CYP 2C9), anti-LKM-3  |                          |                          |                          |
| PBC  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| AMA, anti-PDH-E2   |                          |                          |                          |

|  |                          |                          |                          |
|--|--------------------------|--------------------------|--------------------------|
| PSC  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| p-ANCA, MRC  |                          |                          |                          |
| AIC  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| ANA, SMA   |                          |                          |                          |
| Overlap syndromes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| See AIH, PBC, PSC, and AIC   |                          |                          |                          |
| NASH   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| BMI, insulin resistance, hepatomegaly, echogenicity of the liver   |                          |                          |                          |
| ALD  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Patient's history, clinical and laboratory assessment, sonography  |                          |                          |                          |
| DILI   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Patient's history, clinical and laboratory assessment, sonography, use of the CIOMS scale  |                          |                          |                          |
| Cocaine, ecstasy and other amphetamines  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Toxin screening  |                          |                          |                          |
| Rare intoxications   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Toxin screening for household and occupational toxins  |                          |                          |                          |
| Hereditary hemochromatosis   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Serum ferritin, total iron-binding capacity, genotyping for C2824 and H63D mutation, hepatic iron content  |                          |                          |                          |
| Wilson's disease   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Copper excretion (24 h urine), ceruloplasmin in serum, free copper in serum, Coombs-negative hemolytic anemia, hepatic copper content, Kayser-Fleischer-Ring, neurologic-psychiatric work-up, genotyping                           |                          |                          |                          |
| Porphyria  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Porphobilinogen in urine, total porphyrines in urine   |                          |                          |                          |
| $\alpha$ 1-Antitrypsin deficiency  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| $\alpha$ 1-Antitrypsin in serum  |                          |                          |                          |
| Biliary diseases   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Clinical and laboratory assessment, hepatobiliary sonography, endosonography, CT, MRT, MRC   |                          |                          |                          |
| Pancreatic diseases  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Clinical and laboratory assessment, sonography, CT, MRT  |                          |                          |                          |
| Celiac disease   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| TIG antibodies, endomysium antibodies, duodenal biopsy   |                          |                          |                          |
| Anorexia nervosa   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Clinical context   |                          |                          |                          |
| Parenteral nutrition   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Clinical context   |                          |                          |                          |
| Cardiopulmonary diseases with shock liver (cardiac hepatopathy, ischemic hepatitis)  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Cardiopulmonary assessment of congestive heart disease, myocardial infarction, cardiomyopathy, cardiac valvular dysfunction, pulmonary embolism, pericardial diseases, arrhythmia, hemorrhagic shock, and various other conditions |                          |                          |                          |
| Addison's disease  |                          |                          |                          |
| Plasma cortisol  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Thyroid diseases   |                          |                          |                          |
| TSH basal, T4, T3  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Grand mal seizures   |                          |                          |                          |
| Clinical context of epileptic seizure (duration > 30 min)  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Heat stroke  |                          |                          |                          |
| Shock, hyperthermia  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Polytrauma   |                          |                          |                          |
| Shock, liver injury  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Systemic diseases  |                          |                          |                          |
| Specific assessment of <i>M. Boeck</i> , amyloidosis, lymphoma, other malignant tumors, sepsis and others  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| Other diseases   |                          |                          |                          |
| Clinical context   | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

For each listed item, detailed results obtained for the individual patient are to be supplemented within the checklist. BMI: Body mass index; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; AST: Aspartate aminotransferase; CT: Computer tomography; MRT: Magnetic resonance tomography; MRC: Magnetic resonance cholangiography; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBsAg: Hepatitis B antigen; HBe: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E virus; IgG: Immunoglobulin G; HIV: Human immunodeficiency virus; CMV: Cytomegalovirus; PCR: Polymerase chain reaction; EBV: Epstein Barr virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus; AIH: Autoimmune hepatitis; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; AAA: Anti-actin antibodies; SLA: Soluble liver antigen; LP: Liver-pancreas antigen; LSP: Liver specific protein; ASGPR: Asialo-glycoprotein-receptor; LKM: Liver kidney microsomes; CYP: Cytochrome P450; PBC: Primary biliary cirrhosis; AMA: Antimitochondrial antibodies; PDH: Pyruvate dehydrogenase; PSC: Primary sclerosing cholangitis; p-ANCA: Perinuclear antineutrophil cytoplasmic antibodies; AIC: Autoimmune cholangitis; NASH: Non alcoholic steatohepatitis; ALD: Alcoholic liver disease; DILI: Drug induced liver injury; CIOMS: Council for International Organizations of Medical Sciences; TSH: Thyroid stimulating hormone.

algorithm (Tables 3-6) should be applied early in the unfolding disease, beginning at the day HILI is suspected. Unless this is done in a stringent way, poor data quality will be provided to the scientific community, regulatory

agencies, expert panels, and manufacturers, disabling reevaluation of the case. Initially poor data will produce poor results and is unacceptable. Complete and excellent case data including raw data provided by the physician

**Table 3** Methods of causality assessments for suspected herbal hepatotoxicity

| Methods of causality assessment | Specific criteria of various causality assessment methods |            |             |              |                |                 |
|---------------------------------|---|------------|-------------|--------------|----------------|-----------------|
|                                 | Expert based  | Structured | Qualitative | Quantitative | Liver specific | Liver validated |
| Prospective evaluation          |   |            |             |              |                |                 |
| CIOMS scale                     | No  | Yes        | No          | Yes          | Yes            | Yes             |
| MV scale                        | No  | Yes        | No          | Yes          | Yes            | Yes             |
| Naranjo scale                   | No  | Yes        | No          | Yes          | No             | No              |
| KL method                       | No  | Yes        | Yes         | No           | No             | No              |
| <i>Ad hoc</i> approach          | No  | No         | No          | No           | No             | No              |
| Retrospective evaluation        |   |            |             |              |                |                 |
| DILIN method                    | Yes   | Yes        | Yes         | No           | Yes            | No              |
| WHO method                      | Yes   | Yes        | No          | No           | No             | No              |
| Expert opinion                  | Yes   | No         | No          | No           | Yes            | No              |

Compilation of details are derived from previous reports<sup>[2,3,60-62,76-79,81,89,102]</sup>. Council for International Organizations of Medical Sciences scale (CIOMS scale) refers to both the original scale<sup>[60]</sup> and its update (Tables 5 and 6)<sup>[62]</sup>. Liver-specific and liver-validated criteria reflect hepatotoxicity criteria. Expert based criterion refers to the requirement of several experts for the actual case under consideration. MV scale: Maria and Victorino scale; KL method: Karch and Lasagna method; DILIN method: Drug Induced Liver Injury Network method; WHO method: World Health Organization method.

**Table 4** Details of the various causality assessment methods for herb induced liver injury

| Assessed items with specific scores       | CIOMS | MV | Naranjo | KL | <i>Ad hoc</i> | DILIN | WHO | Expert opinion |
|---|-------|----|---------|----|---------------|-------|-----|----------------|
| Time frame of latency period (score)      | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Time frame of challenge (score)           | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Time frame of dechallenge (score)         | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Recurrent ALT or ALP increase (score)     | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Definition of risk factors (score)        | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Verified alternative diagnoses (score)    | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Assessed HAV, HBV, HCV (score)            | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Assessed CMV, EBV, HSV, VZV (score)       | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Liver and biliary tract imaging (score)   | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Liver vessel Doppler sonography (score)   | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Assessed preexisting diseases (score)     | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Evaluated cardiac hepatopathy (score)     | +     | 0  | 0       | 0  | 0             | 0     | 0   | 0              |
| Excluded alternative diagnoses (score)    | +     | +  | +       | 0  | 0             | 0     | 0   | 0              |
| Comedication (score)                      | +     | 0  | +       | 0  | 0             | 0     | 0   | 0              |
| Prior known herbal hepatotoxicity (score) | +     | +  | +       | 0  | 0             | 0     | 0   | 0              |
| Searched unintended reexposure (score)    | +     | +  | +       | 0  | 0             | 0     | 0   | 0              |
| Defined unintended reexposure (score)     | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Unintended reexposure (score)             | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Laboratory hepatotoxicity criteria        | +     | +  | 0       | 0  | 0             | +     | 0   | +              |
| Laboratory hepatotoxicity pattern         | +     | +  | 0       | 0  | 0             | +     | 0   | +              |
| Liver specific method                     | +     | +  | 0       | 0  | 0             | +     | 0   | +              |
| Structured, liver related method          | +     | +  | 0       | 0  | 0             | +     | 0   | 0              |
| Quantitative, liver related method        | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |
| Validated method for hepatotoxicity       | +     | +  | 0       | 0  | 0             | 0     | 0   | 0              |

Items lacking specific scores were not considered, with the exception of the last six features. The data of the Drug Induced Liver Injury Network method are derived from the report of Rockey *et al*<sup>[102]</sup>, references for the other methods are found in the text. Latency period indicates time from herb start to symptoms, alternatively to abnormal liver tests. The symbol + shows that this item is present and the symbol 0 indicates lack of this item. ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; HAV: Hepatitis A virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus; CIOMS: Council for International Organizations of Medical Sciences scale; MV: Maria and Victorino scale; KL: Karch and Lasagna method; DILIN: Drug-Induced Liver Injury Network method; WHO: World Health Organization method.

are necessary to circumvent later investigative efforts, subsequent discussions, and speculative conclusions.

At each step of the evaluation, full transparency of all data is mandatory. This includes a complete narrative medical history, a causality assessment based on an established algorithm, and presentation of all data as item by item and raw data, ready for reevaluation by other scientists. This is also relevant for case publications and case series analyses, which is indeed feasible as shown in the past<sup>[13,14,25,35-39,58]</sup>. The same transparency is needed for

statements and publications by regulatory agencies and expert panels. Neglecting full transparency will cause concern and uncertainty about the validity of the presented conclusions.

## GENERAL ASPECTS OF CAUSALITY EVALUATION

### Method categories

Some reservations exist about the best method for causal-

**Table 5 Updated Council for International Organizations of Medical Sciences scale for the hepatocellular type of injury with items required for causality assessment in herb induced liver injury cases**

| Items for hepatocellular injury   | Possible score | Patient's score |
|---|----------------|-----------------|
| Time to onset from the beginning of the herb  |                |                 |
| 5-90 d (rechallenge: 1-15 d)  | +2             |                 |
| < 5 or > 90 d (rechallenge: > 15 d)   | +1             |                 |
| Alternative: Time to onset from cessation of the herb   |                |                 |
| ≤ 15 d (except for slowly metabolized herbal chemicals: > 15 d)   | +1             |                 |
| Course of ALT after cessation of the herb   |                |                 |
| Percentage difference between ALT peak and N  |                |                 |
| Decrease ≥ 50% within 8 d   | +3             |                 |
| Decrease ≥ 50% within 30 d  | +2             |                 |
| No information or continued herbal use  | 0              |                 |
| Decrease ≥ 50% after the 30 <sup>th</sup> day   | 0              |                 |
| Decrease < 50% after the 30 <sup>th</sup> day or recurrent increase                                       | -2             |                 |
| Risk factors  |                |                 |
| Alcohol use (drinks/d: > 2 for women, > 3 for men)  | +1             |                 |
| No alcohol use (drinks/d: ≤ 2 for women, ≤ 3 for men)   | 0              |                 |
| Age ≥ 55 yr   | +1             |                 |
| Age < 55 yr   | 0              |                 |
| Concomitant herbs(s) and drug(s)  |                |                 |
| None or no information  | 0              |                 |
| Concomitant herb or drug with incompatible time to onset  | 0              |                 |
| Concomitant herb or drug with compatible or suggestive time to onset                                      | -1             |                 |
| Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset             | -2             |                 |
| Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test) | -3             |                 |
| Search for non drug causes  |                |                 |
| Group I (6 causes)  |                |                 |
| Anti-HAV-IgM  |                |                 |
| HBsAg, anti-HBc-IgM, HBV-DNA  |                |                 |
| Anti-HCV, HCV-RNA   |                |                 |
| Hepatobiliary sonography/colour Doppler sonography of liver vessels/endsonography/CT/MRC                  |                |                 |
| Alcoholism (AST/ALT ≥ 2 IU/L)   |                |                 |
| Acute recent hypotension history (particularly if underlying heart disease)                               |                |                 |
| Group II (6 causes)   |                |                 |
| Complications of underlying disease(s)  |                |                 |
| Infection suggested by PCR and titre change for   |                |                 |
| CMV (anti-CMV-IgM, anti-CMV-IgG)  |                |                 |
| EBV (anti-EBV-IgM, anti-EBV-IgG)  |                |                 |
| HEV (anti-HEV-IgM, anti-HEV-IgG)  |                |                 |
| HSV (anti-HSV-IgM, anti-HSV-IgG)  |                |                 |
| VZV (anti-VZV-IgM, anti-VZV-IgG)  |                |                 |
| Evaluation of group I and II  |                |                 |
| All causes-groups I and II - reasonably ruled out   | +2             |                 |
| The 6 causes of group I ruled out   | +1             |                 |
| 5 or 4 causes of group I ruled out  | 0              |                 |
| Less than 4 causes of group I ruled out   | -2             |                 |
| Non herb cause highly probable  | -3             |                 |
| Previous information on hepatotoxicity of the herb  |                |                 |
| Reaction labelled in the product characteristics  | +2             |                 |
| Reaction published but unlabelled   | +1             |                 |
| Reaction unknown  | 0              |                 |
| Response to readministration  |                |                 |
| Doubling of ALT with the herb alone, provided ALT below 5N before reexposure                              | +3             |                 |
| Doubling of ALT with the herb(s) and drug(s) already given at the time of first reaction                  | +1             |                 |
| Increase of ALT but less than N in the same conditions as for the first administration                    | -2             |                 |
| Other situations  | 0              |                 |
| Total score for patient   |                |                 |

The compilation of the individual items is adapted from the updated version of the Council for International Organizations of Medical Sciences (CIOMS) scale<sup>[62]</sup> and the original CIOMS scale<sup>[60]</sup>. The above items refer to the hepatocellular type of injury, whereas items for the cholestatic (± hepatocellular) type are presented in Table 6. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol<sup>[2,3,90]</sup>. Total score and resulting causality grading: ≤ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥ 9, highly probable. HAV: Hepatitis A virus; IgM: Immunoglobulin M; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; CMV: Cytomegalovirus; CT: Computer tomography; EBV: Epstein Barr virus; HBc: Hepatitis B core; HBsAg: Hepatitis B antigen; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HEV: Hepatitis E; HILL: Herb induced liver injury; HSV: Herpes simplex virus; MRC: Magnetic resonance cholangiography; N: Upper limit of the normal range; VZV: Varicella zoster virus.

**Table 6 Updated Council for International Organizations of Medical Sciences scale for the cholestatic ( $\pm$  hepatocellular) type of injury with items required for causality assessment in herb induced liver injury cases**

| Items for cholestatic ( $\pm$ hepatocellular) injury  | Possible score | Patient's score |
|---|----------------|-----------------|
| Time to onset from the beginning of the herb  |                |                 |
| 5-90 d (rechallenge: 1-90 d)  | +2             |                 |
| < 5 or > 90 d (rechallenge: > 90 d)   | +1             |                 |
| Alternative: Time to onset from cessation of the herb   |                |                 |
| ≤ 30 d (except for slowly metabolized herbal chemicals: > 30 d)   | +1             |                 |
| Course of ALP after cessation of the herb   |                |                 |
| Percentage difference between ALP peak and N  |                |                 |
| Decrease ≥ 50% within 180 d   | +2             |                 |
| Decrease < 50% within 180 d   | +1             |                 |
| No information, persistence, increase, or continued herbal use  | 0              |                 |
| Risk factors  |                |                 |
| Alcohol use (drinks/d: > 2 for women, > 3 for men) and pregnancy  | +1             |                 |
| No alcohol use (drinks/d: ≤ 2 for women, ≤ 3 for men)   | 0              |                 |
| Age ≥ 55 yr   | +1             |                 |
| Age < 55 yr   | 0              |                 |
| Concomitant herbs(s) and drug(s)  |                |                 |
| None or no information  | 0              |                 |
| Concomitant herb or drug with incompatible time to onset  | 0              |                 |
| Concomitant herb or drug with compatible or suggestive time to onset                                      | -1             |                 |
| Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset             | -2             |                 |
| Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test) | -3             |                 |
| Search for non drug causes  |                |                 |
| Group I (6 causes)  |                |                 |
| Anti-HAV-IgM  |                |                 |
| HBsAg, anti-HBc-IgM, HBV-DNA  |                |                 |
| Anti-HCV, HCV-RNA   |                |                 |
| Hepatobiliary sonography/colour Doppler sonography of liver vessels/endsonography/CT/MRC                  |                |                 |
| Alcoholism (AST/ALT ≥ 2 IU/L)   |                |                 |
| Acute recent hypotension history (particularly if underlying heart disease)                               |                |                 |
| Group II (6 causes)   |                |                 |
| Complications of underlying disease(s)  |                |                 |
| Infection suggested by PCR and titre change for   |                |                 |
| CMV (anti-CMV-IgM, anti-CMV-IgG)  |                |                 |
| EBV (anti-EBV-IgM, anti-EBV-IgG)  |                |                 |
| HEV (anti-HEV-IgM, anti-HEV-IgG)  |                |                 |
| HSV (anti-HSV-IgM, anti-HSV-IgG)  |                |                 |
| VZV (anti-VZV-IgM, anti-VZV-IgG)  |                |                 |
| Evaluation of group I and II  |                |                 |
| All causes-groups I and II - reasonably ruled out   | +2             |                 |
| The 6 causes of group I ruled out   | +1             |                 |
| 5 or 4 causes of group I ruled out  | 0              |                 |
| Less than 4 causes of group I ruled out   | -2             |                 |
| Non herb cause highly probable  | -3             |                 |
| Previous information on hepatotoxicity of the herb  |                |                 |
| Reaction labelled in the product characteristics  | +2             |                 |
| Reaction published but unlabelled   | +1             |                 |
| Reaction unknown  | 0              |                 |
| Response to readministration  |                |                 |
| Doubling of ALP with the herb alone, provided ALP below 5N before reexposure                              | +3             |                 |
| Doubling of ALP with the herb(s) and drug(s) already given at the time of first reaction                  | +1             |                 |
| Increase of ALP but less than N in the same conditions as for the first administration                    | -2             |                 |
| Other situations  | 0              |                 |
| Total score for patient   |                |                 |

The compilation of individual items is adapted from the updated version of the Council for International Organizations of Medical Sciences (CIOMS) scale<sup>[62]</sup> and the original CIOMS scale<sup>[60]</sup>. The above items refer to the cholestatic ( $\pm$  hepatocellular) type of injury, whereas items for the hepatocellular type are presented in Table 5. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol<sup>[2,3,90]</sup>. Total score and resulting causality grading: ≤ 0, excluded; 1-2, unlikely; 3-5, possible; 6-8, probable; ≥ 9, highly probable. ALP: Alkaline phosphatase; N: upper limit of the normal range; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBsAg: Hepatitis B antigen; HBc: Hepatitis B core; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CT: Computer tomography; MRC: Magnetic resonance cholangiography; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; PCR: Polymerase chain reaction; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; IgG: Immunoglobulin G; VZV: Varicella zoster virus.

ity assessment in hepatotoxicity cases<sup>[1-4,13,14,17,21-26,34-39,59-64]</sup>. HILI case series reported in 23 publications with 573 HILI cases used various causality assessment meth-

ods<sup>[12-14,23,25,34-36,38,39,53,54,65-75]</sup>. These can be classified into prospective and retrospective analyses (Table 3).

The prospective evaluation focuses on the physician

caring for a patient with suspected liver injury. This setting requires a readily available and time efficient method to evaluate causation that can adapt to further clinical and causality approach necessities. Candidates are the Council for International Organizations of Medical Sciences (CIOMS) scale, also called Roussel Uclaf Causality Assessment Method scale<sup>[60-62]</sup>, the Maria and Victorino (MV) scale<sup>[76]</sup>, the Naranjo scale<sup>[77]</sup>, the Karch and Lasagna (KL) method<sup>[78]</sup>, and the *ad hoc* approach<sup>[79]</sup>.

Retrospective evaluations are based on an expert panel evaluating reported or published case data, sometimes going back for months or years. Examples are the Drug Induced Liver Injury Network (DILIN) method<sup>[73,80]</sup>, the World Health Organization global introspection method (WHO method) as defined by the WHO Collaborating Centre for International Drug Monitoring<sup>[81]</sup>, and the expert opinion<sup>[2,3]</sup>. Major differences exist (Table 3), especially when assessing items that require score attribution (Table 4).

### Usage frequency

Analyzing 23 publications of initially assumed causality but not necessarily confirmed later on<sup>[12-14,23,25,34-36,38,39,53,54,65-75]</sup> with HILI cases by BC, Greater Celandine, Green Tea extracts, some Herbalife products, Hydroxycut, kava, *Pelargonium sidoides*, and various herbs, the CIOMS scale was applied in 52.2%, the WHO method in 17.4%, the *ad hoc* approach in 13.1%, the Naranjo scale in 8.7%, and the KL and DILIN method each in 4.3% of these publications<sup>[82]</sup>. Similar results were obtained when analyzing the frequency for the 573 cases: the CIOMS scale was used in 275 cases (48.0%), the WHO method in 134 cases (23.4%), the Naranjo scale in 64 cases (11.2%), the *ad hoc* approach in 63 cases (11.0%), the KL method in 20 cases (3.5%), and the DILIN method in 20 cases (3.0%)<sup>[82]</sup>. For instance, the CIOMS scale was applied for Kava<sup>[13,14,67]</sup>, BC<sup>[25,34,71,72]</sup>, Greater Celandine<sup>[35,36]</sup>, *Pelargonium sidoides*<sup>[38,39]</sup>, and various herbs<sup>[75]</sup>, the WHO method for Kava<sup>[65,68]</sup> and Herbalife products<sup>[53,54]</sup>, the *ad hoc* approach for Kava<sup>[12,66]</sup> and Greater Celandine<sup>[69]</sup>, the Naranjo scale for BC<sup>[23]</sup> and Green Tea extracts<sup>[70]</sup>, the KL method for Herbalife products<sup>[74]</sup>, and the DILIN method for Hydroxycut<sup>[8]</sup><sup>[73]</sup>.

A systematic analysis of causality methods is also available for DILI cases<sup>[83]</sup>. In 2008, 61 DILI publications in the PubMed database over the last decade were reviewed. It revealed that in 38 publications (62.3%) no specific causality assessment method was mentioned; presumably, the evaluation was based on the *ad hoc* approach. The CIOMS scale, Naranjo scale, and WHO method were used in 10, 8, and 2 publications, respectively<sup>[83]</sup>. Therefore, in HILI and DILI publications the CIOMS scale was the preferred specific causality assessment method if the unstructured *ad hoc* approach is excluded. Physicians are well advised to use the CIOMS scale for HILI causality evaluation, to err on the side of caution.

### NIH PREFERENCE

The NIH LiverTox specifically addressed the item of

causality in hepatotoxicity cases<sup>[2,3]</sup>. It focuses primarily on using the CIOMS scale, which is discussed in detail. Moreover, the MV and Naranjo scales, the Bayesian, and expert opinion assessment are referred to; details of the DILIN causality assessment also are presented. Some strengths and weaknesses of these methods are compiled (Tables 3 and 4).

## PROSPECTIVE CAUSALITY ASSESSMENT METHODS

### CIOMS scale

The method of choice for the causality assessment of suspected HILI is the CIOMS scale in its original form<sup>[60,61]</sup> or preferably its update (Tables 5 and 6)<sup>[62]</sup>, with early starting of the evaluation at the day the physician assumes this diagnosis. The CIOMS scale is intended for prospective use at the time of manifestation; it does not require expert knowledge, is structured, quantitative, liver specific, and validated for hepatotoxicity (Table 3). Its items provide individual scores, which estimate causality levels for the agent(s) under consideration as highly probable, probable, possible, unlikely, and excluded (Tables 5 and 6). The CIOMS scale takes into account all core elements of hepatotoxicity and thereby has advantages over other algorithms (Table 4)<sup>[62]</sup>. Compared to the regulatory used *ad hoc* approach, assessment of HILI cases with the CIOMS scale leads to lower causality grades for the incriminated herb and/or for concomitant medications and to better reproducible results due to greater transparency<sup>[84]</sup>.

CIOMS was developed by an international expert panel and validated by cases with positive reexposure tests serving as a gold standard<sup>[60,61]</sup>. CIOMS based assessment has shown good sensitivity (86%), specificity (89%), positive predictive value (93%), and negative predictive value (78%)<sup>[61]</sup>. The scales differ slightly for the hepatocellular and the cholestatic ( $\pm$  hepatocellular) type of injury (Tables 5 and 6)<sup>[62]</sup>. Differentiation between these types is feasible by comparing the ratio of the serum activities of ALT and ALP at diagnosis of suspected herbal hepatotoxicity<sup>[60,62]</sup>. Enzyme activity is expressed as a multiple of the upper limit of the normal range (N), and the ratio (R) of ALT/ALP is calculated. Liver injury is classified as: (1) hepatocellular, if ALT > 2N alone or R  $\geq$  5; (2) cholestatic, when there is an increase of ALP > 2N alone or when R  $\leq$  2; and (3) mixed cholestatic-hepatocellular, if ALT > 2N, ALP is increased, and R between 2 and 5.

Strengths and weaknesses of the CIOMS scale have been discussed extensively<sup>[2,3,62,73,79,82,85-91]</sup>. This scale clearly compiles liver specific criteria for challenge, dechallenge, risk factors, exclusion of unrelated diseases, and comedication, but does not use liver histology data (Tables 5 and 6)<sup>[60,62]</sup>, agreed upon as less helpful criteria in most cases<sup>[90,91]</sup>. It considers unintentional reexposure results according to criteria as established by previous expert consensus meetings<sup>[92,93]</sup>. For reexposure results of the hepatocellular type of liver injury, ALT levels are as-

sessed before reexposure (designed as baseline ALT or ALTb), and at reexposure (designed as ALT<sub>r</sub>). The reexposure test is positive, if (1) ALT<sub>b</sub> is below 5N with N as the upper limit of the normal value, and (2) ALT<sub>r</sub>  $\geq$  2ALT<sub>b</sub><sup>[92]</sup>.

The test is negative, if only one or no criterion is fulfilled; it is uninterpretable, if ALT data are lacking for one or both times. For reexposure assessments of the cholestatic ( $\pm$  hepatocellular) type of liver injury, ALT has to be replaced by ALP. Criteria for positive reexposure tests are included in the updated CIOMS scale (Tables 5 and 6) and were not previously applied in cases with reported positive reexposure tests<sup>[40-57,59,91]</sup>. When these cases were submitted to retrospective analysis using the reexposure test criteria, a positive reexposure test could be confirmed in only 13/30 cases, the test was negative in 5/30 cases and uninterpretable in 12/30 cases<sup>[91]</sup>. In 8 cases of initially assumed Herbalife hepatotoxicity with a previously reported positive reexposure test result, retrospective evaluation applying the test criteria revealed that criteria for a positive reexposure were fulfilled in only 1/8 cases, whereas the reexposure test was classified as negative in another case or the data were considered as uninterpretable due to missing information to comply adequately with the criteria in the remaining six cases<sup>[94]</sup>.

The CIOMS scale was widely used for hepatotoxicity assessments in epidemiological studies, clinical trials, case reports, case series, regulatory analyses, and genotyping studies<sup>[13,14,24,25,35,36,38,39,58,59,61,64,72,79,84,86,87,90,95-98]</sup>. Proposals for refinement and strengthening of the CIOMS scale focused on the weight of individual parameters and risk factors such as alcohol and age, and other shortcomings were addressed<sup>[24,87,89,90]</sup>. However, there is lack of valid data to verify improvements based on reassessing and reevaluating of published approaches<sup>[87,89,90,98]</sup>, calling for new approaches.

Assessment of suspected HILI cases may be problematic in spontaneous reports with insufficient data. Evaluating these cases requires a sophisticated approach, as undertaken by EMA for 31 EU cases of suspected HILI by BC, using the CIOMS scale<sup>[34]</sup>. This series included 11/31 unassessable cases (35%) due to poor data quality, with causality assessment feasible in 20/31 cases (65%). Among these, EMA specified likely alternative causes in 8/20 cases with diagnoses such as autoimmune hepatitis, DILI, preexisting liver disease, alcoholic hepatitis, and preexisting liver cirrhosis with Stevens Johnson syndrome<sup>[34]</sup>. Causality for BC was unlikely or excluded in another 6/20 cases and 5/20 cases, respectively. In 1/20 cases, causality was judged as possible by EMA<sup>[34]</sup>, but upon further evaluation this particular case with insufficient data quality was attributed with an excluded causality<sup>[71]</sup>. Consequently, in this EMA study group of 31 EU cases there was little evidence of liver injury by BC based on the use of the CIOMS scale, which was most helpful in this particular analysis and provided robust results<sup>[34]</sup>. The approach of EMA to apply the CIOMS scale in hepatotoxicity cases<sup>[34]</sup> should be highly appreciated and is in line with the corresponding recom-

mendation by the NIH for their LiverTox database to prefer the CIOMS scale over other methods<sup>[2,3]</sup>.

At present, we are far away from valid data and strict management in suspected HILI cases, which impedes description of classic HILI by the majority of herbs. Possible or likely alternative diagnoses were evident in 278/573 cases (48.5%) of suspected HILI cases; causality assessment was impeded in 165/573 patients (29.0%) due to missing case data or lack of a temporal association, resulting in diagnostic problems in 77.5% of all cases<sup>[82]</sup>. Given these limitations, actual discussions of validity of reported HILI cases are understandable<sup>[82,90,91,94,98-100]</sup>, and uncertainty also extends to the validity of the type of liver injury reported for some cases lacking a probable or highly probable causality. Considering these restrictions, the hepatocellular type of injury was described for Indian Ayurvedic herbs<sup>[72,98]</sup>, Chaparral (*Larrea tridentata*)<sup>[40,98]</sup>, Dai Saiko To<sup>[47,98]</sup>, Germander<sup>[98]</sup>, Green Tea extract<sup>[98]</sup>, Greater Celandine<sup>[37]</sup>, Hydroxycut<sup>[6,98]</sup>, *Jin Bu Huan* (*Lycopodium serratum*)<sup>[45,98]</sup>, Kava<sup>[13]</sup>, the cholestatic or mixed type for Chaparral<sup>[98]</sup>, Germander<sup>[98]</sup>, Green Tea extract<sup>[98]</sup>, Greater Celandine<sup>[98]</sup>, Hydroxycut<sup>[6,98]</sup>; and the veno-occlusive disease for plants containing pyrrolizidine alkaloids such as *Senecio*, *Heliotropium*, *Crotalaria*, and *Symphytum* species<sup>[98]</sup>.

In clinical practice, the physician will start at the day HILI is suspected with the CIOMS scale to arrive at an initial estimation and to exclude the most frequent alternative causes, provided point by point in the CIOMS questionnaire (Tables 5 and 6). The practical application of the CIOMS scale was published in various case series<sup>[13,25,35,36,38,39,71,72,94]</sup> and is shown by two single cases as examples, one for a case of hepatotoxicity by Indian Ayurvedic herbs (Table 7)<sup>[58]</sup>, and another one for a case of liver injury by a dietary supplement<sup>[97]</sup>. For further refinement, specific information usually is necessary to rule out rare alternative causes (Table 2). This initial approach using the CIOMS scale ensures prospectively the collection of highly qualified case data and enables a sophisticated case evaluation currently and in the future. Information of individual CIOMS items (Tables 5 and 6), the checklist for HILI diagnosis (Table 2), all raw data, and a narrative case report should be presented to regulatory agencies, the scientific community, manufacturers, and expert panels to allow refined use of the CIOMS scale and all other case data, provided causality for the incriminated herb reached a probable or highly probable level.

### MV scale

The MV scale<sup>[76]</sup> was developed in an attempt to improve the CIOMS scale by adding other clinical elements and by simplifying and changing the relative weight of assessment parameters, in detail discussed by the NIH LiverTox<sup>[2,3]</sup> and others<sup>[62,87]</sup>, or briefly referenced<sup>[98]</sup>. As a shortened and modified version of the CIOMS scale<sup>[60]</sup>, the MV scale<sup>[76]</sup> has fewer specific criteria than the original CIOMS scale (Table 4); due to major differences in test cases, however, the equivalency to CIOMS has been debated<sup>[2,3,62,84,87,89,96]</sup>.

Specifically, the MV scale evaluates dechallenge as

**Table 7 Council for International Organizations of Medical Sciences scale as an example with items required for causality assessment in a patient with herb induced liver injury by four Indian Ayurvedic herbs**

| Items for hepatocellular injury   | Possible score | Psoralea corylifolia | Acacia catechu | Eclipta alba | Vetivexia zizanioidis |
|---|----------------|----------------------|----------------|--------------|-----------------------|
| Time to onset from the beginning of the herb  |                |                      |                |              |                       |
| 5-90 d (rechallenge: 1-15 d)  | +2             |                      |                |              |                       |
| < 5 d or > 90 d (rechallenge: > 15 d)   | +1             | +1                   | +1             | +1           | +1                    |
| Alternative: Time to onset from cessation of the herb   |                |                      |                |              |                       |
| ≤ 15 d (except for slowly metabolized herbal chemicals: > 15 d)   | +1             |                      |                |              |                       |
| Course of ALT after cessation of the herb   |                |                      |                |              |                       |
| Percentage difference between ALT peak and N  |                |                      |                |              |                       |
| Decrease ≥ 50% within 8 d   | +3             | +3                   | +3             | +3           | +3                    |
| Decrease ≥ 50% within 30 d  | +2             |                      |                |              |                       |
| No information or continued herbal use  | 0              |                      |                |              |                       |
| Decrease ≥ 50% after the 30 <sup>th</sup> day   | 0              |                      |                |              |                       |
| Decrease < 50% after the 30 <sup>th</sup> day or recurrent increase                                       | -2             |                      |                |              |                       |
| Risk factors  |                |                      |                |              |                       |
| Alcohol use (drinks/d: > 2 for women, > 3 for men)  | +1             |                      |                |              |                       |
| No alcohol use (drinks/d: ≤ 2 for women, ≤ 3 for men)   | 0              | 0                    | 0              | 0            | 0                     |
| Age ≥ 55 yr   | +1             | +1                   | +1             | +1           | +1                    |
| Age < 55 yr   | 0              |                      |                |              |                       |
| Concomitant herbs(s) and drug(s)  |                |                      |                |              |                       |
| None or no information  | 0              |                      |                |              |                       |
| Concomitant herb or drug with incompatible time to onset  | 0              |                      |                |              |                       |
| Concomitant herb or drug with compatible or suggestive time to onset                                      | -1             | -1                   |                |              |                       |
| Concomitant herb or drug known as hepatotoxin and with compatible or suggestive time to onset             | -2             |                      | -2             | -2           | -2                    |
| Concomitant herb or drug with evidence for its role in this case (positive rechallenge or validated test) | -3             |                      |                |              |                       |
| Search for non herb causes  |                |                      |                |              |                       |
| Group I (6 causes)  |                |                      |                |              |                       |
| Anti-HAV-IgM  |                | -                    | -              | -            | -                     |
| HBsAg, anti-HBc-IgM, HBV-DNA  |                | -                    | -              | -            | -                     |
| Anti-HCV, HCV-RNA   |                | -                    | -              | -            | -                     |
| Hepatobiliary sonography/colour Doppler sonography of liver vessels/endsonography/CT/MRC                  |                | -                    | -              | -            | -                     |
| Alcoholism (AST/ALT ≥ 2 IU/L)   |                | -                    | -              | -            | -                     |
| Acute recent hypotension history (particularly if underlying heart disease)                               |                | -                    | -              | -            | -                     |
| Group II (6 causes)   |                |                      |                |              |                       |
| Complications of underlying disease(s)  |                | -                    | -              | -            | -                     |
| Infection suggested by PCR and titre change for   |                |                      |                |              |                       |
| CMV (anti-CMV-IgM, anti-CMV-IgG)  |                | -                    | -              | -            | -                     |
| EBV (anti-EBV-IgM, anti-EBV-IgG)  |                | -                    | -              | -            | -                     |
| HEV (anti-HEV-IgM, anti-HEV-IgG)  |                | -                    | -              | -            | -                     |
| HSV (anti-HSV-IgM, anti-HSV-IgG)  |                | -                    | -              | -            | -                     |
| VZV (anti-VZV-IgM, anti-VZV-IgG)  |                | -                    | -              | -            | -                     |
| Evaluation of group I and II  |                |                      |                |              |                       |
| All causes-groups I and II-reasonably ruled out   | +2             | +2                   | +2             | +2           | +2                    |
| The 6 causes of group I ruled out   | +1             |                      |                |              |                       |
| 5 or 4 causes of group I ruled out  | 0              |                      |                |              |                       |
| Less than 4 causes of group I ruled out   | -2             |                      |                |              |                       |
| Non herb cause highly probable  | -3             |                      |                |              |                       |
| Previous information on hepatotoxicity of the herb  |                |                      |                |              |                       |
| Reaction labelled in the product characteristics  | +2             |                      |                |              |                       |
| Reaction published but unlabelled   | +1             | +1                   |                |              |                       |
| Reaction unknown  | 0              |                      | 0              | 0            | 0                     |
| Response to readministration  |                |                      |                |              |                       |
| Doubling of ALT with the herb alone, provided ALT below 5N before reexposure                              | +3             |                      |                |              |                       |
| Doubling of ALT with the herb(s) and drug(s) already given at the time of first reaction                  | +1             |                      |                |              |                       |
| Increase of ALT but less than N in the same conditions as for the first administration                    | -2             |                      |                |              |                       |
| Other situations  | 0              |                      |                |              |                       |
| Total score for each individual herb used by the patient  |                | +7                   | +5             | +5           | +5                    |

The data of the patient with severe hepatotoxicity by four different Indian Ayurvedic herbs are derived from a published report<sup>[58]</sup>, using the updated Council for International Organizations of Medical Sciences scale for the hepatocellular type of liver injury (Table 5). The symbol - signifies that this particular item has been evaluated and no abnormality was found. Regarding risk factor of alcohol use, 1 drink commonly contains about 10 g ethanol<sup>[2,3,90]</sup>. For the four herbs, the total score was either 5 (possible causality) or 7 (probable causality). ALT: Alanine aminotransferase; N: Upper limit of the normal range; HBsAg: Hepatitis B antigen; HBc: Hepatitis B core; HAV: Hepatitis A virus; IgM: Immunoglobulin M; HBV: Hepatitis B virus; HCV: Hepatitis C virus; CT: Computer tomography; MRC: Magnetic resonance cholangiography; AST: Aspartate aminotransferase; PCR: Polymerase chain reaction; CMV: Cytomegalovirus; EBV: Epstein Barr virus; HEV: Hepatitis E virus; HSV: Herpes simplex virus; VZV: Varicella zoster virus.

the time necessary for ALT or ALP to fall below 2N, considers a shorter latency period, asks for less accurate exclusion criteria of drug-independent causes, ignores concomitant drug use, emphasizes drugs with more than 5 years marketing without published hepatotoxicity, and overestimates extrahepatic manifestations like hypersensitivity reactions<sup>[76]</sup>. The validation used real and fictive cases and as gold standard the opinion of three external experts<sup>[76,87]</sup> and not cases with verified results of positive reexposure tests<sup>[76]</sup>; for initial validation of the CIOMS scale, both a panel of experts and positive reexposure tests were used<sup>[60,61]</sup>. Compared to the CIOMS scale<sup>[60]</sup>, the MV scale was equivalently accurate only in cases of hypersensitivity; otherwise, the CIOMS scale was superior to the MV scale<sup>[89,96]</sup>. A comparison of the two scales for hepatotoxicity cases demonstrated low consistency between the two systems, with agreement between the scales in only 18% of the cases; the CIOMS scale showed better discriminative power and produced assessments closer to those of specialists<sup>[87]</sup>. These limitations restrict the general use of the MV scale in hepatotoxicity cases<sup>[62]</sup>.

A recent HILI study confirmed poor concordance between the MV and CIOMS scales for both the herb and concomitant medication assessment. The CIOMS scale found higher causality levels for the herb and concomitant medications than the MV scale; this was associated with considerably lower causality levels provided by the MV scale compared to the *ad hoc* approach<sup>[84]</sup>. The low MV scores were attributed to various parameters such as prolonged latency and dechallenge periods, the presence of several alternative herb independent causes for the observed liver disease, only partial exclusion of herb unrelated causes due to missing essential case data, and lacking consideration of extrahepatic manifestations like rash, fever, arthralgia, peripheral eosinophilia, and cytopenia. It therefore appeared that various confounders precluded a high level of causality for the herb in a setting of HILI cases assessed by the MV scale.

The MV scale may be useful in some selected hepatotoxicity cases. Nonetheless, little evidence is provided that this scale has advantages over the CIOMS scale and should be the preferred tool<sup>[2,3,62,87,89,95,96]</sup>. It has been criticized by the NIH LiverTox that the elements used in the MV scale and their relative weights were based upon the authors' expert opinion and not by prospective evaluation of a variety of possible elements and different cutoff values and weights<sup>[2,3]</sup>. Additional concern was expressed that the MV scale focuses on hypersensitivity features that are comparatively infrequent in hepatotoxicity cases; it performs poorly in atypical cases, such as unusually long latency periods or residual chronic symptoms after cessation of the culprit<sup>[87]</sup>. Another issue raised was the low numbers of experts and the low degree of validation<sup>[2,3]</sup> of the MV scale<sup>[76]</sup>. Thus, the MV scale is not commonly recommended for assumed HILI cases and certainly is no substitute for the CIOMS scale<sup>[2,3,87,98]</sup>.

### Naranjo scale

The NIH LiverTox summarized the arguments for and against the Naranjo scale<sup>[2,3]</sup>. In detail, while this scale includes all general features important in assessing causality, most critical elements are not weighed in judging the likelihood of liver injury, for example specific time to onset, criteria for recovery time, and list of critical diagnoses to exclude, limiting the use of this scale for assessing hepatotoxicity. The Naranjo scale includes testing for drug levels, which is rarely helpful in idiosyncratic drug induced liver disease. Finally, the scale was designed for use in clinical trials, and points are subtracted if the reaction reappears with administration of placebo, which does not apply to the usual case of drug induced liver disease. Direct comparisons to the CIOMS scale have shown that the Naranjo scale is easier to apply, but has less sensitivity and specificity in assigning causality to cases of liver injury. These statements of the NIH LiverTox<sup>[2,3]</sup> supported other views<sup>[87]</sup>, confirming low sensitivity, and a lower prediction rate of the Naranjo scale in a careful comparison with the CIOMS scale for suspected hepatotoxicity cases<sup>[101]</sup>. These studies concluded that the Naranjo scale lacks validity and reproducibility when evaluating hepatotoxicity<sup>[86,93]</sup>; it was not recommended for hepatotoxicity assessment<sup>[87]</sup>.

The Naranjo scale was designed to assess causality of any adverse drug reaction (ADR), independent from the affected organ<sup>[77]</sup>. It substantially differs from other causality algorithms for hepatotoxicity (Tables 3 and 4)<sup>[2,3,24-26,63,79,87,88,101]</sup>. This scale relates toxic drug reactions to general pharmacological drug actions rather than possibly to idiosyncratic reactions like rare hepatotoxicity<sup>[77]</sup>. Its items include drug concentrations and monitoring, dose relations such as decreasing dose, placebo response, cross-reactivity, and confirmation of ADRs using unidentified objective evidence, which is relevant only for toxic reactions<sup>[77,79,88]</sup>. The general use of the Naranjo scale in hepatotoxicity cases<sup>[23,79]</sup> created concern<sup>[2,3,24-26,63,70,87,88,101]</sup>.

The use of the liver unspecific Naranjo scale<sup>[77]</sup> is unacceptable in suspected HILI cases<sup>[23,79]</sup>, its results are heavily disputed<sup>[24-26,63,70,79,88]</sup>; this pertains especially to the shortened version used by the United States Pharmacopeia (USP)<sup>[23,79]</sup> with only 5 of the original 10 items<sup>[88]</sup>. Lack of liver specificity associated with the Naranjo algorithm is evident by lack of a definition of liver injury as ADR; an unclear time frame and latency period; undefined time frames for dechallenge; no definition of risk factors; insufficient evaluation of alternative diagnoses; inappropriate assessment of comedication; and lacking definition of a positive rechallenge test (Table 4)<sup>[77,88]</sup>. This scale also was considered too insensitive, allowing a possible causality even in the absence of essential data, by virtue of the patient simply having taken the suspected agent<sup>[63,70]</sup>. Most importantly, the modified Naranjo scale as used by USP<sup>[23,70]</sup> did not exclude relevant alternative causes such as idiopathic autoimmune hepatitis, alcoholic or cardiac hepatopathy, other preexisting liver

diseases, DILI, and drug-induced rhabdomyolysis<sup>[24-26]</sup>. Use of this method has raised concern about judgement validity by the USP<sup>[63,88]</sup>. Considering all shortcomings along with the lack of liver specificity and validation for hepatotoxicity, the Naranjo scale should be excluded from use in hepatotoxicity cases. It certainly is no substitute for the CIOMS scale.

#### KL method

The KL method<sup>[78]</sup> is neither liver specific nor validated for hepatotoxicity (Table 3), it also lacks important items for hepatotoxicity assessment (Table 4). It was recently applied for causality assessment of suspected hepatotoxicity for some Herbalife products<sup>[74]</sup>. Subjective judgement is needed for many steps, making this method more prone to bias<sup>[87]</sup>. Though commonly applied by the Spanish Pharmacovigilance Centres<sup>[74]</sup>, the KL method is not used by the Spanish Group for the Study of Drug-induced Liver Disease<sup>[59,85,87,95]</sup>, which applies the CIOMS scale as the preferred assessment tool. The KL method should not be used for assessment of hepatotoxicity cases.

#### Ad hoc approach

Numerous published HILI reports lack any causality method description and presumably are based on the *ad hoc* assessment with its relevant shortcomings (Tables 3 and 4). When using this approach, the physician notes the coincidence of herbal product and chemical drug use, and will estimate the likelihood of a hepatotoxic reaction<sup>[89]</sup>.

After ruling out alternative causes, the *ad hoc* approach is often used to distinguish a probable, possible, or unlikely causality<sup>[89]</sup>. A probable causality is usually attributed when the manifestations of liver disease, temporal association, and dechallenge response seems to fit the typical signature pattern of the product in question. A possible attribution is assigned when one feature is not typical, the product not known to cause the reaction or so rarely that it is difficult to distinguish from background, or an alternative cause is less or equally plausible. An unlikely causality is assigned when most of the features are atypical or an alternative cause is more plausible<sup>[89]</sup>.

Though relevant items such as signature of symptoms, latency period, dechallenge, definitive exclusion of alternative causes, risk factors, alcohol use, and track record of the product are used<sup>[79,89]</sup>, no universally accepted description exists for either the method or its application<sup>[79]</sup>. Due to missing specific criteria (Tables 3 and 4), the *ad hoc* approach is obsolete to validly assess causality in HILI<sup>[79]</sup> or DILI cases<sup>[89]</sup>.

With the *ad hoc* assessment applied prior to the liver specific CIOMS scale, the physician inevitably will postpone an assessment by such a procedure and thereby delay the diagnosis. Since the parameters of the *ad hoc* approach are liver unspecific and not validated (Tables 3 and 4), this method should be replaced by better alterna-

tives. The NIH LiverTox does not even mention the *ad hoc* approach as a possible causality evaluation method for hepatotoxicity cases<sup>[2,3]</sup>.

## RETROSPECTIVE CAUSALITY ASSESSMENT METHODS

#### DILIN method

According to the NIH LiverTox, the DILIN method is based on a narrative summary and a compilation of clinical findings and sequential biochemical abnormalities<sup>[2,3]</sup>. These are extracted from clinical records and entered into a 65-page case report form, but a scoring system was lacking<sup>[102]</sup>, as opposed to the CIOMS scale (Table 4). The DILIN causality adjunction process is outlined in a 12 step flow diagram, using three independently assessing experts in hepatotoxicity who grade the likelihood of a causal relationship between the drug and liver injury in one of five scores<sup>[102]</sup>: (1) Definite (> 95% assurance): the evidence for the drug causing the injury is beyond a reasonable doubt; (2) Highly likely (75% to 95% assurance): the evidence for the drug causing the injury is clear and convincing but not definite; (3) Probable (50% to 74% assurance): the preponderance of the evidence supports the link between the drug and the liver injury; (4) Possible (25% to 49% assurance): the evidence for the drug causing the injury is equivocal but present; and (5) Unlikely (< 25% assurance): there is evidence that an etiological factor other than the drug caused the injury.

While these causality grades appear vague, attempts are made to provide an objective and critical evaluation of the likelihood that the liver injury is due to the suspected agent<sup>[2,3]</sup>. In particular, cases are not considered “probable” merely because there is no other explanation. Similarly, cases are not considered “definite” if another diagnosis is possible. If two or three drugs are implicated, only one can be considered probable, highly likely or definite, the others are assigned “possible” or “unlikely”, so that the total percent assurance does not exceed 100%<sup>[2,3]</sup>. The causality assessment is accepted as initially scored if the three expert reviewers completely agree; if there is disagreement, the reviewers meet to reconcile the differences and reach a final single score<sup>[2,3,102]</sup>. A complete summary of the definitions for each category is provided<sup>[102]</sup>.

The DILIN method requires experts and has shortcomings (Tables 3 and 4)<sup>[2,3,73,80,86,102]</sup>; it is therefore not suitable for the physician who needs assessment results during the early disease. The DILIN method was used for retrospective assessments of case series where time to conclusion is not a crucial issue<sup>[73,86,102]</sup>. In combination with the CIOMS scale, this method is the basis for future DILIN group studies of clinical, genetic, environmental, and immunological risk factors<sup>[80]</sup>. To exclude alternative causes in retrospective analyses by the DILIN method, screening was required for previous liver disease, alcohol use, hepatitis A, B, or C infection, autoantibodies, ceruloplasmin,  $\alpha_1$ -antitrypsin, ferritin, iron, and

imaging data; specific details or appropriate scores for each item were not provided (Table 4)<sup>[102]</sup>. Other possible causes were not considered (Table 2), including specific liver infections like hepatitis E or by cytomegalovirus (CMV), Epstein Barr virus (EBV), herpes simplex virus (HSV), and varicella zoster virus (VZV)<sup>[102]</sup>. At present, questions regarding the actual DILIN method validity remain, and transparent results of all diagnostic items from each individual patient would be preferred rather than a summarizing causality grade.

Another approach of the DILIN group targets a novel Causality Assessment Tool (CAT) specifically for HDS<sup>[103]</sup>. CAT was designed to retrospectively adjudicate multiple products as a single entity using structured causality assessment and expert opinion. The elements of the CAT considered the multiplicity of products consumed, implicated drugs, alternative diagnoses, and published DILI literature on the product or an ingredient<sup>[103]</sup>. In analogy to the scoring system, the DILIN method expresses causality levels as percentage assurance<sup>[102]</sup>; CAT also grades the likelihood of a causal relationship between HDS and liver injury from definitive to unlikely<sup>[103]</sup>. In this preliminary study, CAT was applied in 16 DILI cases, which were initially evaluated by the DILIN method and in which HDS are implicated as a potential cause. Overall agreement and reliability in this study of retrospective analysis requiring an expert panel was moderate<sup>[103]</sup>; this method needs further investigation and validation<sup>[98]</sup>.

### WHO method

In its recent statement, the NIH LiverTox does not mention the WHO method in connection with causality assessment methods for hepatotoxicity cases but rather discusses other methods<sup>[2,3]</sup>. Since the WHO method<sup>[81]</sup> was not developed for hepatotoxicity cases and therefore does not consider hepatotoxicity characteristics<sup>[79,104]</sup>, this omission appears warranted. The shortcomings of the unspecific features of the WHO method (Tables 3 and 4) have been a matter of major concern<sup>[38,39,104-106]</sup> and led to the conclusion that this scale is not appropriate for causality assessment in suspected HILI cases<sup>[79,104]</sup>.

The WHO method consists of two parts, one being the WHO scale to assess causality levels, the other one the global introspection by experts<sup>[81]</sup>. Though not validated for any ADR<sup>[103]</sup>, global introspection surprisingly represented a popular strategy in evaluating the likelihood of drug causality for general ADRs of all organs<sup>[107]</sup>. As early as 1986, however, global introspection by experts has been shown to be neither reproducible nor valid<sup>[107]</sup>. In detail, the assessor considers factors that might support a causal link of one or more drugs to an observed ADR, lists all factors, weighs their importance, and estimates the probability of drug causation; no specific checklist or level of strength is given<sup>[107]</sup>. It has been recognized that both the questions and the answers are ambiguous<sup>[79]</sup>. Though these shortcomings are described for general ADRs, they certainly also apply even more to hepatic ADRs.

The WHO scale has not been based on a gold standard, is not quantitative, not liver specific, and has not been validated for hepatotoxicity (Tables 3 and 4)<sup>[4,38,39,79,104-106]</sup>. In particular, reliability, sensitivity, specificity, positive and negative predictive values are unknown, but likely are low<sup>[79,81,104-106]</sup>. Its scope is also limited since it cannot discriminate between a positive and a negative correlation, thereby resulting in overdiagnosing and overreporting<sup>[104]</sup>.

The WHO method ignores relevant data like uncertainties in daily dose, temporal association, start, duration and end of herbal use, time to onset of ADR, and course of liver values after herbal discontinuation. Insufficiently considered or ignored are comedication, preexisting liver diseases, numerous alternative explanations, and exclusion of virus infections by hepatitis A, B, C and E, CMV, EBV, HSV, and VZV<sup>[38,39]</sup>. Since only a few raw data are evaluated, case duplications and retracted cases remain undetected by the WHO method to a higher degree than by other methods<sup>[38]</sup>. Despite these flaws, the WHO method was used for causality assessment<sup>[17,38,39,53,54]</sup>. Re-evaluation often could not confirm causality in cases of two assessed reports<sup>[38,39]</sup>; therefore, the use of the WHO method in HILI cases has major limitations.

Causality assessment by the WHO method requires a panel of experts rarely available at a hospital or a family physician office. Consequently, analyses based on this method are retrospective; their results are available long after the patient problems of assumed HILI.

### Expert opinion

Expert opinion as an assessment tool is poorly defined (Tables 3 and 4), except that a panel of specialists with clinical expertise in hepatology is available for causality assessment in HILI. For DILI, groups of skilled hepatologists exists without any doubt in most countries including Japan<sup>[108,109]</sup> and in expert projects like the international DILI Expert Working Group<sup>[90]</sup>, the United States DILIN group<sup>[73,80,86,102,103]</sup>, the Spanish Group for the Study of Drug-Induced Liver Disease<sup>[59,85,87,95,101]</sup>, and the Spanish-Latin American network on drug induced liver injury<sup>[110]</sup>. For HILI, the Hong Kong Herb-Induced Liver Injury Network is of importance<sup>[75]</sup>. However, the qualification of assessors is sometimes crucial and may be problematic as discussed in detail<sup>[88,105,106]</sup>. Even with specialists, individual opinion often results in judgement bias.

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## RELEVANCE TO ACTUAL MEDICAL PRACTICE

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For HILI case assessment, strategies need to be developed that are clinically useful and applicable in daily practice. These must meet the expectations of the scientific community, regulatory agencies, and manufacturers, provided the case is going to be reported. At the day when HILI is suspected and criteria of hepatotoxicity are fulfilled, the physician should explore through the internet and regulatory databases how frequently the suspected herb has been associated with hepatotoxic

adverse reactions both in the scientific literature and by regulatory notifications. Publication as an interesting case report should be encouraged, if there are few or even none hepatotoxicity reports of this particular herb. Consequently, the decision will depend on the physician's own interest and clinical experience, resulting in three different levels of assessment intensity. These include first a wait and see approach after cessation of the herbal product, second a strategy aimed at exclusion of the most frequent differential diagnoses, or third an exclusion of even rare alternative causes.

The first approach of wait and see requires little attention and few elements and is cost effective, at least initially but not necessarily in the further course. If for some reasons the correct diagnosis was missed, it will be costly and risky for the patient, the physician, or both. Submitting such an insufficiently documented case as suspected HILI case to scientific journals, regulatory agencies or manufacturers would be difficult to reconcile, leading to overreporting due to overdiagnosing<sup>[68,82,88,104,105,111]</sup>. In detail, diagnostic problems including alternative diagnoses as confounding variables were evident in 77.5% of 573 cases of initially suspected HILI, presented as spontaneous reports or as published case reports<sup>[82]</sup>.

For the second strategy, the elements of the updated CIOMS scale are sufficient, starting with the evaluation of time to onset to verify at least a temporal association between the herbal use and the liver disease (Tables 5 and 6). For instance, if clinical assessment, hepatobiliary sonography, or serology of hepatitis A-C provides an alternative cause as the correct and final diagnosis, the costs will remain low since further diagnostic measures are not warranted. If diagnostic exclusion is unsuccessful so far, parameters of CMV, EBV, HEV, HSV, and VZV are needed (Tables 5 and 6), though in reality these elements are rarely reported in suspected HILI cases<sup>[13,14,17,23-26,38,39,94]</sup>. With complete or even some missing CIOMS elements, the CIOMS scale provided causality for various herbs with levels of probable and highly probable<sup>[35-37]</sup>.

For the third level of evaluation, the physician will have to decide, which of the multiple other and rare differential diagnoses are worth of consideration. The checklist should be valued as a reminder of possible alternatives and as a suggestion for further approaches, depending on the clinical phenotype. Clearly, the number of criteria set for ruling out alternative causes is not required for all cases, the checklist therefore asks selectively whether the information was completely, partially or not obtained (Table 2). A sophisticated strategy is needed, however, if the case is reported to regulatory agencies and the scientific community, which are overflooded by poorly documented suspected and often misdiagnosed HILI cases<sup>[26,34-36,38,39,82]</sup>. For optimum case presentation, the individual items of the updated CIOMS scale should be provided for a single case (Table 7)<sup>[58,97]</sup> as well as for case series. This is feasible as shown in numerous

publications<sup>[13,25,35,36,38,39,71,72,94]</sup> for 26 cases<sup>[13]</sup>, 22 cases<sup>[25]</sup>, 22 cases<sup>[35]</sup>, 21 cases<sup>[36]</sup>, 15 cases<sup>[38]</sup>, 13 cases<sup>[39]</sup>, and 4-9 cases<sup>[71,72,94]</sup>. The presentation of the CIOMS items for the single case should be combined with a detailed report of all relevant case data<sup>[58,97]</sup> and a list of differential diagnoses that were excluded completely or partially, or were not considered<sup>[58]</sup>, similar to the checklist for HILI diagnosis (Table 2). For a case series, basis data for each individual case are to be provided in a single table, focusing on details required for causality assessment; examples are presented in various publications<sup>[14,25,35,36,38,39]</sup>. Presentation of excellent data will lead to valid causality results and appropriate conclusions. This is prerequisite for well founded assessments of further HILI cases, with benefit for patients, physicians, the scientific community, regulatory agencies, and manufacturers.

## FUTURE PERSPECTIVES

Future considerations will have to focus on improvements of causality assessment methods<sup>[90,98]</sup> to obtain prospectively valid HILI diagnoses at the time the patient experiences liver injury, corresponding efforts of retrospective causality assessments of HILI cases are promising and on the way with preliminary data<sup>[103]</sup>. Strategies are to be developed to characterize liver injury by various herbs with all facets. At the day HILI is suspected, causality assessment should be initiated in all cases using the CIOMS scale preferentially in its updated form (Tables 5 and 6). Supported by the checklist for HILI diagnosis (Table 2), this could provide HILI cases with a probable or highly probable causality for a special herb as basis for further evaluation. Overall, this will facilitate characterization of disease entities including phenotype standardization, retrospective reanalysis by expert panels, improvement of pharmacovigilance decisions, safety strategies of manufacturers, and studies directed to assess pathogenetic aspects of HILI.

Studies are needed in the future to assess factors leading to unpredictable HILI in few patients, who experience this disease with a probable or highly probable causality level. As for DILI, future issues for HILI cases with established causality are to define genetic, environmental, and immunological determinants of HILI susceptibility<sup>[80,90,112,113]</sup>. Overall, metabolomics, pharmacogenetics, proteomics, and transcriptomics are areas of potential interest in HILI, as detailed for DILI<sup>[112]</sup>. Since HILI is commonly an unpredictable disease<sup>[91]</sup>, experimental studies dealing with predictive cellular systems as used to identify potentially hepatotoxic synthetic drugs<sup>[114]</sup> will be of limited if any relevance for herbs. Similarly, applying well-defined primary cultures of human hepatocytes and measuring a panel of signals directly linked to key mechanisms of liver injury to predict drugs, which can cause liver injury<sup>[114]</sup>, will be restricted to drugs and not be applicable to herbs. Recent advances of the early pre-clinical assessment of the potential intrinsic hepatotoxicity of candidate drugs has been reviewed

in detail, focusing on cell-based models such as cell cultures with outcome and detection methods, on profiling technologies, and emerging technologies including stem cell technologies and 3D as compared to 2D culturing techniques<sup>[115]</sup>. However, it is unlikely that the results of these *in vitro* studies of intrinsic and predictable hepatotoxicity induced by synthetic drugs are transferable to a clinical setting of HILI that commonly represents the idiosyncratic and unpredictable form of liver injury by one or more herbs, each with multiple chemical constituents. More important seems the search for biomarkers in HILI patients with clearly established causality<sup>[116]</sup>.

## CONCLUSION

The rare liver injury by herbs, herbal drugs, and herbal supplements may present itself with numerous facets, providing challenging issues for causality assessment. The physician is responsible to make available all necessary data for a high quality judgement; otherwise, causality evaluation will be problematic. Timely causality assessment is mandatory when the disease is unfolding to base prospective diagnostic and therapeutic decisions. The most appropriate causality assessment method is the liver specific CIOMS scale, which should prospectively be applied by the physician. If used, other methods have pitfalls and cause ambiguous results debated on reasons of imprecision, liver unspecificity, and limitations to retrospective analyses, or they are unavailable due to requirements for expert panels.

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**P- Reviewers** Devarbhavi H, Helling TS, Taye A  
**S- Editor** Wen LL **L- Editor** A **E- Editor** Xiong L



## Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver

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Supported by Australian National Health and Medical Research Council Grant 512282 (to Gorrell MD); Rebecca L Cooper Foundation Equipment Grants (to Gorrell MD); University of Sydney International Scholarship (to Chen Y); Australian Postgraduate Scholarship (to Yao TW); and Grant NIH U19 AI066313 (to Schuppan D)

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Received: November 7, 2012 Revised: January 17, 2013

Accepted: February 2, 2013

Published online: May 21, 2013

(B220<sup>+</sup>), human lymphoma cell lines and mouse splenocytes stimulated with pokeweed mitogen (PWM) or lipopolysaccharide (LPS), and in dithiothreitol (DTT) and mitomycin-C treated Raji cells. DPP8 and DPP9 expression were measured in epidermal growth factor (EGF) treated Huh7 hepatoma cells, in fibrotic liver samples from mice treated with carbon tetrachloride (CCl<sub>4</sub>) and from multidrug resistance gene 2 (*Mdr2/Abcb4*) gene knockout (gko) mice with biliary fibrosis, and in human end stage primary biliary cirrhosis (PBC).

**RESULTS:** All three lymphocyte subsets expressed DPP8 and DPP9 mRNA. DPP8 and DPP9 expression were upregulated in both PWM and LPS stimulated mouse splenocytes and in both Jurkat T- and Raji B-cell lines. DPP8 and DPP9 were downregulated in DTT treated and upregulated in mitomycin-C treated Raji cells. DPP9-transfected Raji cells exhibited more annexin V<sup>+</sup> cells and associated apoptosis. DPP8 and DPP9 mRNA were upregulated in CCl<sub>4</sub> induced fibrotic livers but not in the lymphocytes isolated from such livers, while DPP9 was upregulated in EGF stimulated Huh7 cells. In contrast, intrahepatic DPP8 and DPP9 mRNA expression levels were low in the *Mdr2* gko mouse and in human PBC compared to non-diseased livers.

**CONCLUSION:** These expression patterns point to biological roles for DPP8 and DPP9 in lymphocyte activation and apoptosis and in hepatocytes during liver disease pathogenesis.

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### Abstract

**AIM:** To investigate the expression of dipeptidyl peptidase (DPP) 8 and DPP9 in lymphocytes and various models of liver fibrosis.

**METHODS:** DPP8 and DPP9 expression were measured in mouse splenic CD4<sup>+</sup> T-cells, CD8<sup>+</sup> T-cells and B-cells

**Key words:** Dipeptidyl peptidase; CD26; Lymphocytes; Liver fibrosis; Biliary fibrosis

Chowdhury S, Chen Y, Yao TW, Ajami K, Wang XM, Popov Y, Schuppan D, Bertolino P, McCaughan GW, Yu DMT, Gorrell MD. Regulation of dipeptidyl peptidase 8 and 9 expression in activated lymphocytes and injured liver. *World J Gastroenterol*

2013; 19(19): 2883-2893 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2883.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2883>

## INTRODUCTION

The four enzyme members of the dipeptidyl peptidase (*DPP*) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9*, have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes<sup>[1,2]</sup>. *FAP* is a potential cancer therapeutic target<sup>[2,3]</sup>. *DPP4*, the most well characterized family member, has ubiquitous cell surface and extracellular expression<sup>[2,4-7]</sup>. *DPP8* and *DPP9* are the most recently discovered members of the *DPP4* gene family<sup>[8-11]</sup>. *DPP4*, *DPP8* and *DPP9* are ubiquitously expressed cytosolic enzymes with *DPP4*-like activity<sup>[8,11,12]</sup>. They are expressed by major epithelial organs including liver, colon, small intestine, stomach, lung, skin, tongue, kidney, testis and the lymphoid cells of lymph node, blood, thymus, and spleen<sup>[13]</sup>. The biological functions of *DPP8* and *DPP9* are largely uncharacterized.

*DPP4* is also known as *CD26* and has important roles in the immune system. It is a costimulatory molecule in T cell activation and proliferation and is critical in the development of T helper 1 responses to foreign antigens. It is expressed at detectable levels by some resting T cells but the cell surface expression increases 5-10 fold following stimulation with antigen or anti-CD3<sup>+</sup> interleukin-2 or with mitogens such as phytohaemagglutinin<sup>[14-19]</sup>. However, the costimulatory role of *DPP4/CD26* is mediated by extra-enzymatic activities<sup>[20-22]</sup>. Hence, some of the immunological effects observed in early *DPP4* inhibitor studies are now thought to be due to off-target non-selective inhibition of *DPP8* and *DPP9*<sup>[2,23,24]</sup>. In support of this viewpoint, there is some evidence that *DPP8* and *DPP9* are functionally significant in the immune system. Their mRNA levels are elevated in activated human leukocytes<sup>[25,26]</sup>. An inhibitor of *DPP8* and *DPP9* attenuates proliferation in *in vitro* models of human T-cell activation<sup>[23]</sup>. An inhibitor selective for *DPP8* and *DPP9* vs related proteases can suppress DNA synthesis in mitogen-stimulated splenocytes from both wildtype *DPP4*<sup>+/+</sup> and *DPP4*<sup>-/-</sup> gene knockout (gko) mice<sup>[27]</sup>. Moreover, *DPP8* and *DPP9* have been implicated in hematopoiesis and in inflammatory diseases including arthritis<sup>[2,28,29]</sup>. Most importantly, *DPP8* and *DPP9* are involved in processing and degradation of peptides involved in antigen presentation by Major histocompatibility complex class I<sup>[30]</sup>.

Inflammatory and immune responses are important in liver injury. Improved understanding of immune response, inflammation and fibrogenic progression is needed to advance the understanding of liver disorders. *DPP8* and *DPP9* are expressed in hepatocytes and lymphocytes of human cirrhotic liver<sup>[13]</sup>. Hepatocytes in the periportal area of regenerative nodules and lymphocytes in the portal tracts are strongly positive for *DPP8* and *DPP9* *in situ*

hybridization (ISH)<sup>[13]</sup>. Bile ducts and ductular reactions are ISH positive for *DPP9* but not for *DPP8*<sup>[13]</sup>. However, the role of *DPP8* and *DPP9* in liver is unknown. Other members of this protease family, *DPP4* and *FAP*, are altered in liver diseases and are potential disease markers and therapeutic targets<sup>[31-36]</sup>. Despite the pleiotropic roles of *DPP4* and *FAP* in various biological processes, *DPP4* and *FAP* gko mice exhibit no spontaneous defects, suggesting that *DPP4* and *FAP* are not essential for normal functions, and hence, targeting them is likely to lack adverse side effects<sup>[37,38]</sup>.

*DPP8* and *DPP9* have interesting properties in cell biological processes that may contribute to disease pathogenesis, such as apoptosis and cell migration<sup>[39,40]</sup>. Their biological functions, especially in the immune system, are important considerations for the selectivity of *DPP4* inhibitors over *DPP8* and *DPP9* in clinical development of *DPP* antagonists. Here we studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury to elucidate their potential biological roles in the immune system and in liver diseases.

## MATERIALS AND METHODS

### Materials

Antibodies are detailed in Table 1. Other materials were from Sigma-Aldrich (St Louis, MO, United States) unless stated.

### Animal studies

Mice were maintained in the Centenary Institute animal facility under specific pathogen-free conditions. The Animal Ethics Committee of the University of Sydney approved experimental procedures and housing arrangements. *FAP* gko<sup>[38]</sup> and *DPP4* gko mice<sup>[37]</sup> (C57BL/6J background) were bred at the Animal Resource Centre (Perth, Australia). Female multidrug resistance gene 2 (*Mdr2/Abcb4*) gko mice (FVB/N background) with targeted disruption of *Mdr2*, were obtained from Jackson Laboratory (Jackson Laboratory, Bar Harbor, ME, United States)<sup>[35]</sup>. Liver samples from the *Mdr2* gko and wild type (wt) mice were obtained at 4, 8 and 12 wk after birth, the time points that span the most active fibrosis progression<sup>[35]</sup>. RNA were obtained as previously described<sup>[41]</sup>. Lymphocytes from wt, *DPP4* gko and *FAP* gko mouse spleen, liver and lymph nodes were isolated as previously described<sup>[42]</sup>.

For the liver fibrosis mouse model, 8-wk-old female wt, *DPP4* gko and *FAP* gko mice were injected intraperitoneally with carbon tetrachloride (CCl<sub>4</sub>) twice weekly for 3 wk. Each dose comprised 5.36  $\mu$ L of 12% CCl<sub>4</sub> (in paraffin oil) per gram of initial weight of each mouse. Significantly elevated alanine aminotransferase (ALT) (68  $\pm$  11.1 U/L vs untreated controls 32  $\pm$  1.2 U/L) indicated liver injury. ALT was performed by an auto-analyzer at the Clinical Biochemistry Department of the Royal Prince Alfred Hospital. Organs were collected 3 d after the final CCl<sub>4</sub> treatment.

**Table 1** Antibodies used in immunoblot and flow cytometry

| Name                  | Isotype                            | Conjugate | Dilution | Supplier                               | Catalogue no. |
|-----------------------|------------------------------------|-----------|----------|--|---------------|
| Primary antibodies    |                                    |           |          |  |               |
| CD4                   | Rat IgG <sub>2b</sub>              | FITC      | 1:50     | BD Pharmingen, NJ, United States       | 557307        |
| B220                  | Rat IgG                            | PE        | 1:50     | Caltag Laboratories, CA, United States | RM2604-3      |
| CD8                   | Rat IgG <sub>2a</sub>              | APC       | 1:50     | BD Pharmingen, NJ, United States       | 553035        |
| Annexin V             |                                    | APC       | 1:50     | BD Pharmingen, NJ, United States       | 550474        |
| V5                    | Mouse monoclonal IgG <sub>2a</sub> |           | 1:5000   | Invitrogen                             | R960-25       |
| DPP8-catalytic domain | Rabbit polyclonal                  |           | 1:2000   | Abcam Inc                              | Ab42077       |
| DPP8-catalytic domain | Rabbit polyclonal                  |           | 1:2000   | Abcam Inc.                             | Ab42076       |
| DPP9-catalytic domain | Rabbit polyclonal                  |           | 1:2000   | Abcam Inc.                             | Ab42080       |
| GAPDH                 | Mouse monoclonal                   |           | 1:1000   | EnCor biotechnology                    | MCA-1D4       |
| β-actin               | Rabbit polyclonal                  |           | 1:1000   | Sigma                                  | A2103         |
| Secondary antibodies  |                                    |           |          |  |               |
| Anti-rabbit IgG       | Goat                               | HRP       | 1:3000   | DAKO, Carpinteria, CA, United States   | PO448         |
| Anti-mouse            | Goat IgG                           | R-PE      | 1:400    | Molecular Probes                       | P852          |

DPP: Dipeptidyl peptidase; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; IgG: Immunoglobulin G; FITC: Fluorescein; PE: Phycoerythrin; APC: Allophycocyanin; HRP: Horse radish peroxidase; R-PE: R-phycoerythrin.

### Human liver samples

Human liver tissues were obtained from liver transplant recipients in accordance with National Health and Medical Research Council guidelines under Royal Prince Alfred Hospital Human Ethics Committee approvals. Non-diseased liver donors had an age range of 56-58 years and mixed genders. Cirrhotic livers were from primary biliary cirrhosis (PBC) patients of average age  $51.7 \pm 13.3$  years (range 27-67 years; 10 females, 2 males) and end stage alcoholic liver disease patients of average age  $49.3 \pm 8$  years (range 34-60 years, 9 males) as described previously.<sup>[40]</sup>

### In vitro stimulation assays

Human B lymphocyte Burkitt's lymphoma cell line (Raji) (ATCC, CCL-86) and human T cell leukemia cell line (Jurkat) (ATCC, TIB-153) were cultured in Roswell Park Memorial Institute (RPMI) Medium 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal calf serum (FCS) and Penicillin-Streptomycin (100 units of penicillin and 100 µg/mL of streptomycin) (1 × P/S) and human liver hepatocellular carcinoma cell line Huh7 were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% FCS and 1 × P/S.

Lymphocytes at  $1 \times 10^6$  cells/mL RPMI were treated with either 5 µg/mL pokeweed mitogen (PWM), 20 µg/mL lipopolysaccharide (LPS), 50 µg/mL Mitomycin C or 10 mmol/L dithiothreitol (DTT). Human liver hepatocellular carcinoma cell line, Huh7 cells were serum starved for 20 h before stimulation with 0, 1, 10, 100 ng/mL epidermal growth factor (EGF; R-D Systems, MN, United States) for 4 h.

### Apoptosis assay

To determine if DPP9 overexpression induces apoptosis, Raji cells were transiently transfected with wtDPP9-V5-His, mutDPP9-V5-His or vector control (pcDNA3.1/V5-HisA; Invitrogen, Carlsbad, CA, United States) as described previously,<sup>[39]</sup> then cultured. The lymphocytes

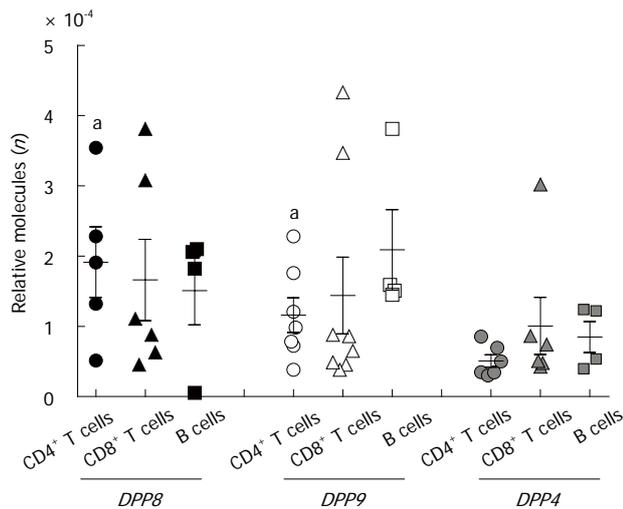
were transfected by electroporation using Amaxa® Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland) on a Lonza-amaxa Nucleofector device (Lonza). Forty hours post transfection, cells were washed with annexin binding buffer (10 mmol/L HEPES, 140 mmol/L NaCl, 2.5 mmol/L CaCl<sub>2</sub>, pH 7.4). Staining involved incubating cells with annexin V antibody (Table 1) for 30 min at room temperature in the dark followed by 4',6-diamidino-2-phenylindole (DAPI), Sigma Aldrich) at 100 ng/mL. Cells were enumerated using flow cytometry. Analysis was performed using FlowJo software (Tree Star Inc., Ashland, OH, United States).

### Fluorescence activated cell sorting

To isolate mouse lymphocyte subsets,  $3 \times 10^7$  splenocytes were resuspended in primary antibody diluted in phosphate buffered saline (PBS) containing 1% FCS and incubated in the dark, on ice for 30 min. The primary antibodies used are listed in Table 1. Following antibody staining, cells were washed with PBS containing 1% FCS. Cells underwent a final resuspension of  $2 \times 10^7$  cells/mL of PBS with 5% FCS and 2 mmol/L ethylene diamine tetraacetic acid (EDTA) to minimize clumping of cells. Twenty-five µL/mL of DAPI was added prior to cell sorting. Cell sorting was performed using the Fluorescence Activated Cell Sorting Vantage™ SE (BD Bioscience, NJ, United States). Cells were gated to exclude doublets and DAPI<sup>+</sup> (dead) cells. Three-way sort was performed to collect CD4<sup>+</sup> cells, CD8<sup>+</sup> cells and B220<sup>+</sup> cells into separate collection tubes.

### Real time quantitative polymerase chain reaction

RNA from cells was extracted using the RNAqueous-Micro™ kit (Ambion, TX, United States) following manufacturer's instructions. Total RNA (1 µg) was then reverse-transcribed to cDNA using 10 pmol of oligo(dT)<sub>12-18</sub> primer (Invitrogen, Carlsbad, CA, United States), 10 mmol/L deoxyribonucleotide triphosphates and SuperScript III reverse transcriptase (Invitrogen). Real time quantitative poly-



**Figure 1** Dipeptidyl peptidase mRNA expression in C57BL/6 mouse splenic lymphocyte subpopulations. Number of molecules relative to 18S RNA ( $n = 4-6$  mice). <sup>a</sup> $P < 0.05$  vs dipeptidyl peptidase (DPP) 4.

merase chain reaction (PCR) by Taqman<sup>®</sup> gene expression assays was performed using the Stratagene<sup>®</sup> Mx3000PTM System (La Jolla, CA, United States) according to manufacturer's recommendations. Taqman primers used for the assays were mouse *DPP4* (Mm00494548\_mL), *DPP8* (Mm00547049\_mL) and *DPP9* (Mm00841122\_mL). The samples were run in duplicates. The gene expression level was analyzed using a standard curve of serially diluted known numbers of molecules of the same gene and then normalized relative to 18S (Hs99999901\_s1). Quantitative PCR on human samples were performed using sequence detector (Prism, model 7700; Life Technologies, NY, United States) and were analyzed using sequence detector software (Prism, Version.1.6.3; Applied Biosystems Inc.). Primers used for human *DPP8* were forward: 5' CCAGATGGACCTCATTCAGACAG-3' and reverse: 5'GGTTGTTGCGTAAATCCTTGTGG-3' and for human *DPP9* were forward: 5'AGAAGCACCCACCGTCCTCTTTG-3' and reverse: 5'AGGACCAGCCATGGATGGCAACTC-3'. The number of molecules was normalized with human aldolase B (forward: 5'-CCTC-GCTATCCAGGAAAAC-3' and reverse: 5'TTGTAGA-CAGCAGCCAGGAC-3').

### Immunoblotting assay

Cells were washed with ice-cold PBS three times and then lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, 1 mmol/L EDTA, 1mmol/L MgCl<sub>2</sub>, 300  $\mu$ L of 150 mmol/L NaCl, 1% Triton-114, 10% glycerol and 1  $\times$  Roche complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and stored at -80  $^{\circ}$ C. Protein concentration was determined using the micro BCA protein assay kit (Thermo Scientific, CA, United States) following the manufacturer's protocol. 50  $\mu$ g total of each cell lysate in LDS sample buffer (catalogue No. NP0007, Invitrogen) with reducing agent (catalogue No. NP0004, Invitrogen) in conditions that retain *DPP8* and *DPP9* dimerization<sup>[18,9,40]</sup> was resolved on 3%-8%

Tris-acetate sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Invitrogen) followed by immunoblotting. Antibodies for immunoblotting are listed in Table 1. Relative band intensities were quantified using Image J and normalized against control proteins as indicated<sup>[40]</sup>.

### Statistical analysis

Results are expressed as individual replicates. Horizontal lines represent mean and error bars represent standard error. Differences among groups were analyzed using Mann-Whitney *t*-test by GraphPad Prism 5 software. *P* values  $< 0.05$  were considered significant.

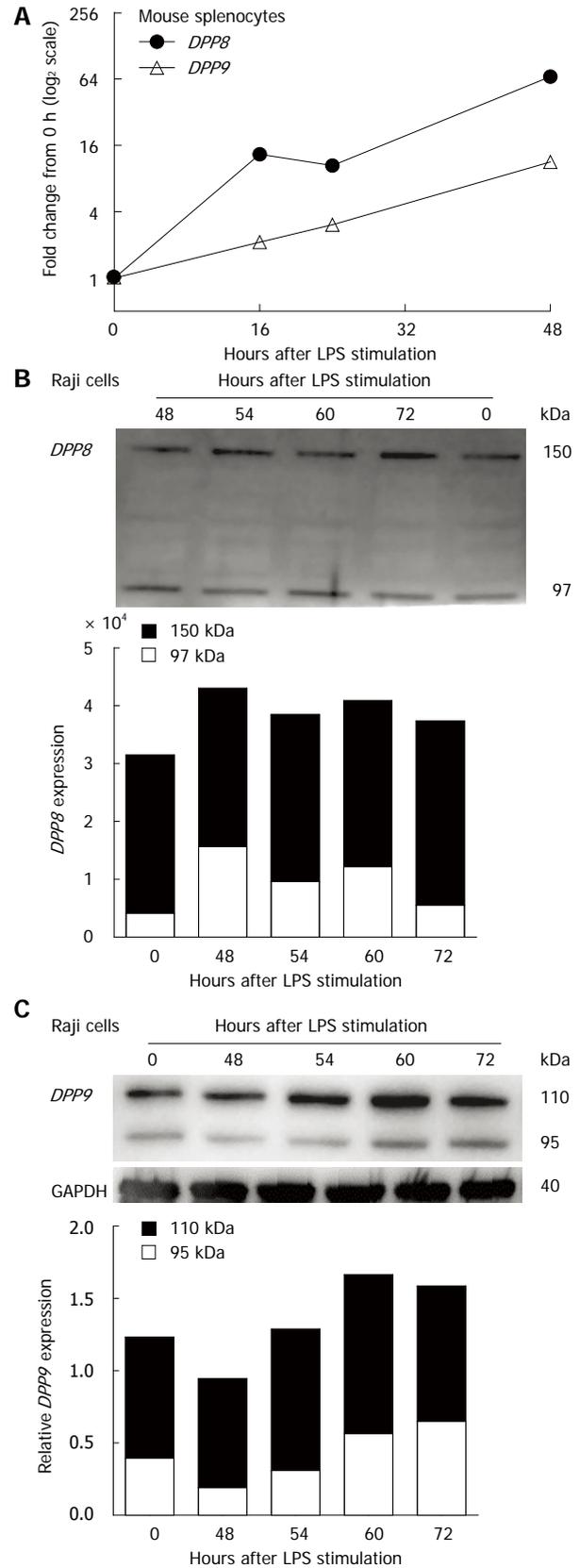
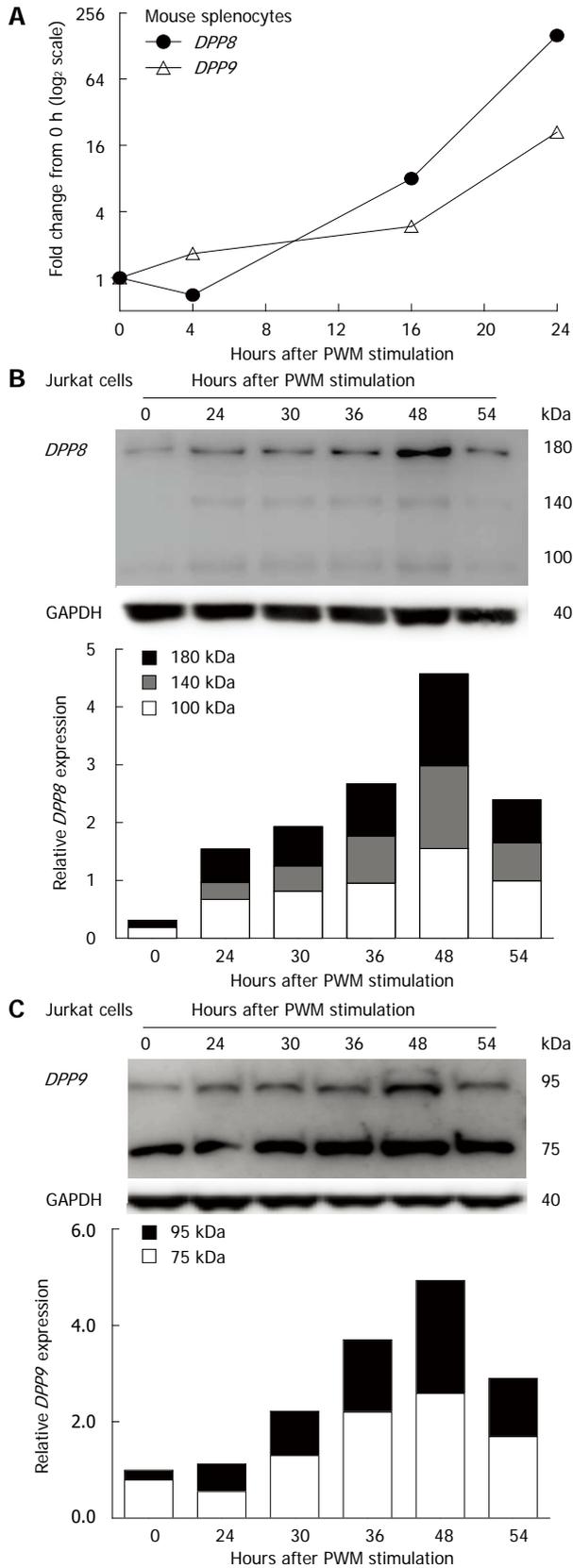
## RESULTS

To investigate which lymphocyte subsets express *DPP8* and *DPP9*, their transcript levels were quantified in the major lymphocyte subpopulations, CD4<sup>+</sup> (helper) and CD8<sup>+</sup> (cytotoxic) T cells and B220<sup>+</sup> (B cells) from normal C57BL/6 mouse splenocytes. All three lymphocyte subsets expressed *DPP8* and *DPP9* mRNA (Figure 1). *DPP8* and *DPP9* transcripts were expressed to significantly greater levels than *DPP4* transcripts in CD4<sup>+</sup> T cell subpopulation ( $P = 0.02$ ) and *DPP9* mRNA was significantly more abundant than *DPP4* mRNA in B cells ( $P = 0.03$ ).

To examine whether, like *DPP4*<sup>[7]</sup>, *DPP8* and *DPP9* are upregulated upon lymphocyte activation, mouse splenocytes were stimulated *in vitro* with PWM<sup>[43-45]</sup> and LPS<sup>[46,47]</sup>. *DPP8* and *DPP9* mRNA was markedly upregulated in PWM stimulated mouse splenocytes in a time dependent manner (Figure 2A). To examine whether the increased mRNA levels corresponded to protein expression, *DPP8* and *DPP9* proteins were measured in Jurkat (T cells) stimulated *in-vitro* with PWM. Both *DPP8* and *DPP9* were upregulated in a time dependent manner (Figure 2B and C).

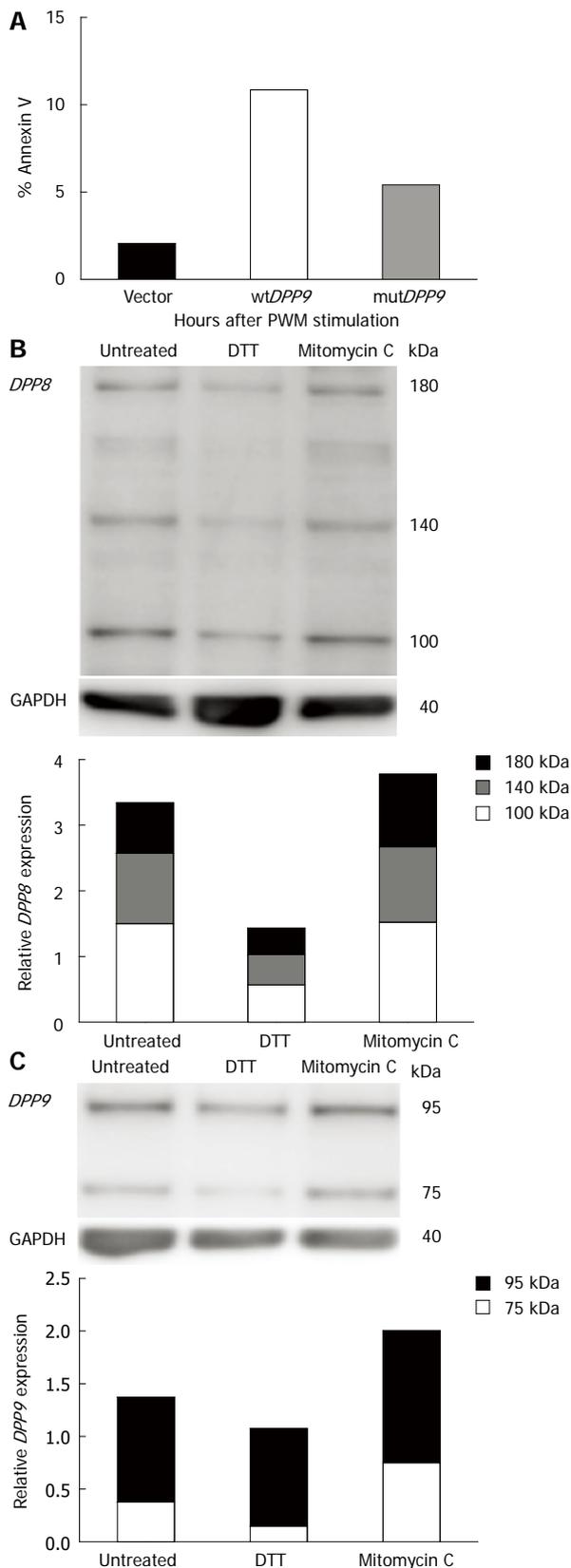
Similarly, mRNA levels of *DPP8* and *DPP9* were upregulated in LPS stimulated mouse splenocytes (Figure 3A). Also, LPS stimulated Raji (B cells) had upregulated *DPP8* and *DPP9* protein expression in a time dependent manner (Figure 3B and C).

Immunoblots of *DPP8* exhibited a slow mobility band at 150-180 kDa, which probably represents dimer or processed dimer, in addition to the faster mobility bands at 95-100 kDa that are likely to be monomer and truncated or trimmed monomer (Figure 2B and 3B). *DPP9* showed a slow mobility band of monomer at 110 kDa and faster mobility bands, which are possibly truncated or trimmed monomers at 75-95 kDa (Figure 2C and 3C)<sup>[18,9,40,48]</sup>. The intensity of all three *DPP8* bands increased in a time dependent manner with PWM stimulation in Jurkat cells (Figure 2B). However, in LPS stimulated Raji cells the intensity of only the 150 kDa band increased in a time dependent manner (Figure 3B). The intensity of all the *DPP9* bands increased with time in both PWM stimulated Jurkat cells and LPS stimulated Raji cells (Figure 2C and 3C). In PWM stimulated Jurkat cells, *DPP8* and *DPP9* expression both peaked at 48 h (Figure 2B and C). In Raji cells, increased expression of *DPP8* was observed at 72 h post LPS stimulation (the longest time point of the

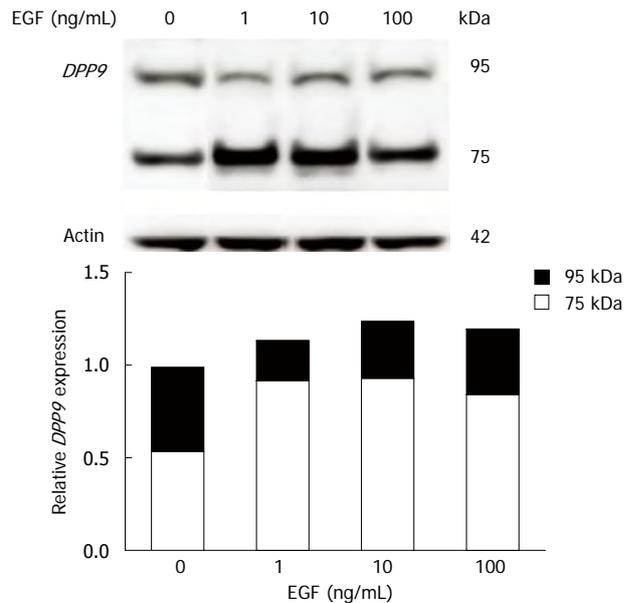


**Figure 2** Diptidyl peptidase 8 and diptidyl peptidase 9 upregulation in pokeweed mitogen stimulated lymphocytes. **A:** Diptidyl peptidase (*DPP*) 8 and *DPP9* mRNA in mouse splenocytes (representative data from one of three mice); *DPP8* and *DPP9* proteins from Jurkat cells; **B:** Immunoblot of *DPP8* and densitometry analysis of *DPP8* bands; **C:** Immunoblot of *DPP9* and densitometry analysis of bands. Densitometry data shown are relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PWM: Pokeweed mitogen.

**Figure 3** Diptidyl peptidase 8 and diptidyl peptidase 9 upregulation in lipopolysaccharide stimulated lymphocytes. **A:** Diptidyl peptidase (*DPP*) 8 and *DPP9* mRNA in mouse splenocytes (representative data from one of three mice); **B:** Immunoblot of *DPP8* and densitometry analysis of *DPP8* bands; **C:** *DPP9* immunoblot and densitometry analysis of *DPP9* bands relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). LPS: Lipopolysaccharide.



**Figure 4** Dipeptidyl peptidase 8 and dipeptidyl peptidase 9 were associated with lymphocyte apoptosis. **A:** Percentage of annexin V + Raji cells 40 h after transfection with vector, wild type (wt) dipeptidyl peptidase (*DPP*) 9-V5-His or enzyme-negative mutant (mut) *DPP*9-V5-His. Annexin V staining was enumerated by flow cytometry; **B:** Immunoblot of *DPP*8 and its densitometry (**C**) immunoblot of *DPP*9 and its densitometry in Raji cells untreated and treated with dithiothreitol (DTT) or mitomycin C for 24 h. Densitometry are shown as relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).



**Figure 5** Dipeptidyl peptidase 9 upregulation in epidermal growth factor treated Huh7 cells. Dipeptidyl peptidase (*DPP*) 9 immunoblot of untreated and epidermal growth factor (EGF)-treated Huh7 cells at 4 h. Cells were serum starved overnight before EGF treatment. Densitometry of *DPP*9 is shown relative to actin.

study) (Figure 3B), and *DPP*9 expression peaked at 60 h (Figure 3C).

### *DPP*8 and *DPP*9 in lymphocyte apoptosis

We have previously shown that *DPP*9 overexpression induces intrinsic cell apoptosis in human hepatoma and embryonic kidney cell lines<sup>[39,40]</sup>. Similar to epithelial cells, *DPP*9 overexpression induced increased cell death in Raji cells (Figure 4A). This effect was less pronounced when Raji cells were transfected with mutant *DPP*9 lacking *DPP* activity (Figure 4A), suggesting that the enzyme activity of *DPP*9 influences lymphocyte apoptosis.

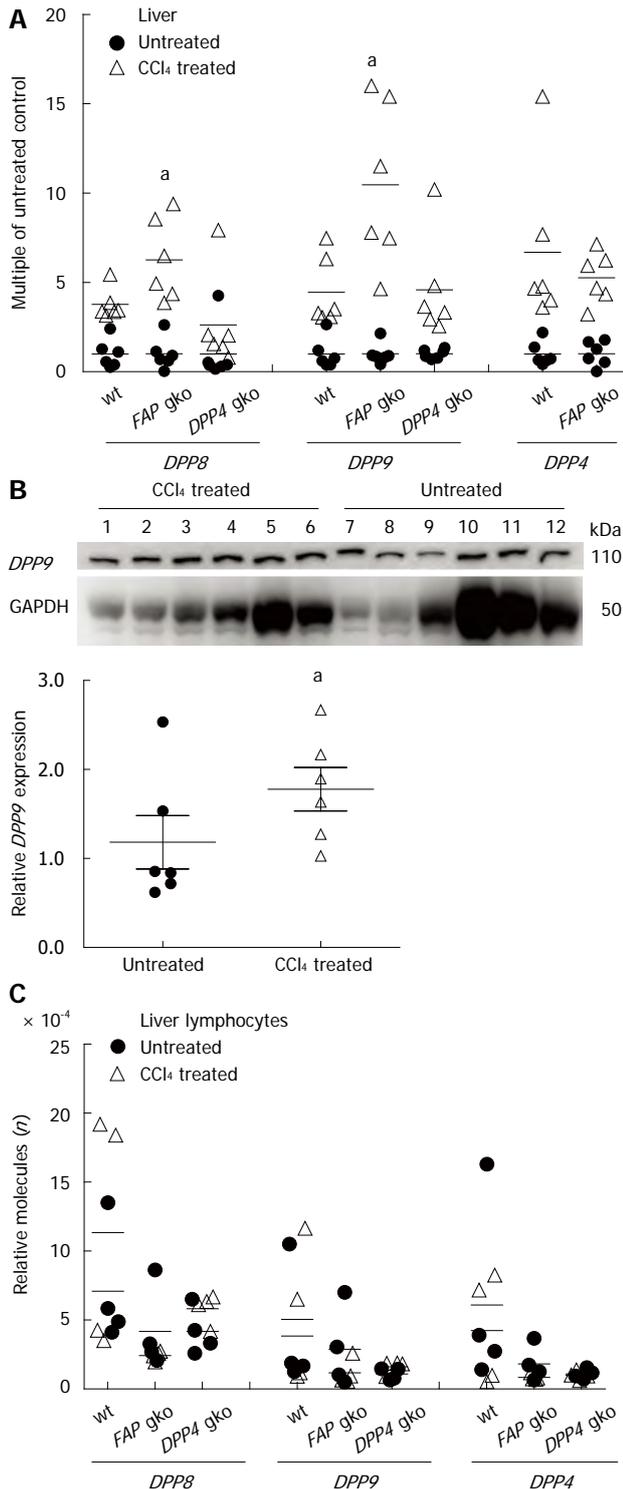
Interestingly, Raji cells treated with DTT, an antioxidant that impairs cell apoptosis, had less *DPP*8 and *DPP*9 expression compared to untreated cells (Figure 4B and C). Conversely, treatment of Raji cells with mitomycin C, a lectin that impairs cell proliferation, resulted in increased *DPP*8 and *DPP*9 expression in Raji cells (Figure 4B and C). Intensities of all *DPP*8 and *DPP*9 band sizes were less with DTT treatment and greater with Mitomycin C treatment compared to untreated cells (Figure 4B and C).

### *DPP*9 in EGF stimulated hepatocytes

EGF is a regulatory factor in cell survival, growth, proliferation and differentiation<sup>[49]</sup>. Previously, we have shown that *DPP*9 overexpression impairs EGF-stimulated cell proliferation in HepG2 and Huh7 human hepatoma cell lines<sup>[40]</sup>. *DPP*9 expression at 75 kDa was greater in Huh7 cells after EGF stimulation (Figure 5). This study expands the association of *DPP*9 with EGF in this hepatoma cell line.

### Intrahepatic *DPP*8 and *DPP*9 upregulation in CCl<sub>4</sub> induced liver injury

To examine *DPP*8 and *DPP*9 expression in liver injury,



**Figure 6** Dipeptidyl peptidase mRNA upregulation in carbon tetrachloride induced liver injury. **A:** Multiple of intrahepatic mRNA in carbon tetrachloride (CCl<sub>4</sub>) treated mice to mean of untreated control mice; <sup>a</sup>*P* < 0.05 in CCl<sub>4</sub> treated fibroblast activation protein (*FAP*) gene knockout (*gko*) vs wild type (*wt*); **B:** Dipeptidyl peptidase (*DPP*) 9 immunoblot of livers from CCl<sub>4</sub> treated (lanes 1-6) and untreated mice (lanes 7-12) (*n* = 6 per group). Densitometry of intrahepatic *DPP9* relative to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). <sup>a</sup>*P* < 0.05 vs untreated controls; **C:** mRNA quantitation from isolated hepatic lymphocytes relative to 18S.

CCl<sub>4</sub> was used to induce liver fibrosis in *wt*, *DPP4 gko* and *FAP gko* mice. *DPP4*, *DPP8* and *DPP9* mRNA were

significantly more abundant in the livers from CCl<sub>4</sub> treated mice of all three genotypes compared to the untreated controls (Figure 6A). *DPP8* and *DPP9* mRNA expression in the CCl<sub>4</sub> treated livers were greater in the *FAP gko* mice compared to *wt* (*DPP8 P* = 0.02; *DPP9 P* = 0.02), suggesting that *DPP8* and *DPP9* might have compensatory roles in the absence of *FAP* (Figure 6A). The increase in *DPP9* mRNA in the fibrotic livers was consistent with protein expression in *wt* mice (*P* = 0.05) (Figure 6B). An appropriate antibody to mouse *DPP8* is not available.

Since *DPP8* and *DPP9* are expressed by human hepatic lymphocytes<sup>[13]</sup> and because there is an increase of infiltrating lymphocytes in liver fibrosis, we examined whether the mouse hepatic lymphocytes were likely to contribute to the observed upregulation of *DPP* expression. However, *DPP* mRNA in the mouse hepatic lymphocytes was similar in the fibrotic and normal livers (Figure 6C).

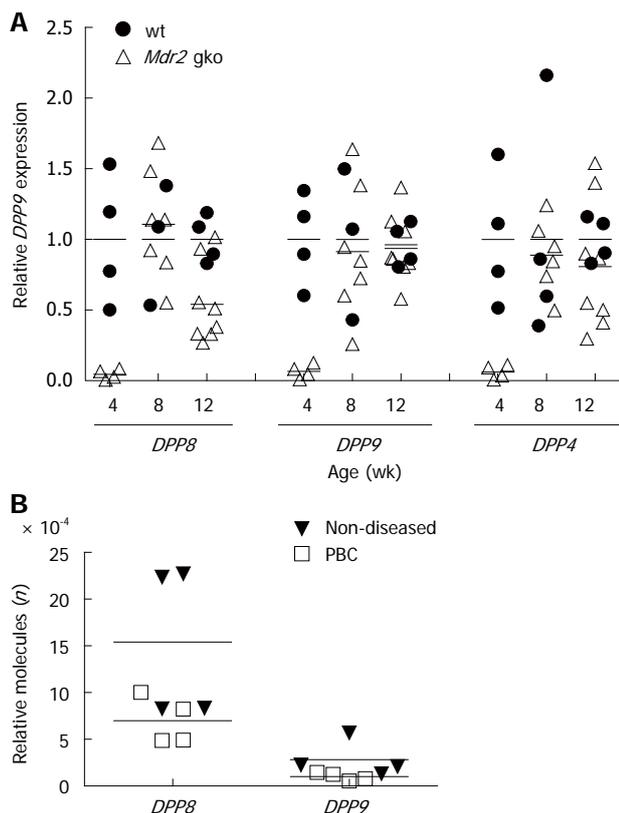
### Intrahepatic *DPP8* and *DPP9* downregulation in biliary liver disease

The *Mdr2 gko* mouse strain is deficient in the canalicular phospholipid flippase and is a model of periportal biliary fibrosis resembling primary sclerosing cholangitis<sup>[41]</sup>. These mice develop spontaneous hepatomegaly as early as 2 wk after birth and significant biliary fibrosis with a fivefold increased liver collagen content by 12 wk of age, when no further fibrosis progression occurs<sup>[41]</sup>. Measuring *DPPs* in these mice at 4, 8 and 12 wk of age showed that *DPP* mRNA expression was surprisingly very low at wk 4, significantly lower than in *wt* (*DPP8 P* = 0.03; *DPP9 P* = 0.03; *DPP4 P* = 0.03) (Figure 7A). At 8 and 12 wk of age, *DPP* expression levels were similar to *wt*.

In human end-stage PBC livers, *DPP9* mRNA expression was significantly less than in the non-diseased livers (*P* = 0.03) (Figure 7B). This finding is consistent with the results in the *Mdr2 gko* mice and with the human *DPP9* Western blot data<sup>[40]</sup>. *DPP8* mRNA expression levels in the non-diseased and PBC livers were not statistically different (*P* = 0.057).

## DISCUSSION

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. We showed that *DPP8* and *DPP9* are widely expressed in lymphocyte subpopulations and upregulated in mitogen activated lymphocytes in a time dependent manner. Besides lymphocyte activation, we demonstrated their potential involvement in lymphocyte apoptosis. In liver, we showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of EGF in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration. The interestingly variable expression patterns of *DPP8* and *DPP9* in different conditions in lymphocytes and in liver injury suggest that these proteases may have important regulatory roles in the immune system and in liver disease pathogenesis.



**Figure 7** Dipeptidyl peptidase mRNA in mouse and human biliary liver diseases. **A:** Multiple of Intrahepatic dipeptidyl peptidase (*DPP*) mRNA in multidrug resistance gene 2 (*Mdr2*) gene knockout (gko) female mice to mean of wild type (wt) controls; **B:** Human end-stage primary biliary cirrhosis (PBC) and non-diseased control livers. Data from each individual is shown as the number of molecules relative to aldolase B (*n* = 4 per group).

*DPP8/9* activity and expression in lymphocytes have been reported previously<sup>[8,30,50]</sup>, but which lymphocyte subpopulations express *DPP8* and *DPP9* remained unknown. Here we show that all the lymphocyte subpopulations tested, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and B220<sup>+</sup> B cells express *DPP8* and *DPP9*. The wide expression of *DPP8* and *DPP9* in lymphocyte subpopulations suggests that these proteases have essential roles in the immune system. As it is now known that immune roles of *DPP4* are mainly extraenzymatic (such as protein-protein interaction), greater abundance of *DPP8* and *DPP9* compared to *DPP4/CD26* in the lymphocytes further supports the hypothesis that the immune effects with non-selective *DPP4* inhibitors in earlier studies were more likely due to *DPP8* and *DPP9* inhibition<sup>[2]</sup>.

We demonstrated a quantitative time-dependent up-regulation of *DPP8* and *DPP9* in mitogen-stimulated mouse splenocytes and human Jurkat CD4<sup>+</sup> T cells, as well as in polyclonally activated Raji B cells. Therefore, *DPP8* and *DPP9* might have roles in both T and B cell activation. *DPP8* and *DPP9* were upregulated in lymphocytes following acute mitogen stimulation, but with prolonged stimulation, they were downregulated. Hence, the role of *DPP8* and *DPP9* perhaps differ in recently activated lymphocytes compared to persistently activated lymphocytes.

*DPP9* enzyme activity induces intrinsic cell apoptosis in epithelial cells through the phosphatidylinositol-3-kinase/protein kinase B (Akt) signaling pathway<sup>[39,40]</sup>. Our data on Raji cells suggest that *DPP9* could similarly have a role in intrinsic lymphocyte apoptosis. Moreover the increase in *DPP8* and *DPP9* expression in mitomycin C treated cells is perhaps a hallmark of increased apoptosis in the absence of cell proliferation<sup>[51,52]</sup>. *DPP9* substrates and ligands involved in these processes have not been identified.

The modulation of *DPP8* and *DPP9* expression with varying lymphocyte activation, proliferation and apoptosis, implies that *DPP8* and *DPP9* have important regulatory roles in lymphocytes that deserve further investigation. Their role in lymphocyte activation is likely to differ from that of *DPP4*. While the role of cell surface *DPP4* in lymphocyte proliferation appears to be mainly extra-enzymatic<sup>[22]</sup>, enzyme inhibition of intracellular *DPP8* and *DPP9* affects lymphocyte proliferation<sup>[23]</sup>. The observation of less annexin V staining in Raji cells overexpressing *DPP9* enzyme mutant compared to wild type *DPP9* suggests that enzyme activity of *DPP9* is important for its role in apoptosis. *DPP9* modulates Akt phosphorylation in hepatoma cell lines<sup>[40]</sup>, so *DPP8* and *DPP9* might similarly modulate the activity of signaling molecules that are crucial in lymphocyte activation pathways. *DPP8* can cleave several chemokines, stromal cell-derived factor (SDF)-1 $\alpha$ , SDF-1 $\beta$ , inflammatory protein 10 and interferon-inducible T-cell alpha chemoattractant, *in vitro*<sup>[12]</sup>, however since *DPP8* is an intracellular protease, the biological relevance of this cleavage is unknown.

The association of *DPP4* and *FAP* with liver fibrosis is well documented<sup>[24,53]</sup>. Here we have demonstrated possible involvement of *DPP8* and *DPP9* in liver fibrosis, too. Treatment of mice with CCl<sub>4</sub> for 3 wk, which represents early fibrosis with mild hepatic injury, increased intrahepatic *DPP8* and *DPP9* expression. This association with early stage disease may suggest pro-fibrogenic roles of *DPP8* and *DPP9*. Though *DPPs* have been implicated in inflammation and inflammatory diseases<sup>[28,29,54]</sup>, no change in *DPP* expression was observed in hepatic lymphocytes in this early stage fibrosis, suggesting that hepatocytes, which constitute more than 80% of the liver cell population, are probably the major source of upregulated *DPP8* and *DPP9* in this liver fibrosis model.

Unlike the CCl<sub>4</sub> induced liver fibrosis model, *DPP8* and *DPP9* were downregulated in end stage human PBC and in the *Mdr2* gko mice. This suggests that *DPP8* and *DPP9* expression varies with the pathophysiology of liver diseases. The mouse CCl<sub>4</sub> model represents zone 3 fibrosis whereas *Mdr2* gko represents a zone 1 fibrosis model<sup>[41,55]</sup>. *DPP8* and *DPP9* show a zonal distribution pattern, with stronger staining in zone 3, the periportal hepatocytes and periportal lymphocytes<sup>[13]</sup>. Hence, the zonal injury pattern may be important for *DPP8* and *DPP9* expression. Another possibility could be that activated cholangiocytes downregulate *DPP8* and *DPP9* expression. In the *Mdr2* gko mice, *DPP8* and *DPP9* expression was least at week 4, when the cholangiocytes are

most active<sup>[41]</sup>. Hence, this could be the reason why *DPP8* and *DPP9* expression was downregulated in human PBC and *Mdr2* gko mice.

Alternatively, the differential expression of *DPP* in the different liver diseases could be due to acute *vs* chronic stimuli.  $\text{CCl}_4$  induces acute liver injury with hepatocyte damage followed by a repair phase that involves increased collagen deposition<sup>[55]</sup>. Administration of  $\text{CCl}_4$  twice per week for 3 wk leads to repeated cycles of injury and repair that results in fibrosis. We collected liver samples from the  $\text{CCl}_4$  treated mice at day 3 after the last  $\text{CCl}_4$  injection. At day 3, hepatocyte apoptosis is waning whereas fibrosis is developing<sup>[55]</sup>. In contrast, the *Mdr2* gko mice and human end stage PBC represent chronic liver injury, whereby there is persistent (mild) hepatocyte damage, a fibrogenic cholangiocyte/progenitor cell response and downregulation of collagenolytic activity resulting in continuing progression of biliary fibrosis until week 12 of age<sup>[41]</sup>. Thus, our data are consistent with the paradigm that *DPP8* and *DPP9* are upregulated in acute disease states then downregulated with progression to chronic disease states.

This distinctive *DPP* expression pattern in different liver diseases suggests that *DPP8* and *DPP9* have important regulatory roles in the pathogenesis of liver diseases, perhaps in modulating liver regeneration and apoptosis, which are important processes in liver disease progression. *DPP9* impairs EGF-stimulated hepatoma proliferation<sup>[40]</sup>. Our observation that *DPP9* is upregulated in the presence of EGF is perhaps part of a regulatory mechanism of *DPP9* in hepatocyte proliferation. *DPP9* can induce intrinsic apoptosis in hepatoma cell lines *via* the Akt signaling pathway<sup>[40]</sup>. Furthermore, *DPP8* and *DPP9* influence cell-extracellular matrix (ECM) interactions *in vitro*<sup>[39]</sup> and in liver fibrogenesis, cell-ECM interaction is responsible for disrupting wound healing and progressive scarring in liver disease<sup>[56]</sup>.

The upregulated expression of *DPP8* and *DPP9* in acute conditions and less expression in chronic or persistent conditions in the immune system and in liver injury suggests that *DPP8* and *DPP9* are crucial for early cellular responses to stimuli. The mechanisms of *DPP8* and *DPP9* are yet to be elucidated. One obstacle in *DPP8* and *DPP9* studies is the poor availability of appropriate tools, such as monoclonal antibodies and selective inhibitors<sup>[57]</sup>.

In conclusion, our study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in liver fibrogenesis. A better understanding of the biological functions of *DPP8* and *DPP9* could help reveal their therapeutic potential for liver diseases, cancer, inflammatory and autoimmune diseases.

## ACKNOWLEDGMENTS

We thank the Royal Prince Alfred Hospital National Liver Transplant Unit for providing human liver samples, Michelle Vo for advice, Adrian Smith and Robert Salomon

of the Centenary Institute Flow Cytometry Facility for their expert cell sorting.

## COMMENTS

### Background

The four enzyme members of the dipeptidyl peptidase (*DPP*) 4 gene family, *DPP4*, fibroblast activation protein (*FAP*), *DPP8* and *DPP9* have attracted considerable research interest in recent years since *DPP4* inhibitors became a successful therapy for type 2 diabetes and *FAP* a potential cancer therapeutic target. *DPP8* and *DPP9* are the more recently discovered members of the *DPP4* gene family. They are ubiquitously expressed cytoplasmic enzymes with *DPP4* like enzyme activity. Many compounds intended to inhibit *DPP4* or *FAP* also inhibit *DPP8* and *DPP9*, but the compounds that became successful diabetes drugs are *DPP4*-selective.

### Research frontiers

*DPP4* is also known as CD26 T cell differentiation marker and has roles in T cell activation and proliferation. *DPP8* and *DPP9* are in lymphoid tissues and may have functional significance in the immune system. *DPP8* and *DPP9* are expressed in hepatocytes and expression is elevated in damaged hepatocytes near the septum of human cirrhotic liver. However, potential roles of *DPP8* and *DPP9* in liver disease are unknown. Here the authors studied the expression of *DPP8* and *DPP9* in lymphocyte activation, proliferation and apoptosis and in liver injury models to elucidate their potential biological roles in the immune system and in liver diseases. Models included hepatotoxicity from  $\text{CCl}_4$ , and the multidrug resistance gene 2 knockout mouse that spontaneously develops biliary fibrosis.

### Innovations and breakthroughs

This study significantly promotes our understanding of the novel proteases *DPP8* and *DPP9* in lymphocytes, hepatocytes and liver injury. The authors showed that *DPP8* and *DPP9* were widely expressed in lymphocyte subpopulations and were upregulated in activated lymphocytes in a time dependent manner. The authors also demonstrated potential involvement of *DPP8* and *DPP9* in lymphocyte apoptosis. In liver, the authors showed that *DPP8* and *DPP9* expression levels were altered in liver injury and confirmed their role in the regulation of epidermal growth factor in hepatocytes, a mitogen that is considered crucial for hepatocyte proliferation and liver regeneration.

### Applications

This study suggests that *DPP8* and *DPP9* have fundamental roles in the immune system, in lymphocyte activation and in apoptosis and they could be involved in chronic liver injury pathogenesis.

### Terminology

*DPP4* enzyme activity is a specialized proteolytic enzyme activity that cuts two amino acids from the N-terminus of each target peptide, usually cutting after a proline residue; Lymphocyte activation is a cellular process that leads to a radical shift in cell behavior to a more active and proliferative one. The activation of lymphocytes serves two purposes, augmenting the number of cells to respond to a particular antigen (clonal expression), and specializing to produce cytokines, and produce antibodies against a pathogen; cell apoptosis is the process of cell death mediated by an intracellular program. Apoptosis is important for normal cell turnover and organ remodeling.

### Peer review

The manuscript deals with regulation of *DPP8* and *DPP9* expression in activated lymphocytes and injured liver. Here the authors focus on the expression levels of *DPP8* and *DPP9* in lymphocyte subpopulations in a time dependent manner. The authors have confirmed the altered expression level of *DPP8* and *DPP9* in liver injury and also confirmed their role in the regulation of epidermal growth factor in hepatocytes. The work has been carefully conducted and the experiments are clearly described in the vast majority of the cases.

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P- Reviewer Shembade ND S- Editor Jiang L  
L- Editor A E- Editor Xiong L



## Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats

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Supported by The Istanbul University Department of Scientific Research Projects, Grant No. 3101

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Received: November 14, 2012 Revised: January 23, 2013

Accepted: February 5, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To investigate the effects of long term pretreatment with low-, medium- and high-dose aspirin (acetylsalicylic acid, ASA) on a model of acute pancreatitis (AP) induced in rats.

**METHODS:** Forty male Wistar rats were used. Three experimental groups, each consisting of eight animals,

received low- (5 mg/kg per day), medium- (150 mg/kg per day) and high-dose (350 mg/kg per day) ASA in supplemented pellet chow for 100 d. Eight animals, serving as the AP-control group, and another eight, serving as reference value (RV) group, were fed with standard pellet chow for the same period. After pretreatment, AP was induced in the experimental animals by intraperitoneal administration of cerulein ( $2 \times 50 \mu\text{g}/\text{kg}$ ), while the RV group received saline in the same way. Twelve hours after the second injection, the animals were sacrificed. Pancreatic tissue and plasma samples were collected. One part of the collected pancreatic tissues was used for histopathological evaluation, and the remaining portion was homogenized. Cytokine levels [tumor necrosis factor, interleukin (IL)-1 $\beta$ , IL-6], hemogram parameters, biochemical parameters (amylase and lipase), nuclear factor- $\kappa$ B, aspirin triggered lipoxins and parameters related to the antioxidant system (malondialdehyde, nitric oxide, hemeoxygenase-1, catalase and superoxide dismutase) were measured.

**RESULTS:** Cerulein administration induced mild pancreatitis, characterized by interstitial edema (total histopathological score of  $5.88 \pm 0.44$  vs  $0.25 \pm 0.16$ ,  $P < 0.001$ ). Subsequent pancreatic tissue damage resulted in an increase in amylase ( $2829.71 \pm 772.48$  vs  $984.57 \pm 49.22$  U/L,  $P = 0.001$ ) and lipase ( $110.14 \pm 75.84$  U/L vs  $4.71 \pm 0.78$  U/L,  $P < 0.001$ ) in plasma, and leucocytes ( $6.89 \pm 0.48$  vs  $4.36 \pm 0.23$ ,  $P = 0.001$ ) in peripheral blood. Cytokines, IL-1 $\beta$  ( $18.81 \pm 2.55$  pg/ $\mu\text{g}$  vs  $6.65 \pm 0.24$  pg/ $\mu\text{g}$ ,  $P = 0.002$ ) and IL-6 ( $14.62 \pm 1.98$  pg/ $\mu\text{g}$  vs  $9.09 \pm 1.36$  pg/ $\mu\text{g}$ ,  $P = 0.04$ ) in pancreatic tissue also increased. Aspirin pretreatment reduced the increase in the aforementioned parameters to a certain degree and partially improved the histopathological alterations caused by cerulein. No evidence of side effects related to chronic ASA administration (*e.g.*, inflammation or bleeding) was observed in the gastrointestinal tract in macroscopic and histopathological examination.

**CONCLUSION:** Long term ASA pretreatment could prevent and/or ameliorate certain hematological, serological and histological alterations caused by cerulein-induced AP.

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**Key words:** Aspirin; Acute pancreatitis; Cerulein; Antioxidant; Cytokines

**Core tip:** Acute pancreatitis (AP) is an inflammatory and potentially life-threatening disease. An estimated 80000 cases of AP occur each year in the United States. There is no specific cure for AP; therefore, research interest has focused on prevention strategies. In the present study, the effects of a long-term pretreatment with different doses of aspirin, the oldest and most widely used non-steroidal anti-inflammatory drug, were investigated on a AP model in rats. Our results indicated that aspirin pretreatment dose-dependently prevents or ameliorates some hematological, serological and histological alterations caused by cerulein-induced AP.

Akyazi I, Eraslan E, Gülçubuk A, Ekiz EE, Çıraklı ZL, Haktanir D, Bala DA, Özkurt M, Matur E, Özcan M. Long-term aspirin pretreatment in the prevention of cerulein-induced acute pancreatitis in rats. *World J Gastroenterol* 2013; 19(19): 2894-2903 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2894.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2894>

## INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease with broad clinical variation, ranging from a mild and self-limiting condition to a severe, life-threatening necrotizing inflammation<sup>[1,2]</sup>. Furthermore, it can lead to the development of systemic inflammatory response syndrome (SIRS) and multisystem organ failure<sup>[3,4]</sup>.

AP may have numerous causes, such as bile duct obstructions, alcohol abuse, metabolic abnormalities, various toxins and infections<sup>[5]</sup>. One of the aforementioned incidents may trigger pancreatic acinar cell injury and premature activation of pancreatic zymogens<sup>[6]</sup>. The initial acinar cell damage is followed by local activation of the immune system and induction of transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B)<sup>[4,7]</sup>. Activation of inflammatory cells and transcription factors leads to elaboration of various proinflammatory mediators, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6<sup>[8]</sup>. If this proinflammatory response to acinar cell damage is balanced by an anti-inflammatory response, the pancreatitis and local inflammation resolve at this stage. However, in some cases, an overwhelming proinflammatory response upsets this balance and the proinflammatory mediators migrate into systemic circulation leading to a generalized inflammation and SIRS<sup>[4,8]</sup>.

In this context, proinflammatory mediators and NF- $\kappa$ B, which play a key role in expression of these mediators, emerge as potential therapeutic targets<sup>[6,8]</sup>.

Acetylsalicylic acid (ASA) exerts analgesic, antipyretic and antiplatelet effects, and is the oldest and most widely used nonsteroidal anti-inflammatory drug<sup>[9]</sup>. In addition to its conventional effects, ASA has a preventative effect on a wide range of diseases, including gastrointestinal cancer<sup>[10]</sup>, ischemic stroke<sup>[11]</sup>, myocardial infarction<sup>[12]</sup> and Alzheimer's disease<sup>[13]</sup>. The anti-inflammatory, analgesic and antipyretic efficacy of ASA is attributed mainly to its inhibitory impact on the enzymatic activity of cyclooxygenases (COX), which convert arachidonic acid to prostaglandins (PGs)<sup>[14]</sup>. On the other hand, it has been speculated that simple inhibition of PG production cannot fully account for the wide spectrum of effects of ASA<sup>[9,15]</sup>. Indeed, substantial data have been gathered, indicating that COX-independent mechanisms play a significant role in ASA's efficacy<sup>[15]</sup>. Kopp *et al.*<sup>[16]</sup> discovered that ASA inhibits NF- $\kappa$ B activation, which is a pivotal transcription factor in cytokine network. NF- $\kappa$ B regulates the expression of proinflammatory enzymes, cytokines, chemokines, immunoreceptors, acute phase proteins and cell adhesion molecules; therefore, it has often been termed a "central mediator of the immune system"<sup>[15,17,18]</sup>. In this regard, it has been stated that even partial inhibition of NF- $\kappa$ B by ASA could have a substantial effect on inflammation<sup>[16]</sup>. Another major finding was the discovery that acetylation of COX-2 by ASA can lead to transcellular biosynthesis of a new class of eicosanoids, the 15-epi-lipoxins or so-called aspirin-triggered lipoxins (ATL), which promote the resolution of inflammation<sup>[19,20]</sup>. Lipoxins have potent counter-regulatory effects *in vivo* and *in vitro* on proinflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-4<sup>[19,21,22]</sup>. Furthermore it has been speculated that ASA's unique ability to trigger the synthesis of ATLs causes an increase in nitric oxide (NO) synthesis and this aspirin-elicited NO exerts anti-inflammatory effects<sup>[23]</sup>. Grosser *et al.*<sup>[24]</sup> found that ASA stimulates the expression and enzymatic activity of hemeoxygenase-1 (HO-1) protein in a COX-independent manner. HO-1 is a crucial mediator of the cellular antioxidant defense system and has anti-inflammatory, anti-apoptotic, and antiproliferative effects<sup>[25,26]</sup>. Recent data<sup>[27]</sup> elucidated the underlying mechanism of HO-1 expression stimulated by ASA: ATL is mainly responsible for the aforementioned stimulation.

Taken together, this wide spectrum of therapeutic effects of ASA is a consequence of its efficacy in regulating a network of biochemical and cellular events in a more complex manner than was initially thought<sup>[9,28]</sup>.

The significant role of proinflammatory mediators (*e.g.*, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and platelet activating factor) and transcription factors (*e.g.*, NF- $\kappa$ B and AP-1) in the pathogenesis and complications of AP are well documented in the literature<sup>[6,8]</sup>. Considering the inhibitory efficacy of ASA on these agents, it would be reasonable to suggest that ASA may be efficient in preventing or at-

**Table 1** Grouping and experimental design

| Group No. | <i>n</i> | Group name                 | ASA pretreatment (mg/kg) | AP induction |
|-----------|----------|----------------------------|--------------------------|--------------|
| 1         | 8        | Reference value            | No                       | No           |
| 2         | 8        | Acute pancreatitis control | No                       | Yes          |
| 3         | 8        | Low-dose ASA               | 5                        | Yes          |
| 4         | 8        | Medium-dose ASA            | 150                      | Yes          |
| 5         | 8        | High-dose ASA              | 350                      | Yes          |

ASA: Acetylsalicylic acid; AP: Acute pancreatitis.

tenuating AP and its subsequent complications. Furthermore, ASA's antioxidant efficacy exerted *via* the stimulation of HO-1 expression and the anti-inflammatory efficacy of ATL supports and strengthens the aforementioned hypothesis that ASA may be a therapeutic agent for the prevention and/or treatment of AP. However, to the best of our knowledge, there are no studies investigating the preventive and/or therapeutic effects of ASA on AP. Therefore, this study aimed to investigate the effects of ASA pretreatment on experimental AP in rats. By designing an experimental study with a long-term pretreatment, we focused on the preventive effects of ASA, rather than the curative ones, because the multiple and diverse mechanisms of action of ASA seem to be most effective on the initial proinflammatory progress in the pathogenesis of AP.

## MATERIALS AND METHODS

### Animals and grouping

Studies were performed on 40 male Wistar rats weighing 350-400 g. Animals were housed in polycarbonate cages (four rats/cage) with wood chip bedding and fed standard laboratory chow (supplemented with ASA for treatment groups) and tap water *ad libitum*. They were maintained in a climate-controlled animal room (temperature: 22 ± 3 °C; relative humidity: 60% ± 5%) with a 12 h/12 h light/dark cycle.

The Istanbul University's Local Ethics Committee approved all the experimental procedures. The animals were randomly allocated to five groups as shown in Table 1.

### ASA pretreatment and dosing

Low, medium and high doses of ASA pretreatment were performed as diet supplements for 100 d<sup>[29]</sup>. Doses of 80 mg/d, 2-4 and 6-8 g/d ASA have been regarded as low, medium and high doses for humans, respectively<sup>[30]</sup>. Based on average human body weight of 70 kg, these doses correspond to 1.1, 28-56 and 86-114 mg/kg per day, respectively<sup>[31]</sup>. These human doses were scaled to rats according to Kleiber's rule<sup>[32]</sup> using the following equation: dose (rat)/dose (human) = BW<sup>0.25</sup> (rat)/BW<sup>0.25</sup> (human) (BW = body weight)<sup>[33]</sup>. Based on the dose intervals derived from the above equation, the following doses were chosen for ASA pretreatment: 5 mg/kg (low-dose), 150 mg/kg (medium-dose) and 350 mg/kg (high-dose). Considering the daily food consumption of rats, stan-

dard rat chow material was supplemented with the corresponding amounts of ASA before pelleting to achieve the aforementioned low, medium and high doses. Groups 3-5 were fed these ASA supplemented pellets, while the other groups (Groups 1 and 2) received standard chow during the 100-d pretreatment.

### Induction of AP

After pretreatment, all the animals, except those in the reference value (RV) group (Group 1), received two intraperitoneal injections of cerulein in 0.9% NaCl at an hourly interval at a dose of 50 µg/kg to induce AP<sup>[34]</sup>. Animals in the RV group received injections of the same volume of sterile saline solution (0.9% NaCl) in the same way.

### Sample collection and preparation

Twelve hours after the induction of AP, all animals were anesthetized (xylazine/ketamine, 10/75 mg/kg) and exsanguinated *via* cardiac puncture. Blood samples were collected into ethylene diamine tetraacetic acid-coated tubes and plasma samples were separated *via* centrifugation after performing a complete blood count. The plasma samples were aliquoted and frozen at -80 °C. After sacrificing the animals, necropsies were performed and pancreatic tissues were removed. One part of the pancreas of each animal was used for homogenization, while the remaining portion was fixed in formol-saline (10%) for histopathological examination.

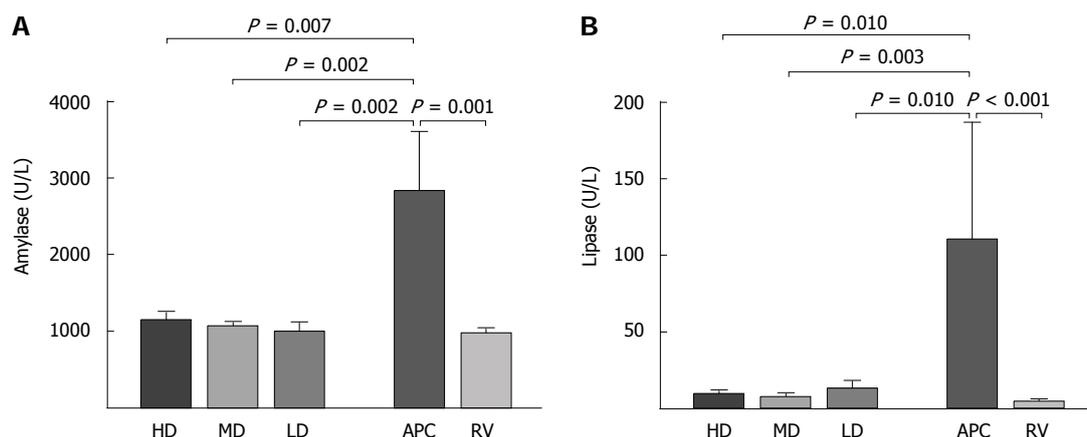
Pancreas samples were homogenized in a 20 mmol/L Tris-HCl buffer (pH 7.4) containing 0.5 mol/L sucrose, 25 mmol/L KCl and 5 mmol/L MgCl<sub>2</sub> using a rotor-stator homogenizer. The homogenates were centrifuged at 1000 *g* for 10 min at 4 °C, and the supernatants containing the cytosolic fraction were removed, aliquoted and frozen at -80 °C until assayed. Sedimented pellets containing the nuclear fraction were used to obtain nuclear protein extracts using a commercial protein extraction kit (Intron Biotechnology Inc., Sungnam, South Korea).

### Statistical analysis

TNF-α, IL-1β and IL-6 levels were determined in tissue homogenates containing the cytosolic fraction and in plasma samples using commercial enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Camarillo, CA, United States). NF-κB levels were measured in nuclear protein extractions, using a commercial ELISA kit (USCN Life Science Inc., Wuhan, Hubei Province, China).

Catalase (CAT) activities (Cell Biolabs, San Diego, CA, United States), superoxide dismutase (SOD) activities (Assay Designs, Ann Arbor, MI, United States), and malondialdehyde (MDA) levels (Cell Biolabs) were measured in pancreas homogenates and in plasma using commercial test kits. HO-1 levels were determined in pancreas homogenates and plasma using commercial ELISA kits (Assay Designs).

Amylase and Lipase levels in plasma were measured using an automated analyzer (Architect 16200, Abbott,



**Figure 1 Plasma amylase (A) and lipase (B) activities.** Columns show the mean and the error bars represent SEM. All groups are compared with the acute pancreatitis control (APC) group and the statistical significance is expressed as a vertical  $P$  value over the column. LD: Low-dose; MD: Medium-dose; HD: High-dose; RV: Reference values.

IL, United States). Total NO levels were determined in plasma and pancreas homogenates using commercial test kits (Assay Designs). Plasma ATL levels were measured using commercial ELISA kits (Neogen, Lexington, KY, United States). Total protein contents of homogenates were determined using the method described by Lowry *et al.*<sup>[35]</sup> and all parameters measured in homogenates were proportioned to the total protein content of the homogenate in mg.

Tissue samples fixed in formol-saline were embedded in paraffin blocks, sectioned using a microtome and stained with hematoxylin-eosin. Histopathological scoring was performed as described by Gülçubuk *et al.*<sup>[36]</sup>, graded on a score of 0 to 3.

Statistical analysis of the obtained data was performed using the SPSS-software package (Version 11.5.2.1, SPSS Inc., Chicago, IL, United States). Results are expressed as mean  $\pm$  SEM. Data for all groups were first tested for normality using the Shapiro-Wilk test. Data of groups found to be normally distributed were then compared using one-way analysis of variance. If the normality assumption was found to be violated, data were analyzed using the non-parametric Kruskal-Wallis test. Planned (a priori) contrasts and Mann Whitney  $U$  tests were used for pairwise comparisons following parametric and non-parametric tests, respectively. The ordinal data of histopathological scoring were analyzed using aforementioned non-parametric tests.

## RESULTS

Intraperitoneal administration of cerulein ( $2 \times 50 \mu\text{g}/\text{kg}$ ) caused AP in all tested rats, as indicated by the marked increase in serum amylase and lipase levels (Figure 1) and histopathological changes (Figure 2).

Cerulein induced AP caused almost 3- and 23-fold increases in plasma amylase and lipase levels, respectively. ASA pretreatment significantly decreased these levels to close to those of the RV group (Figure 1). Cerulein-induced AP increased the peripheral white blood cell (WBC)

count significantly compared to the RV group ( $6.89 \pm 0.48$  and  $4.36 \pm 0.23$ , respectively,  $P = 0.001$ ). This increase was abolished by medium- and low-dose ASA.

### Cytokine levels, lipid peroxidation and WBCs

Columns show the mean and error bars represent SEM in all figures. All groups were compared with the APC group and statistical significance was expressed as a vertical  $P$  value over the column.

The histopathological scores are shown in Table 2. Marked interstitial edema was observed in the APC group, with a score of  $2.75 \pm 0.16$ . In contrast, the edema score of the RV group was  $0.25 \pm 0.16$  and the difference was significant ( $P < 0.001$ ). Concerning the total score (Figure 2), which indicates the overall level of pathological changes, a marked difference was found between the APC and RV group scores ( $5.88 \pm 0.44$  and  $0.25 \pm 0.160$ , respectively) with a high level of significance ( $P < 0.001$ ). Considering the histopathological scores, ASA pretreatment generally improved the histopathological changes. However, only the effect of the medium-dose was statistically significant ( $P < 0.001$  for the total score). No evidence of side effects related to chronic ASA administration (e.g., inflammation or bleeding) for any of the three doses was observed in the gastrointestinal tract by macroscopic and histopathological examinations.

### Histopathological scores

All groups were compared with the APC group and statistical significance was expressed as  $P$  values under the corresponding mean  $\pm$  SEM values.

As shown in Figure 3A, cerulein-induced AP caused a marked elevation of the IL-1 $\beta$  level in pancreatic tissue compared to that of the RV group ( $18.81 \pm 2.55 \text{ pg}/\mu\text{g}$  and  $6.65 \pm 0.24 \text{ pg}/\mu\text{g}$ , respectively,  $P = 0.002$ ). This elevation was suppressed significantly by ASA in all treatment groups. A similar increase was observed in the pancreatic IL-6 level ( $14.62 \pm 1.98 \text{ pg}/\mu\text{g}$  vs  $9.09 \pm 1.36 \text{ pg}/\mu\text{g}$ ,  $P = 0.04$ , Figure 3B); however, the low-dose could not prevent this increase, whereas the medium- and

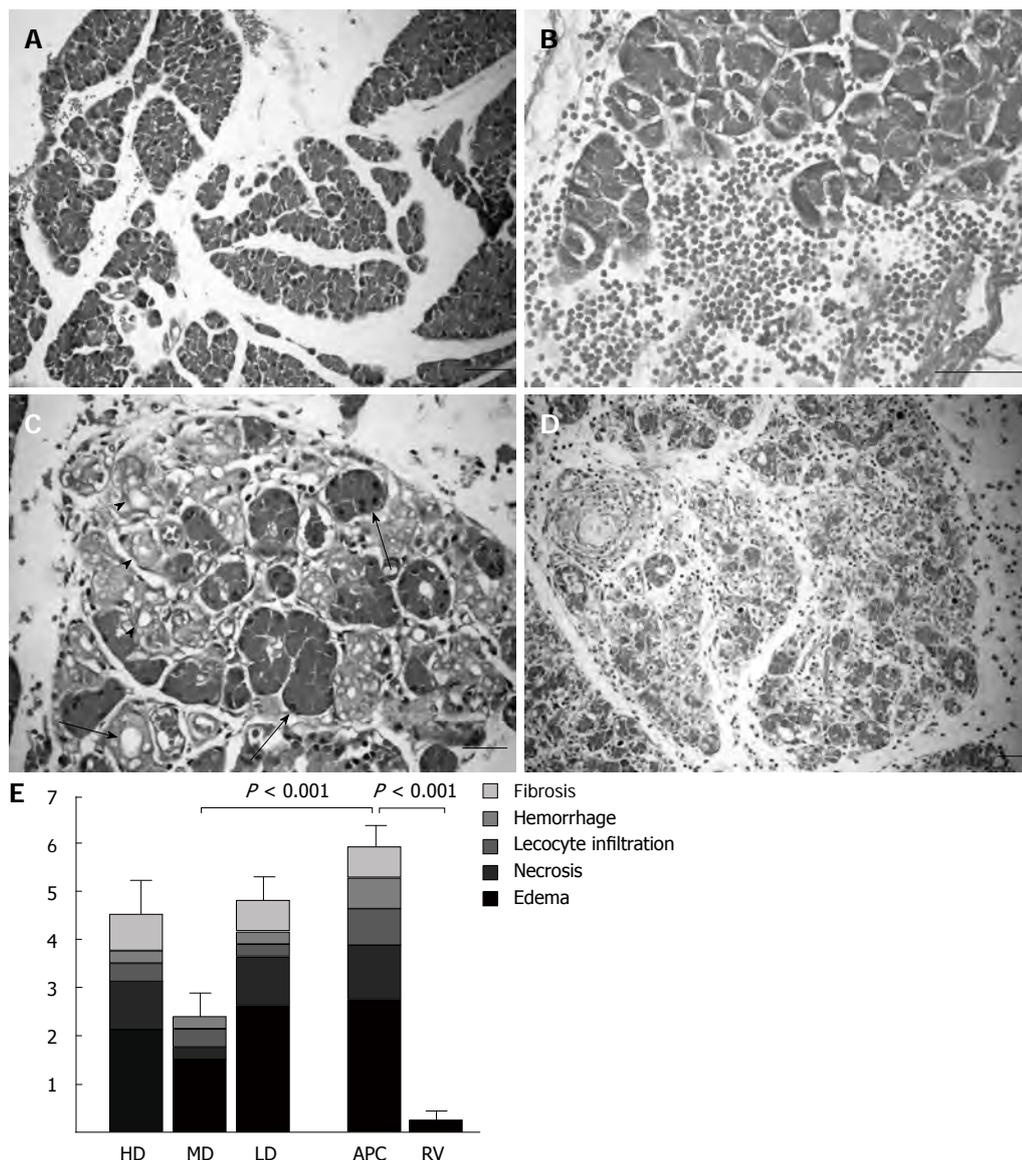


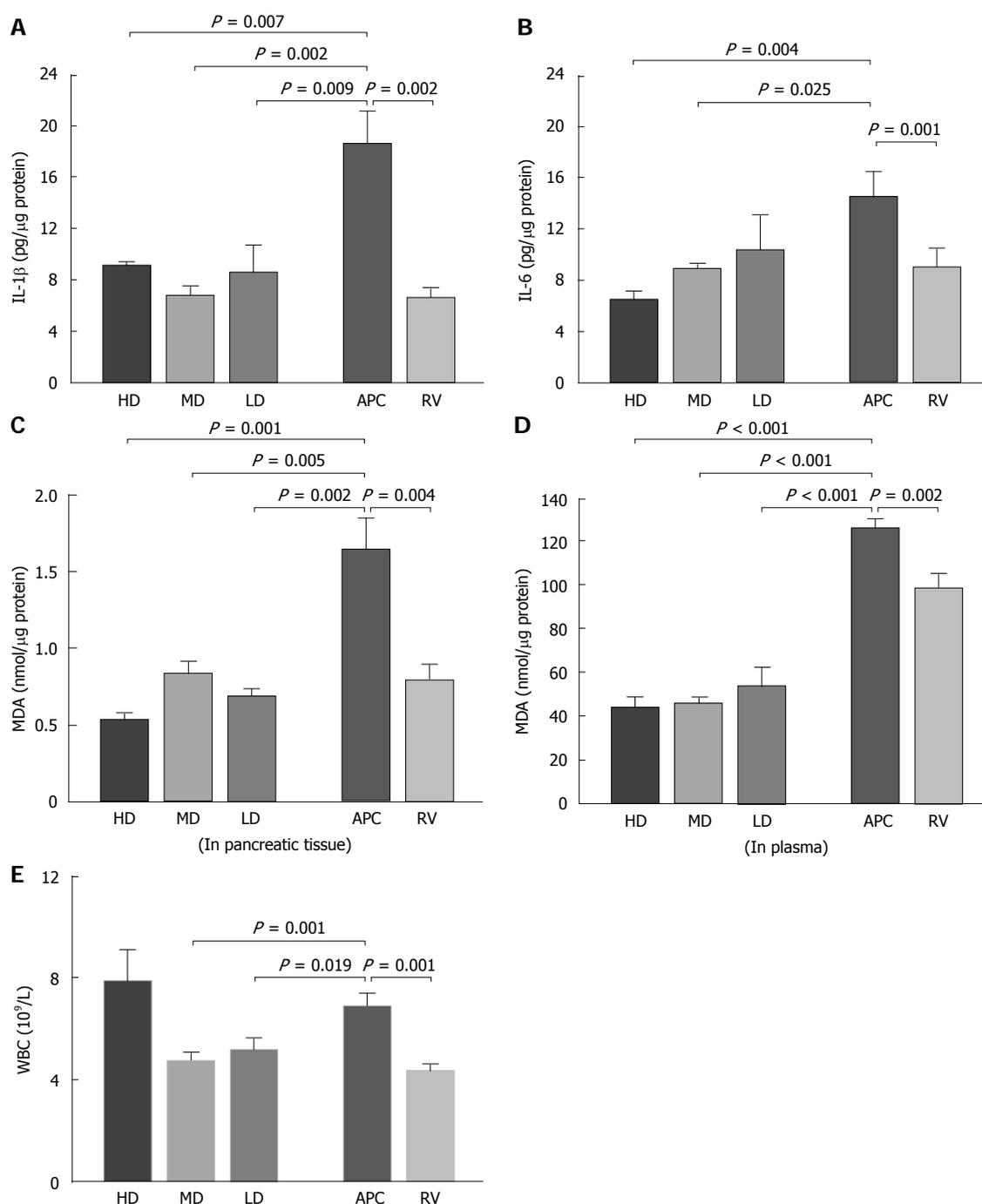
Figure 2 Histopathological alterations in rat pancreas caused by cerulein-induced acute pancreatitis and histopathological scores. A: Pancreatic acini were separated because of interlobular edema in cerulein treated animals (bar = 100 μm); B: Occasionally mild hemorrhages were observed (bar = 50 μm); C: Several acinar cells lost their zymogen granules (arrows) and ductus-like structures (arrowheads) occurred (bar = 50 μm); D: In some animals, leucocyte, fibrocyte and fibroblast infiltrations and collagen bands were detected (bar = 200 μm); E: Histopathological scores of each group are shown as stacked columns representing means. The whole column corresponds to the mean of the total score and the error bars represent the SEM of the total score. All groups are compared with the APC group and the statistical significance is expressed as a vertical *P* value over the column. HD: High-dose; MD: Medium-dose; LD: Low-dose; APC: Acute pancreatitis control; RV: Reference values.

|                        | HD-ASA (n = 8) | MD-ASA (n = 8) | <i>P</i> value | LD-ASA (n = 8) | APC (n = 8) | RV (n = 8)  | <i>P</i> value |
|------------------------|----------------|----------------|----------------|----------------|-------------|-------------|----------------|
| Edema                  | 2.13 ± 0.30    | 1.50 ± 0.19    | 0.002          | 2.63 ± 0.18    | 2.75 ± 0.16 | 0.25 ± 0.16 | 0.001          |
| Hemorrhage             | 0.25 ± 0.16    | 0.25 ± 0.16    |                | 0.25 ± 0.16    | 0.63 ± 0.18 | 0.00 ± 0.00 | 0.01           |
| Leukocyte infiltration | 0.38 ± 0.18    | 0.38 ± 0.18    |                | 0.25 ± 0.16    | 0.75 ± 0.16 | 0.00 ± 0.00 |                |
| Necrosis               | 1.00 ± 0.19    | 0.25 ± 0.16    | 0.007          | 1.00 ± 0.19    | 1.13 ± 0.13 | 0.00 ± 0.00 | 0.001          |
| Fibrosis               | 0.75 ± 0.25    | 0.00 ± 0.00    |                | 0.63 ± 0.26    | 0.63 ± 0.18 | 0.00 ± 0.00 |                |
| Total score            | 4.50 ± 0.68    | 2.38 ± 0.50    | 0.001          | 4.75 ± 0.49    | 5.88 ± 0.44 | 0.25 ± 0.16 | 0.001          |

ASA: Acetylsalicylic acid; RV: Reference values; APC: Acute pancreatitis control; LD: Low-dose; MD: Medium-dose; HD: High-dose.

high-dose ASA pretreatments could suppress the IL-6 elevation. There were no statistical differences between the groups regarding the TNF-α and NF-κB levels.

Cerulein-induced AP increased MDA levels in both plasma and pancreatic tissue compared to that of the RV group (Figure 3C and D). ASA pretreatment at all three



**Figure 3** Cytokine levels, lipid peroxidation and white blood cells. **A:** Cerulein induced acute pancreatitis (AP) caused a marked elevation of interleukin (IL)-1 $\beta$  level in pancreatic tissue compared to that of the reference values (RV) group ( $18.81 \pm 2.55$  and  $6.65 \pm 0.24$  pg/ $\mu$ g, respectively,  $P = 0.002$ ); **B:** This elevation was suppressed significantly by acetylsalicylic acid (ASA) in all treatment groups. A similar increase was observed in the pancreatic IL-6 level. However, this time, the low-dose seemed to be ineffective against it, while medium- and high-dose ASA pretreatments suppressed the IL-6 elevation. There was no statistical difference between the groups regarding the tumor necrosis factor- $\alpha$  and nuclear factor- $\kappa$ B levels; **C, D:** Cerulein induced AP increased malondialdehyde (MDA) levels in both pancreatic tissue and plasma compared to that of the RV group; **E:** Cerulein induced AP increased the peripheral white blood cell (WBC) count significantly compared to the RV group. HD: High-dose; MD: Medium-dose; LD: Low-dose; APC: Acute pancreatitis control.

doses inhibited this increase. Cerulein-induced AP increased the peripheral WBC count (Figure 3E). Other antioxidant system parameters, including NO, SOD, HO-1 and CAT, were not affected by cerulein-induced AP and ASA treatment; there were no significant differences between the treatment, APC and RV groups regarding these parameters.

## DISCUSSION

To the best of our knowledge, the present study is the first investigation of the effect of long-term ASA pretreatment on a cerulein-induced pancreatitis model. Our findings indicate that long term ASA pretreatment dose-dependently prevents or ameliorates certain hematologi-

cal, serological and histological alterations caused by cerulein-induced AP.

Cerulein-induced pancreatitis is the most preferred animal model of AP, because it is non-invasive, easily applicable and highly reproducible<sup>[37]</sup>. The similarity of the cerulein-induced histopathology to human AP, especially in the early phase, has substantially increased the preference for this model<sup>[38]</sup>. Administration of cerulein, a cholecystokinin (CCK) analog, stimulates the pancreatic acinar cells *via* CCK receptors, which leads to prematuration of proteolytic enzymes<sup>[39]</sup>. The activation of proteases triggers an autodigestion of pancreatic tissue, causing vascular damage, edema, fibrosis and necrosis, which constitute the histopathological profile of AP<sup>[6]</sup>. The markedly higher edema, hemorrhage, necrosis and the total histopathological scores of the APC group compared to the RV group, observed in our present study, are the expected results of the cerulein-induced AP model and are consistent with the literature.

The serum amylase level has been the most widely used parameter for the diagnosis of AP<sup>[40]</sup>, since 1929, when its diagnostic value was demonstrated for the first time<sup>[41]</sup>. The serum lipase level, another widely accepted marker of AP, rises after the onset of AP in parallel with the amylase level, although its rise starts slightly later and lasts longer than that of amylase<sup>[42]</sup>. The plasma levels of both enzymes have substantial sensitivity and specificity for the diagnosis of AP<sup>[43]</sup>. As expected, in the present study, both the amylase and lipase levels rose markedly in the AP group. In the pretreatment groups, ASA prevented the elevation of both enzyme levels. This observation constitutes additional evidence supporting the preventive effect of ASA against cerulein-induced AP.

There is a positive correlation between the severity of AP and the increase in the peripheral WBCs<sup>[44]</sup> and the WBC count is one of the parameters included in most of the scoring systems used for the assessment of the severity of AP<sup>[45]</sup>. The increased WBC in the AP group is an expected result of the inflammation induced by cerulein. Our observation that the WBC count of medium- and low-dose groups was significantly lower than that of the AP group and close to that of the RV animals, suggests that ASA pretreatment ameliorates the inflammation induced in the pancreas.

Cytokines are a group of low-molecular weight proteins that play a crucial role in induction and progression of inflammatory processes, including AP. Thus, they have been subjected to a wide range of studies in this context<sup>[18,46,47]</sup>. Consequently there is no doubt about the constitutive role of many cytokines in progression of local tissue damage and distant complications in AP<sup>[46]</sup>.

Cytokines can be functionally divided into two groups: pro- and anti-inflammatory cytokines<sup>[46]</sup>. In the proinflammatory group TNF- $\alpha$  and IL-1 $\beta$  are especially prominent and are regarded as "first-line" cytokines<sup>[48]</sup>. IL-1 $\beta$  levels are elevated in the cerulein-induced models of AP<sup>[4,8,49]</sup>. Furthermore, a strong, positive correlation was found between the increase in IL-1 $\beta$  level and the severity of

inflammation<sup>[46,50]</sup>. In the present study, the IL-1 $\beta$  level in pancreatic tissue increased nearly threefold in the AP group compared with the RV group, whereas the difference between plasma levels showed no statistical significance. This rise in the IL-1 $\beta$  level in the AP group is an expected result of cerulein-induced AP. In addition, the contrast observed between the tissue and plasma levels of IL-1 $\beta$  is consistent with previous reports<sup>[50,51]</sup>. Considering the tissue levels of IL-1 $\beta$  in the pretreatment groups, ASA pretreatment had a significant diminishing effect. This finding is consistent with the amylase, lipase and MDA levels. Thus, this represents further evidence of the protective effect of ASA pretreatment against AP. IL-6 is another proinflammatory cytokine that increases in cerulein-induced pancreatitis<sup>[52]</sup>. IL-6 levels correlate with the clinical scenario and severity of AP; therefore, IL-6 has been attributed as a marker of the disease<sup>[4,53]</sup>. Thus, the increase in the IL-6 level in the APC group in the present study is an expected result of cerulein-induced AP. ASA pretreatment in the medium- and high-dose groups decreased the pancreatic IL-6 level significantly. This effect is consistent with the other findings of our study. The numerical decrease in the low-dose group was not statistically significant.

Sanfey *et al.*<sup>[54]</sup> suggested a possible involvement of reactive oxygen species (ROS) in the pathogenesis of AP and since then, observations from increasing numbers of experimental studies have supported this suggestion<sup>[55,56]</sup>. Consequently, there is currently no doubt about the detrimental role of oxidative stress in the pathogenesis of AP and this makes it a therapeutic target. Yu *et al.*<sup>[52]</sup> reported that, in the cerulein-induced AP model, administration of cerulein increased ROS formation and oxidative stress, and caused an increase in IL-1 $\beta$  expression. In the present study, the MDA level, an indicator of oxidative stress, was elevated in the cerulein administered AP group, both in plasma and in pancreatic tissue. This high level of MDA in the AP group, taken together with increased pancreatic IL-1 $\beta$  expression, is a consequence of cerulein-induced AP and these data are consistent with the findings of Yu *et al.*<sup>[52]</sup>. ASA reduces oxidative stress by exerting free radical scavenging activity and antioxidant efficacy<sup>[57-60]</sup>. The findings of Shi *et al.*<sup>[61]</sup> support ASA's free radicals scavenging efficacy and also suggest that it is more potent than several well established antioxidants, such as ascorbate, glutathione and cysteine, with respect to the reaction rate. In the present study, oxidative stress, indicated by the MDA levels, decreased in all the ASA treated groups compared with the AP-induced groups, in both the plasma and pancreatic tissue homogenates. Moreover, considering the plasma, these levels were even below the levels of the reference group, which was fed with commercial diet without ASA supplementation. These findings can be explained by potent antioxidant effect of ASA and are in accordance with the other results presented in this study. Nevertheless, parameters related to the enzymatic antioxidant system, including NO, SOD, HO-1 and CAT, showed no significant changes. Thus,

the reduced oxidative stress induced by ASA in the treatment groups seems not to involve the classic enzymatic antioxidant system and could be attributed to alternative mechanisms<sup>[60]</sup>.

When examining the histopathological scores numerically, the ASA pretreatment generally attenuated the alterations caused by AP. Nevertheless, these numerical changes could not be confirmed statistically for all treatment doses or for all histopathological parameters. Only the reducing effect of the medium dose (150 mg/kg) on edema, necrosis and total score values was found to be statistically significant ( $P = 0.002$ ,  $P = 0.007$  and  $P < 0.001$ , respectively). These histopathological scoring results seem to be inconsistent with the previously discussed data for amylase, lipase, MDA and IL-1 $\beta$ , which were reduced significantly by ASA pretreatment at all three doses. Although there is evidence showing that serum amylase and lipase levels do not correlate with the histopathological alterations and severity of AP<sup>[62-64]</sup>, we believe the aforementioned inconsistency resulted from the high variance of our data set and the small sample size and should be considered as a limitation of our study.

The analgesic, antipyretic, antiplatelet and antiinflammatory effects of ASA have been known for a long time, and most of these effects have been attributed to its COX-inhibitory activity<sup>[14]</sup>. However, it has been speculated that the therapeutic potential of ASA cannot be completely explained by COX inhibition<sup>[15,65]</sup>. Previous results from a wide variety of studies revealed different mechanisms of action of ASA, including inhibition of proinflammatory cytokines, such as IL-1 $\beta$ <sup>[65]</sup>, scavenging of ROS<sup>[59]</sup>, triggering the production of antiinflammatory mediators, such as ATL<sup>[21,22]</sup>, and inhibition of transcription factors, such as NF- $\kappa$ B<sup>[66]</sup>.

In conclusion, our findings indicate that the ASA pretreatment exerted preventive and/or ameliorating effects against AP by normalizing some of the hematological and biochemical indicators of the disease to close to those of the reference value group. These beneficial effects of the pretreatment were confirmed partially by the histopathological findings.

Our findings suggest that these beneficial effects of ASA can be explained by its free radical scavenging efficacy and the inhibitory effect on proinflammatory cytokines IL-1 $\beta$  and IL-6. The ATL pathway, involving the stimulation of NO and HO-1 expression, seemed not to play a role in this preventive effect, as there was no difference between groups with respect to the ATL levels.

Beside its conventional use as an analgesic, antiinflammatory and antipyretic agent, daily intake of ASA is recommended by a large group of physicians as a preventive therapy against cardiovascular diseases<sup>[67,68]</sup>. Furthermore, several studies have indicated the efficacy of long-term ASA use in prevention of colorectal cancer<sup>[69]</sup>, and the long-term use of ASA as a chemopreventive agent against other cancer types has attracted substantial research interest<sup>[70]</sup>. Our results may provide another perspective on the effects of long-term ASA pretreatment.

## ACKNOWLEDGMENTS

We thank Turgay Cakmak, Gizem Kutun, Dilara Yilmaz and Hayriye Guler Tarinci for their assistance in laboratory work.

## COMMENTS

### Background

Acute pancreatitis (AP) is an inflammatory disease with broad clinical variation, ranging from a mild and self-limiting condition to a severe, life-threatening necrotizing inflammation. Aspirin (acetylsalicylic acid, ASA) is the oldest and most widely used non-steroidal anti-inflammatory drug. In addition to its conventional effects, ASA is effective in the prevention of a wide range of diseases, including gastrointestinal cancer, ischemic stroke, myocardial infarction and Alzheimer's disease. This broad range of effectiveness has led to the daily intake of aspirin being recommended by a wide group of physicians as a preventive therapy for the aforementioned diseases. Considering the early events in pathophysiology of AP and the broad variety of aspirin's mechanisms of action, it is reasonable to hypothesize that long term aspirin pretreatment can effectively prevent AP.

### Research frontiers

An estimated 80000 cases of AP occur each year in the United States. Much research interest has focused on prevention strategies because there is no specific cure for AP.

### Innovations and breakthroughs

In the present study, the effects of ASA pretreatment on a pancreatitis model were investigated for the first time. The findings indicate that long term ASA pretreatment dose-dependently prevents or ameliorates some hematological, serological and histological alterations caused by cerulein-induced AP.

### Applications

The experimental data obtained in the present study point out another aspect of aspirin's preventive effectiveness and could be used in further studies of preventive strategies against AP.

### Peer review

This is a very interesting, well-structured and original study, being the first reported study on this topic. It is methodologically well planned and performed with well-designed cohorts. The paper is well written and clear.

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**P- Reviewers** Dambrauskas Z, Ko SBH, Pan WS, Rerknimitr R  
**S- Editor** Song XX **L- Editor** Stewart G **E- Editor** Xiong L



## Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa

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Received: November 5, 2012 Revised: December 13, 2012

Accepted: January 11, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To investigate the role of growth hormone (GH), hyperbaric oxygen therapy (HBOT) and combined therapy on the intestinal neomucosa formation of the gastric serosa.

**METHODS:** Forty-eight male Wistar-albino rats, weighing 250-280 g, were used in this study. The rats were divided into four groups ( $n = 12$ ): Group 1, control, gastric serosal patch; Group 2, gastric serosal patch +

GH; Group 3, gastric serosal patch + HBOT; and Group 4, gastric serosal patch + GH + HBOT. Abdominal access was achieved through a midline incision, and after the 1-cm-long defect was created in the jejunum, a 1 cm × 1 cm patch of the gastric corpus was anastomosed to the jejunal defect. Venous blood samples were taken to determine the insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein 3 (IGFBP-3) basal levels. HBOT was performed in Groups 3 and 4. In Groups 2 and 4, human GH was given subcutaneously at a dose of 2 mg per kg/d for 28 d, beginning on the operation day. All animals were sacrificed 60 d after surgery. The jejunal segment and the gastric anastomotic area were excised for histological examination. The inflammatory process, granulation, collagen deposition and fibroblast activity at the neomucosa formation were studied and scored. Additionally, the villus density, villus height, and crypt depth were counted and recorded. The measurements of villus height and crypt depth were calculated with an ocular micrometer. New vessel growth was determined by calculating each new vessel in a 1 mm<sup>2</sup> area.

**RESULTS:** In the histological comparison of groups, no significant differences were observed between the control group and Groups 2 and 3 with respect to epithelialization, granulation, fibroblastic activity and the inflammatory process, but significant differences were present between the control group and all others groups (Groups 2-4) with respect to angiogenesis ( $P < 0.01$ ) and collagen deposition ( $P < 0.05$ ,  $P < 0.01$ ). Significant differences between the control group and Group 4 were also observed with respect to epithelialization and fibroblastic activity ( $P < 0.01$  and  $P < 0.05$ , respectively). There were significant differences in villus density in all of groups compared with the control group ( $P < 0.05$ ). Crypt depth was significantly greater in Group 4 than in the control group ( $P < 0.05$ ), but no other groups had deeper crypts. However, villus height was significantly longer in Groups 2 and 4 than in

the control group ( $P < 0.05$ ). The comparison of groups revealed, significant difference between control group and Groups 2 and 4) with respect to the levels of IGF-1 and IGFBP-3 ( $P < 0.01$ ) 3 wk after the operation.

**CONCLUSION:** HBOT or GH and combined therapy augmented on neomucosal formation. The use of combined therapy produced a synergistic effect on the histological, morphological and functional parameters.

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**Key words:** Growth hormone; Hyperbaric oxygen; Neomucosa; Short bowel syndrome; Hypoxia

Adas G, Adas M, Arıkan S, Sarvan AK, Toklu AS, Mert S, Barut G, Kamali S, Koc B, Tural F. Effect of growth hormone, hyperbaric oxygen and combined therapy on the gastric serosa. *World J Gastroenterol* 2013; 19(19): 2904-2912 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2904.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2904>

## INTRODUCTION

Short bowel syndrome (SBS) is a significant problem in clinical medicine that emerged at the beginning of the last century, when the first resections of the gastrointestinal tract were performed<sup>[1]</sup>. SBS a malabsorptive disorder characterized by the loss of intestinal length, occurs when patients have  $< 200$  cm of the post-duodenal small intestine, resulting in inadequate digestion and/or nutrient absorption<sup>[2-5]</sup>. Depending on the extent, degree, and location of the intestinal resection, patients may experience severe malabsorption of fluids, electrolytes, and other nutrients. Many become dependent on long-term parenteral nutrition, which has been a life maintenance therapy<sup>[6]</sup>. Even in the best hands, this treatment can be associated with nutritional deficiencies, septic complications and life-threatening organ failure<sup>[7-9]</sup>. Another important factor is the time allowed for post-enterectomy or in utero bowel loss adaptation, which is the compensatory process in the remnant small intestine that includes mucosal regeneration, villous hypertrophy, bowel dilatation and lengthening, and delayed motility<sup>[10]</sup>. Growth hormone (GH), glutamine, and dietary modification have been proposed as a regimen to enhance bowel adaptation<sup>[3,6,7]</sup>. GH administration produced a positive nitrogen balance at all levels of energy intake<sup>[4,5,11,12]</sup>. Evidence supporting the use of GH in the SBS includes the observation that exogenous GH stimulates structural and functional intestinal adaptation<sup>[13,14]</sup>.

The treatment consists of surgery to slow intestinal transit or increase the area of absorption. Reconstructive procedures on the remnant bowel and intestinal transplantation are areas of special interest to surgeons working in this field<sup>[4,10,15]</sup>. Another potential technique for increasing the intestinal surface area is the growth of new intestinal mucosa, which takes advantage of the regenera-

tive capability of the intestine<sup>[10]</sup>. Some researchers made gastric anastomosis and, colonic and abdominal wall flaps between intestinal defects in experimental SBS to expand the mucosal surface. The regenerated intestine develops by lateral ingrowth from the surrounding mucosa and becomes functionally normal intestinal mucosa<sup>[16-19]</sup>.

In gastrointestinal surgery, if the degree of hypoxia is sufficient to interfere with tissue viability, the tissues become necrosed, resulting in delayed wound healing<sup>[20]</sup>. Ischemic wounds heal poorly and become infected. Tissue hypoxia can be reversed using hyperbaric oxygen therapy (HBOT). The effects of HBOT result from increased pressure and hyperoxia. Several studies have shown that increased oxygen tension with HBOT not only prevents the adverse effects of ischemia but also accelerates healing in different types of wounds<sup>[21]</sup>.

The serosal patch technique is one of the most popular methods. However, in many cases, only short segments of the small intestine can be patched because of the limited serosal surface and anatomical factors. In this experimental study, we used a gastric serosal patch to form neomucosa in ileum defects. Our aim in the present work was to investigate the role of GH, HBOT and combined therapy on the intestinal neomucosa formation of the gastric serosa.

## MATERIALS AND METHODS

### Animals

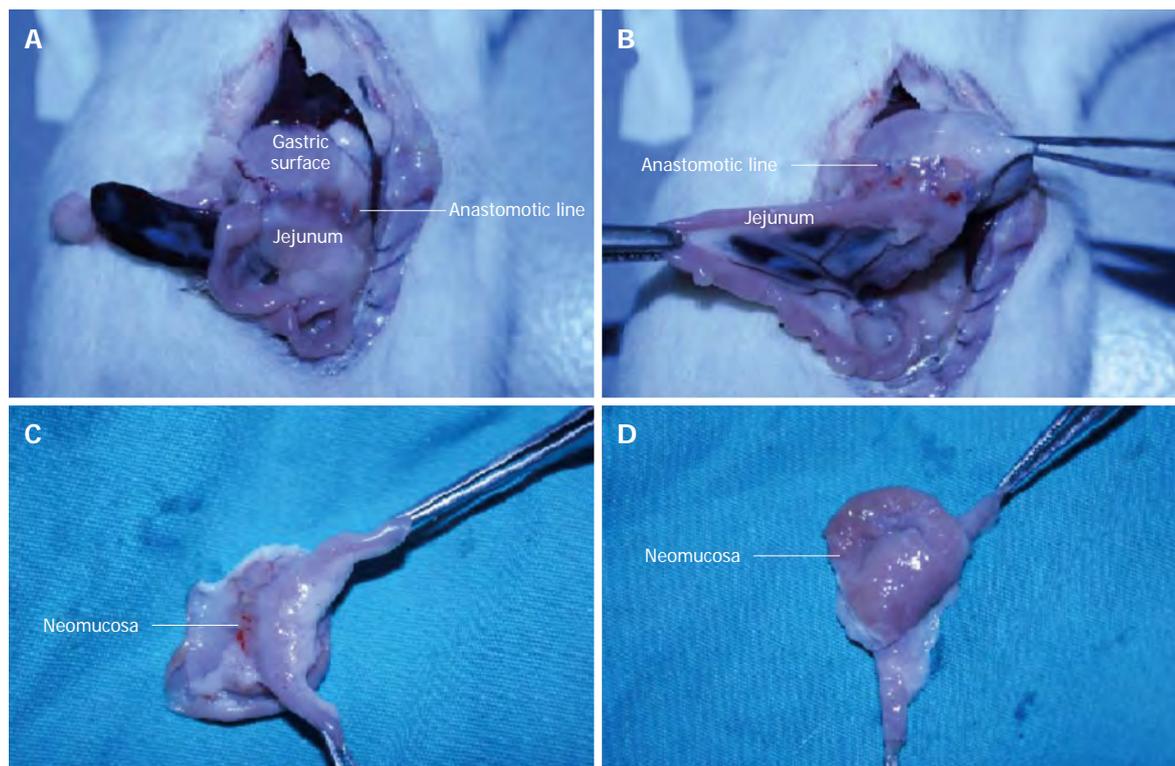
Forty-eight male Wistar-albino rats (Istanbul University, Institute of Experimental Medicine and Research, Turkey), weighing 250-280 g, were used in the study. The study was approved by the ethics committee of Istanbul University, Istanbul Medical School. All animals were housed in cages in a room at a constant temperature of  $22 \pm 2$  °C. The rats were fed a standard chow diet and tap water.

### Study design

The rats were divided into four groups ( $n = 12$ ): Group 1, control, gastric serosal patch; Group 2, gastric serosal-patch + GH; Group 3, gastric serosal patch + HBOT; Group 4, gastric serosal patch + GH + HBOT.

### Surgical procedure

After one night of fasting, the animals were anaesthetized with an intramuscular injection of ketamine hydrochloride (50-100 mg per kg of body weight). Abdominal access was achieved through a midline incision, and the jejunum was incised at 1 cm longitudinally. After the 1-cm-long defect was created in the jejunum, a 1 cm  $\times$  1 cm patch of the gastric corpus was anastomosed to the jejunal defect with interrupted 6/0 polypropylene sutures (Figure 1A and B). During the operation, venous blood samples (portal vein) were taken to determine the insulin-like growth factor 1 (IGF-1) and insulin like growth factor binding protein 3 (IGFBP-3) basal levels. After the bleeding control had been performed, 2 cc of 0.9% NaCl was injected into the intraperitoneal area, and the abdo-



**Figure 1** Surgical procedure and histopathological assessment. A, B: Anastomotic line is shown between the gastric surface and jejunum, C: Outer surface of the neomucosa formation is shown with the jejunal segment; D: Inner surface of the neomucosa is shown, and the neomucosa has a typical small intestinal phenotype.

men was closed with 3/0 continuous silk sutures. Twelve hours after the surgery, water was given; twenty-four hours after the surgery, food was given.

### Treatment

HBOT was started 12 h after the surgery and completed after 12 d. The HBOT was performed 3 times per day for the first 4 d, 2 times per day for the following 4 d and then once per day for the remaining 4 d. This therapy was applied in the Istanbul Faculty of Medicine, Department of Underwater and Hyperbaric Medicine. The total number of HBOT sessions was 24. The treatments were conducted in a small research chamber (0.4 m<sup>3</sup>). The chamber was flushed with oxygen for 10 min to vent the air inside before compression, and thus the animals could be pressurized with 100% oxygen. The HBOT sessions consisted of 10 min of compression to 2.5 atmosphere absolute (ATA), 60 min at 2.5 ATA and 10 min of decompression to the surface pressure.

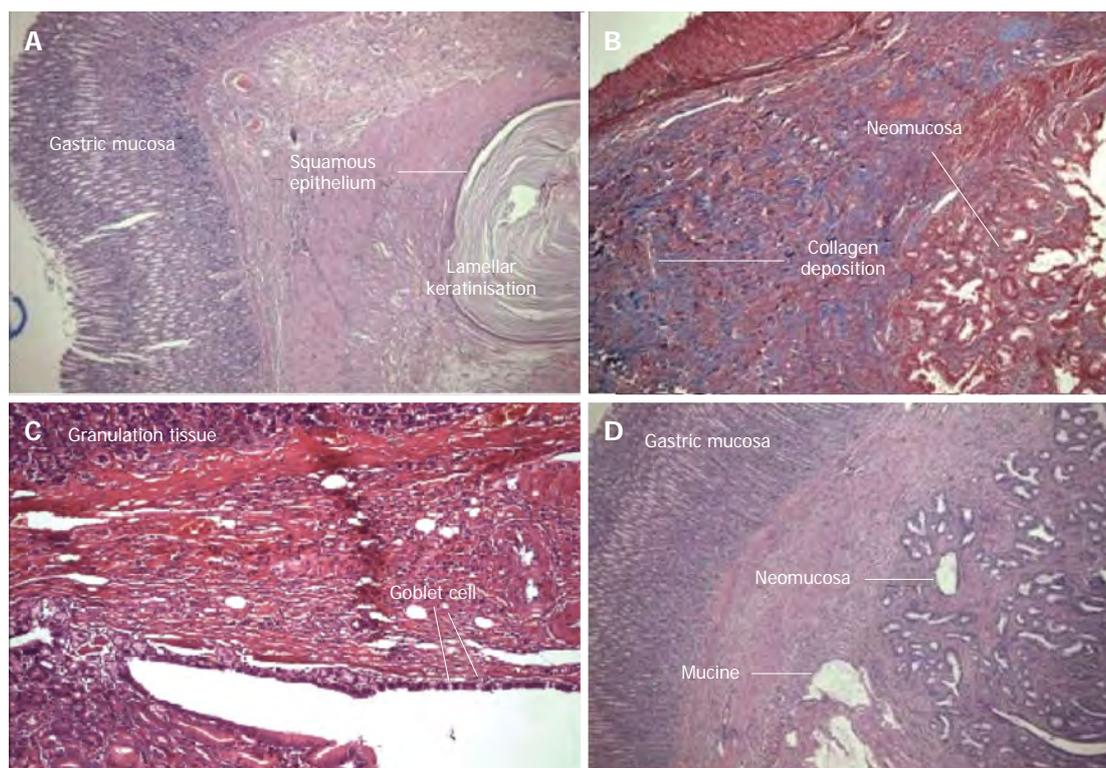
In Groups 2 and 4, human GH was given [Norditropin 4 IU (1.3 mg), Novo Nordisk-Denmark] subcutaneously at a dose of 2 mg per kg/d<sup>[22-25]</sup> for 28 d, beginning on the operation day.

### Histopathological assessment

All of the rats were sacrificed 60 d after the operation. The anastomosis, including the jejunal segment and the gastric anastomotic area, was excised (Figure 1C and D). To clean the fat tissues, the anastomotic area was washed with distilled water. The edges of the anastomotic area

were determined by following the subject line of the non-absorbable suture. Then, the area was fixed in 10% formalin for approximately 24 h and embedded in a paraffin block. Transverse sections of the embedded tissue, 4 μm in thickness, were stained with hematoxylin and eosin, and the histological assessment was performed in a blinded manner. The intestinal neomucosa, inflammatory process, granulation and fibroblast activity at the neomucosa formation were studied and scored (from 0 to 3; 0, none; 1, slight; 2, moderate; 3, dense). Masson's trichrome staining was performed to distinguish the cells from the surrounding connective tissue. Three dyes were employed, and solution C was used to stain for collagen. Collagen deposition in the neomucosa was scored according to the density in the tissue (from 0 to 3; 0, none; 1, slight; 2, moderate; 3, dense). Small intestinal epithelial cell lineages (goblet cells and enteroendocrine cells) were identified within the regenerated intestinal mucosa. The goblet cells were stained by the periodic acid-Schiff stain, and mucin was identified with alcian blue staining, pH 2.5. The alcian blue staining at pH 2.5 was used because the acid mucins of the small intestine are primarily sialo mucins<sup>[26]</sup>. The enteroendocrine cells were identified by immune histochemical staining.

Additionally, the villus density, villus height, and crypt depth were determined and recorded. The villus density was scored (from 0 to 3). The measurements of villus height and crypt depth were calculated with an ocular micrometer. New vessel growth was determined by calculating each new vessel in a 1 mm<sup>2</sup> area.



**Figure 2 Histological and morphologic evaluation.** A: Unexpected neomucosal formation. The gastric corpus mucosa can be seen. The squamous epithelium and lamellar keratinization formed from the anastomosis [Hematoxylin and eosin (HE)  $\times$  100] (Group 3); B: Granulation tissue and newly formed neomucosa. The blue area is connective tissue (Masson Trichrome  $\times$  100) (Group 4); C: In the gastric mucosa of the large granulation tissue, newly formed goblet cells can be seen (HE  $\times$  100) (Group 4); D: The left side shows the gastric mucosa, and the right side shows newly formed neomucosa that contains mucin. The granulation tissue is reduced (HE  $\times$  100) (Group 4).

### ***GH assessment***

To determine the GH efficacy, we assessed the venous IGF-1 and IGFBP-3 levels at the beginning of treatment and on postoperative day 21. Blood samples were taken from the tail vein of the rats. The analyses were performed in a biochemistry laboratory, at the Cerrahpasa Medical Faculty, University of Istanbul. The levels of IGF-1 and IGFBP-3 were measured by double-antibody, immune-radiometric assays. The IGF-1 antibody was from Immunotech France, and the IGFBP-3 antibody was from Diagnostic Systems Laboratories. The mean intra batch coefficients of variation calculated from the quality-control samples in this study were 5.6% and 2.7% for IGF-1 and IGFBP-3, respectively.

### ***Statistical analysis***

The statistical analysis was performed using SPSS 16.0 for Windows. Spearman's test was used for the intergroup correlations. Differences in the histological parameters between the treatment and control groups were analyzed non-parametrically with the Student-*t* test. All data were expressed as the mean  $\pm$  SD, and  $P < 0.05$  was accepted as significant.

## **RESULTS**

### ***Mortality analysis***

Seven rats died in the early phase (in the first week) of

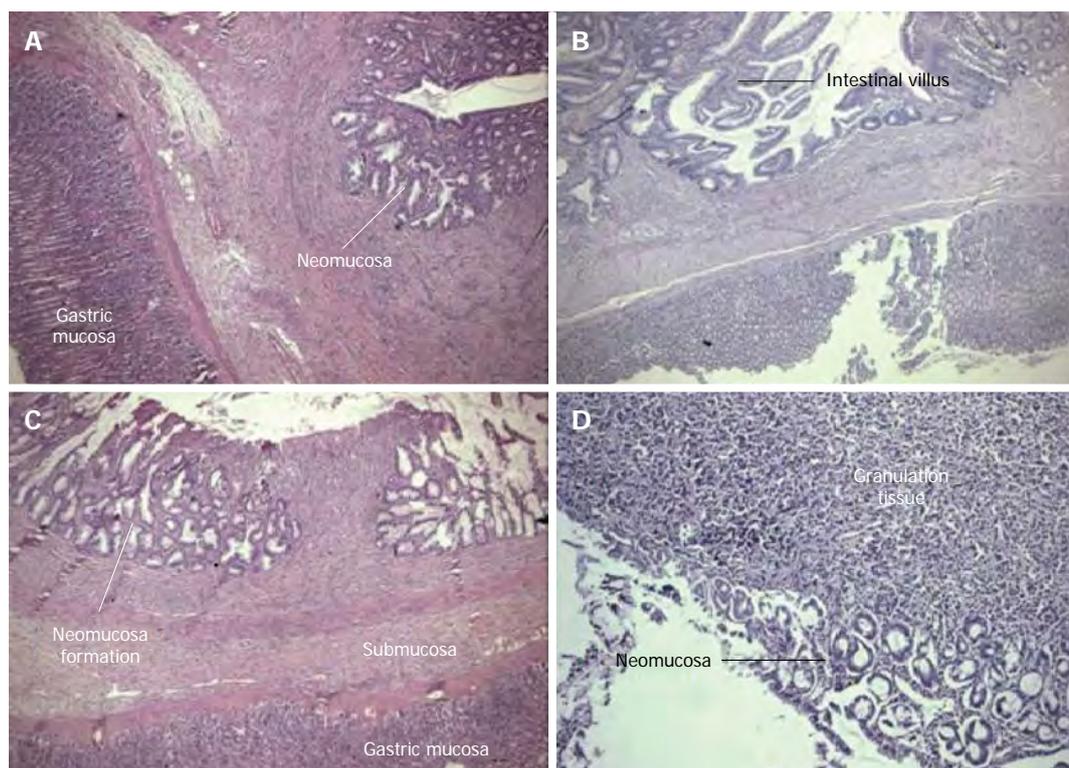
the study. Upon post-mortem examination, we discovered that two had anastomotic leakage and sepsis, two had ileus, and three had pneumothorax.

### ***Histological evaluation***

In the histological comparison of the groups, no significant differences were observed between the control group and Groups 2 and 3 with respect to epithelialization, granulation, fibroblastic activity and the inflammatory process ( $P > 0.05$ ), but significant differences were observed between the control group and all other groups (Groups 2-4) with respect to angiogenesis ( $P < 0.01$ ) and collagen deposition ( $P < 0.05$ ,  $P < 0.01$ ) (Figure 2). We also found significant differences between the control group and Group 4 with respect to epithelialization and fibroblastic activity ( $P < 0.01$  and  $P < 0.05$ , respectively). Two histological parameters were significantly different in Groups 2 and 3, and four histological parameters were significantly different in Group 4. These parameters in Group 4 were epithelialization, fibroblastic activity, angiogenesis and collagen deposition. The histological features and results are given in Table 1.

### ***Morphologic evaluation***

For the morphologic evaluation, we measured the villus density ( $\text{mm}^2$ ), villus height and crypt depth in the neomucosa. By 60 d, the luminal surface of the neomucosa tissue was nearly covered by mucosal epithelium. The



**Figure 3** There were significant differences in the villus density in all of the groups compared with the control group. A: Gastric corpus mucosa on the left, newly formed thin neomucosa on the right. The muscularispropria has not yet formed. The granulation tissue regressed [Hematoxylin and eosin (HE) × 100] (Group 3); B: Neomucosa formation is observed at the bottom of the gastric mucosa (HE × 125) (Group 4); C: The granulation tissue is in the middle, with newly formed neomucosa on either side. At the bottom, the stomach tissue is visible (HE × 100) (Group 2); D: The early development of the mucosal layer and granulation tissue (HE × 125) (Group 1).

**Table 1** Histological features of healing, morphological and functional findings and neomucosa formation

|  | Group 1<br>(control) | Group 2<br>(GH) | Group 3<br>(HBOT) | Group 4<br>(GH + HBOT) | P <sup>1</sup><br>(Groups 1-2) | P <sup>2</sup><br>(Groups 1-3) | P <sup>3</sup><br>(Groups 1-4) |
|--|----------------------|-----------------|-------------------|------------------------|--------------------------------|--------------------------------|--------------------------------|
| Histological findings of wound healing |                      |                 |                   |                        |                                |                                |                                |
| Epithelialization                      | 0.3                  | 1.3             | 1.0               | 1.7                    | > 0.05                         | > 0.05                         | < 0.01                         |
| Granulation                            | 2.4                  | 1.4             | 1.9               | 1.1                    | > 0.05                         | > 0.05                         | > 0.05                         |
| Fibroblast                             | 1.7                  | 1.8             | 2.0               | 2.4                    | > 0.05                         | > 0.05                         | < 0.05                         |
| Inflammation                           | 1.4                  | 1.1             | 1.2               | 1.2                    | > 0.05                         | > 0.05                         | > 0.05                         |
| Angiogenesis                           | 4.4                  | 7.5             | 7.2               | 8.9                    | < 0.01                         | < 0.01                         | < 0.01                         |
| Collagen deposition                    | 0.9                  | 2.2             | 1.6               | 2.6                    | < 0.01                         | < 0.05                         | < 0.01                         |
| Morphological and functional findings  |                      |                 |                   |                        |                                |                                |                                |
| Villus density                         | 0.2                  | 1.3             | 1.1               | 2.0                    | < 0.01                         | < 0.01                         | < 0.01                         |
| Villus height (µm)                     | 101 (70-109)         | 135 (98-153)    | 118 (81-129)      | 153 (119-181)          | < 0.05                         | > 0.05                         | < 0.05                         |
| Crypt depth (µm)                       | 83 (74-88)           | 102 (82-114)    | 93 (76-101)       | 120 (101-133)          | > 0.05                         | > 0.05                         | < 0.05                         |
| Goblet cells                           | 0.2                  | 1.4             | 1.1               | 2.2                    | < 0.01                         | < 0.01                         | < 0.01                         |
| Mucin                                  | 0.2                  | 1.7             | 1.2               | 2.2                    | < 0.01                         | < 0.01                         | < 0.01                         |

All parameters scored from 0 to 3, angiogenesis counted within 1 mm<sup>2</sup>, villus density, goblet cells, mucin secretion and collagen deposition, scored from 0 to 3. The P<sup>1</sup> value compares Groups 1 and 2, P<sup>2</sup> value compares Groups 1 and 3, and P<sup>3</sup> value compares Groups 1 and 4. All values are expressed as the mean ± SD. GH: Growth hormone; HBOT: Hyperbaric oxygen therapy.

neomucosa had a typical small intestinal phenotype (Figure 1C and D). There were significant differences in the villus density in all of the groups compared with the control group ( $P < 0.05$ ). The crypt depth was significantly greater in group 4 than in the control group ( $P < 0.05$ ), but no other groups had deeper crypts. However, the villus height was significantly longer in Groups 2 and 4 than in the control group ( $P < 0.05$ ) (Figure 3). The comparison of the morphological characteristics and activation of the

neomucosa between the groups revealed significant differences between the control group and Groups 2-4, with respect to the number of goblet cells and mucin secretion, respectively (both  $P < 0.01$ ) (Figure 2C and D). When we analyzed all of the groups, we obtained the highest significant differences in Group 4. The morphological and functional features in all groups are given in Table 1. We could not make any comparisons between in groups, because the immunohistochemical staining was too weak.

**Table 2** The per-operative and 3-wk post-operative venous levels of like growth factor and insulin like growth factor binding protein 3 (nmol/L)

|                          | Group 1<br>(control) | Group 2<br>(GH therapy) | Group 4<br>(GH + HBO therapy) | P <sup>1</sup><br>(Groups 1-2) | P <sup>2</sup><br>(Groups 1-4) |
|--------------------------|----------------------|-------------------------|-------------------------------|--------------------------------|--------------------------------|
| Before the surgery       |                      |                         |                               |                                |                                |
| IGF-1 (nmol/L)           | 445 (402-482)        | 421 (405-460)           | 431 (395-470)                 | NS                             | NS                             |
| IGFBP-3 (nmol/L)         | 2250 (2100-2350)     | 2170 (2000-2300)        | 2147 (2025-2350)              | NS                             | NS                             |
| After the surgery (3 wk) |                      |                         |                               |                                |                                |
| IGF-1 (nmol/L)           | 445 (415-500)        | 799 (755-875)           | 813 (775-900)                 | < 0.05                         | < 0.05                         |
| IGFBP-3 (nmol/L)         | 2290 (2050-2500)     | 3300 (3000-3600)        | 3328 (3000-3650)              | < 0.05                         | < 0.05                         |
| P                        | NS                   | < 0.05                  | < 0.05                        |                                |                                |

The *P* value compares the per- and post-operative levels within the same group, *P*<sup>1</sup> value compares the levels between Groups 1 and 2, and *P*<sup>2</sup> value compares the levels between Groups 1 and 4. All values are expressed as the mean ± SD. NS: Non-significant; GH: Growth hormone; HBO: Hyperbaric oxygen therapy; IGF-1: Like growth factor; IGFBP-3: Insulin like growth factor binding protein 3.

### GH determination

To determine the effect of the GH, we measured the IGF-1 and IGFBP-3 blood levels in the rats. The comparisons revealed no significant differences between the control group and Groups 2 and 4 in the blood levels of IGF-1 and IGFBP-3 (*P* > 0.05) per-operatively but significant differences between the control group and the other groups (Groups 2 and 4) in the levels of IGF-1 and IGFBP-3 (*P* < 0.01) 3 wk after the operation (Table 2). We also compared the per- and post-operative results within each group. There were significant differences in Groups 2 and 4 (*P* < 0.05, Table 2).

## DISCUSSION

The use of serosal patching to grow new intestinal mucosa is a technique for enlarging the intestinal surface. In the literature, different animal models have been utilized to study the growth of intestinal neomucosa in full thickness defects patched with a variety of surfaces, including colonic serosa, abdominal wall, pedicle flaps, and prosthetic material. The serosal patch technique is one of the most popular methods. However, in many cases, only short segments of small intestine can be patched because of the limited serosal surface and anatomical factors<sup>[10,27]</sup>. The peritoneal surface has also been utilized to develop epithelial cells. In a rat model, researchers folded the colon to form a seromuscular tunnel and sutured the two ends to the transected ileum. After 6-12 wk, a pouch in which a single layer of cylindrical epithelium had developed showed evidence of disaccharidase activity<sup>[10]</sup>. Erez *et al*<sup>[28]</sup> showed that using pigs and rats, one could successfully enlarge the small bowel surface by growing new mucosa on the parietal peritoneum following entero-peritoneal anastomosis. Some important advantages of this technique include the absorption of fluids and electrolytes through the peritoneum, and slowing of the bowel transit time. Bragg *et al*<sup>[29]</sup> used colonic serosa for patching, and by 8 wk, the defects were completely covered by neomucosa. In this study, we used the gastric serosal surface as a patch. An extensive literature search did not reveal a previous report of the use of the gastric serosal surface for this technique. We selected the gastric serosal-

surface because the gastric serosa constitutes a large area and because it is anatomically close to the small intestine. Furthermore, the stomach tissue is thick and has a wide network of collateral blood vessels. The fitness for surgery is also influenced by the interaction of the main vein with the easily protected neomucosa structure, the ability of the serosal flap of the small bowel to easily move to the surface, and the quick self-renewal and large surface area of the gastric surface, enabling the surgical operation to be repeated multiple times. In our study, we demonstrated that neomucosal formation occurred two months after the operation. To prevent SBS, it is important that the surface area of the bowel increases rapidly after the surgery to allow the absorption of nutrients to occur. To increase the rate of neomucosal formation, we used GH and HBO together. To our knowledge, this study is the first to report the use of both these techniques together to encourage neomucosal formation.

There are a number of peptide growth factors, such as IGF-1, or general growth factors, such as GH, that are used as promoting factors for intestinal hyperplasia and adaptation<sup>[1,2,6,14,30,31]</sup>. GH is expressed throughout the intestinal epithelium and in the lamina propria, muscularis mucosa, submucosa, and muscularispropria, indicating the potential for direct GH action within the intestine. GH has been shown to directly promote wound healing and growth of the intestine by increasing cell proliferation and collagen deposition<sup>[14]</sup>. In neomucosa formation, Thompson investigated the effect of epidermal growth factor (EGF), and octreotide-induced enterocyte apoptosis. Compared with the controls, the EGF group had decreased apoptosis in the crypt and villus<sup>[32]</sup>. Circulating GH binds to the GH receptor in the target cells and stimulates the production of IGF-1 in the liver and other target tissues, including the intestine<sup>[14]</sup>. Clinical trials of GH treatment have reported significantly increased levels of IGF-1 and IGF-binding protein-3<sup>[14,33-35]</sup>. In the GH treatment group, to control the efficacy of the GH, we assessed the level of the IGF-1 and IGF-binding protein at the end of 3 wk. Groups 2 and 4 had significantly higher levels of these hormones at the end of week three compared with the control group (*P* < 0.01).

Oxygen is an essential material for cell metabolism,

and reparative processes, such as cell proliferation and collagen synthesis<sup>[21,36,37]</sup>, have an especially increased demand for oxygen. Evidence from animal and cell line studies has shown that HBOT, the administration of pure oxygen at pressures greater than 1 ATA, results in increased growth factor production, such as platelet-related growth factor, transforming growth factor- $\beta$ 1 and vascular endothelial growth factor, and improved wound healing<sup>[38,39]</sup>. Hyperoxygenation can also increase collagen production, enabling these rapidly migrating fibroblasts to lay down larger, stronger beds of collagen for the advancing capillary beds, leading to increased granulation tissue formation and enhanced overall healing<sup>[40]</sup>. A search of the literature did not reveal any data on HBOT regarding neomucosa formation. In plastic surgery, hyperbaric oxygen has also been used for the management of wounds requiring skin grafting and for the treatment of ischemic flaps<sup>[39]</sup>. Additionally, HBOT has been reported to improve the healing of foot ulcers and glucose metabolism in patients with diabetes mellitus<sup>[56]</sup>. Angiogenesis is a process in which new blood vessels originate by budding or spouting from pre-existing vessels. HBOT increases angiogenesis, which is an important step in wound healing<sup>[21]</sup>. Huddy *et al*<sup>[41]</sup> published the results of giving HBOT to a patient who had suffered from SBS with stomal complications. After the therapy, the patient rapidly made natural adaptations. Neovascularization has been suggested as the mechanism by which HBOT acts. In our study, angiogenesis was significantly different in Group 3, which was given HBOT, compared with the control group ( $P < 0.01$ ). Moreover, collagen (Masson trichrome) was significantly different in Group 3 compared with the control group ( $P < 0.05$ ). This situation is consistent with other data reported in the literature in which HBOT increased the formation of collagen and angiogenesis. The exact mechanism by which HBOT enhances neomucosal formation is not known. There is no direct evidence regarding the mechanism by which HBOT improved neomucosa formation in our study. We thought our use of HBOT directly increased angiogenesis and collagen formation. Furthermore, HBOT may have increased the levels of some growth factors. To further examine the role of HBOT in wound healing, a more in-depth analysis of growth factors is warranted. In our study, we found some adverse side effects of HBOT. Three animals died of pneumo-thorax with the use of HBOT at the beginning. We believe that during the HBOT therapy, the tension pneumo-thorax complication can occur rarely in experimental animals; this condition was a cause of animal deaths in our study. The pneumo-thorax, could be caused directly by the high pressure of the HBOT. Murphy *et al*<sup>[42]</sup> showed that tension pneumo-thorax occurred in 3 patients who underwent HBOT. The GH and HBOT combined treatment had positive effects in all 4 groups.

Studies on the management of SBS have continued worldwide. Early attempts at increasing the surface area by serosal patching with regeneration from the margins of the wound were limited by the marked contraction of

the defects created, and thus only a modest gain in the surface area was achieved. The use of stem cells isolated from the intestine should lead to further progression of intestinal regeneration<sup>[43]</sup>. The current techniques for experimental intestinal tissue engineering employ artificial biodegradable scaffolds in a 3-dimensional structure in which organoid units are seeded<sup>[43,44]</sup>. Although these results have been reported as experimental studies, they have not been tested in clinical studies yet. Our study is a pilot study and also an experimental study model. We suggest that using this method in appropriate cases is easy. These adaptations will be increased with the combined use of GH and HBOT.

In conclusion, this study demonstrated that intestinal neomucosa can be successfully produced on a gastric serosal surface. In addition, HBOT, GH and their combined therapy augmented neomucosal formation. The combination therapy appears to be more effective. The simultaneous use of both therapies produced a synergistic effect on the histological, morphologic and functional parameters.

## COMMENTS

### Background

Short bowel syndrome (SBS) is a malabsorptive disorder characterized by loss of intestinal length and occurs when patients have < 200 cm post-duodenal small intestine, resulting in inadequate digestion and/or nutrient. Treatment consists of surgery to slow intestinal transit or to increase the area of absorption. Reconstructive procedures on the remnant bowel and intestinal transplantation are areas of special interest to surgeons working in this field. Another potential technique for increasing the intestinal surface area is the growth of new intestinal mucosa, which takes advantage of the regenerative capability of the intestine. The regenerated intestine develops by lateral in growth from the surrounding mucosa and is functionally to normal intestinal mucosa.

### Research frontiers

The serosal patch technique is one of the most popular technique to treat SBS. In this experimental study, authors used gastric serosal patch to form neomucosa in ileum defects. In literature search did not reveal a previous report of the use of the gastric serosal surface for this technique. Moreover in the present work is to investigate the role of growth hormone, hyperbaric oxygen therapy (HBOT) and combined therapy on intestinal neomucosa formation of the gastric serosa. Evidence supporting the use of growth hormone (GH) in the SBS includes the fact that exogenous GH stimulates structural and functional intestinal adaptation. Several studies have shown that increased oxygen tension with HBOT not only prevents adverse effects of ischemia but also accelerates healing in different types of wounds.

### Innovations and breakthroughs

In this experimental study, 1 cm-long defect was created in the jejunum, after that a 1 cm  $\times$  1 cm patch of the gastric corpus was anastomosed to the jejunal defect with interrupted 6/0 polypropylene sutures. HBOT was started 12 h after the surgery and completed to 12 d. GH were given subcutaneously at a dose of 2 mg per kg/d for 28 d beginning on the operation day. In order to increase the rate of neomucosal formation, we used the GH and HBOT together. This is the first study to report using these two techniques together to encourage neomucosal formation.

### Applications

Their study is a surface expander research and also is an experimental study model. Authors suggest that it is easy to use of this method in the appropriate cases. These adaptation will be increased with the togetherness use of GH and HBOT.

### Terminology

SBS, also short gut syndrome or simply short gut, is a malabsorption disorder caused by the surgical removal of the small intestine, or rarely due to the complete dysfunction of a large segment of bowel.

**Peer review**

This study demonstrated that intestinal neomucosa can be successfully raised on a gastric serosal surface. In addition, HBOT or GH and combined therapy augmented on neomucosal formation. Combination therapies seem to be more effective. Simultaneous use of combined therapy produced synergistic effect on histological, morphologic and functional parameters.

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## Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma

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Supported by The Beijing Natural Science foundation, No. 5102018; National Bio-Tech 86-3, No. 2006AA02A402 and No. 2012AA02A504

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Received: January 20, 2013 Revised: April 2, 2013

Accepted: April 9, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To investigate the association of p42.3 expression with clinicopathological characteristics and the biological function of p42.3 in human hepatocellular carcinoma (HCC).

**METHODS:** We used reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time RT-PCR and western blotting to detect p42.3 mRNA and protein expression in hepatic cell lines. We examined primary HCC samples and matched adjacent normal tissue by

immunohistochemistry to investigate the correlation between p42.3 expression and clinicopathological features. HepG2 cells were transfected with a pIRES2-EGFP-p42.3 expression vector to examine the function of the *p42.3* gene. Transfected cells were analyzed for their viability and malignant transformation abilities by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, colony formation assay, and tumorigenicity assay in nude mice.

**RESULTS:** p42.3 is differentially expressed in primary HCC tumors and cell lines. Approximately 69.6% (96/138) of cells were p42.3-positive in hepatic tumor tissues, while 30.7% (35/114) were p42.3-positive in tumor-adjacent normal tissues. Clinicopathological characteristics of the HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation ( $P = 0.031$ ). However, p42.3 positivity was not related to tumor tumor-node-metastasis classification, hepatitis B virus status, or hepatoma type. Regarding p42.3 overexpression in stably transfected HepG2 cells, we discovered significant enhancement of cancer cell growth and colony formation *in vitro*, and significantly enhanced tumorigenicity in nude mice. Western blot analysis of cell cycle proteins revealed that enhanced p42.3 levels promote upregulation of proliferating cell nuclear antigen, cyclin B1 and mitotic arrest deficient 2.

**CONCLUSION:** p42.3 promotes tumorigenicity and tumor growth in HCC and may be a potential target for future clinical cancer therapeutics.

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**Key words:** p42.3; Hepatocellular carcinoma; HepG2; Overexpression; Tumorigenicity

**Core tip:** p42.3 is a novel tumor-specific and mitosis phase-dependent expression gene. It is believed to be involved in tumorigenesis in gastric and colorectal can-

cer. To the best of our knowledge, this is the first study to investigate the expression and function of p42.3 in hepatocellular carcinoma (HCC). We found that p42.3 promotes tumorigenicity and tumor growth in HepG2 cells and is overexpressed in HCC. These results suggest that p42.3 may act as a novel tumor biomarker and aid in the development of improved therapeutic strategies.

Sun W, Dong WW, Mao LL, Li WM, Cui JT, Xing R, Lu YY. Overexpression of p42.3 promotes cell growth and tumorigenicity in hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(19): 2913-2920 Available from: URL: <http://www.wjnet.com/1007-9327/full/v19/i19/2913.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2913>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. The annual number of new HCC cases worldwide is over one million, making it the 5<sup>th</sup> most common cancer and the 3<sup>rd</sup> leading cause of cancer-related deaths<sup>[1]</sup>, accounting for more than 1 million deaths annually<sup>[2]</sup>. Despite improvements in monitoring and clinical treatment strategies, HCC prognosis remains poor<sup>[3,4]</sup>. Discovering novel biomarkers that correlate with HCC development or progression may present opportunities to reduce the severity of this disease through early and novel therapeutic interventions.

In our previous research, we cloned the full-length cDNA of the *p42.3* gene by using mRNA differential display in a synchronized gastric cancer (GC) cell lines. We found that p42.3 expression is frequently upregulated in primary tumors and embryonic tissues but not in normal tissues from adult organs. Moreover, stable silencing of p42.3 in BGC823 cells suppresses tumorigenicity and cell proliferation with accumulation of cells at G2/M stage of the cell cycle<sup>[5]</sup>. In addition, Jung *et al*<sup>[6]</sup> reported that the expression of p42.3 mRNA was significantly elevated in colorectal cancer (CRC) tissues compared to normal tissues. All these data indicate that p42.3 plays an important role in tumorigenesis, suggesting that it may be a potential tumor biomarker. In order to elucidate the role of p42.3 in tumorigenesis, we characterized p42.3 expression and validated its biologic significance in HCC.

## MATERIALS AND METHODS

### Patients and tissues

HCC specimens ( $n = 138$ ) were collected from 98 men and 40 women (age, 31-74 years; mean  $\pm$  SD, 52.6  $\pm$  8.7 years) who were inpatients at Beijing Cancer Hospital, Beijing, China, from January 2006 to September 2009. Patient data are shown in Table 1. All patients underwent a radical resection with curative intent and had sufficient clinical information available. No patients had received

**Table 1 p42.3 status in relation to clinicopathological features in patients with hepatocellular carcinoma ( $n = 138$ )  $n$  (%)**

| Tissues parameters            | No. of cases | Positive  | Negative  | P value |
|-------------------------------|--------------|-----------|-----------|---------|
| Gender                        |              |           |           | NS      |
| Male                          | 98 (71.0)    | 42 (42.9) | 56 (57.1) |         |
| Female                        | 40 (29.0)    | 23 (58.0) | 17 (42.0) |         |
| Age at diagnosis (yr)         |              |           |           | NS      |
| < 60                          | 117 (84.8)   | 52 (44.4) | 63 (55.6) |         |
| $\geq$ 60                     | 21 (15.2)    | 12 (57.1) | 9 (42.9)  |         |
| Carcinoma and adjacent tissue |              |           |           | 0.0008  |
| Carcinoma tissue              | 138 (54.8)   | 96 (69.6) | 42 (30.4) |         |
| Adjacent tissue               | 114 (45.2)   | 35 (30.7) | 79 (69.3) |         |
| Degree of differentiation     |              |           |           | 0.031   |
| Well                          | 42 (30.4)    | 11 (26.2) | 31 (73.8) |         |
| Moderate                      | 87 (63.0)    | 39 (44.8) | 48 (55.2) |         |
| Poor                          | 9 (6.5)      | 6 (66.7)  | 3 (33.3)  |         |
| TNM classification            |              |           |           | NS      |
| Stage I / II                  | 101 (73.2)   | 43 (42.6) | 58 (57.4) |         |
| Stage III / IV                | 37 (26.8)    | 19 (51.4) | 18 (48.6) |         |
| HBV                           |              |           |           | NS      |
| Negative                      | 41 (29.7)    | 15 (36.6) | 26 (63.4) |         |
| Positive                      | 97 (70.3)    | 47 (48.5) | 50 (51.5) |         |
| Type of hepatoma              |              |           |           | NS      |
| Nodular                       | 94 (68.1)    | 44 (46.8) | 50 (53.2) |         |
| Massive                       | 35 (25.4)    | 13 (37.1) | 22 (62.9) |         |
| Diffuse                       | 9 (6.5)      | 5 (55.6)  | 4 (44.4)  |         |

TNM: Tumor-node-metastasis; NS: Not significant; HBV: Hepatitis B virus.

chemotherapy or radiation therapy. Moreover, 114 adjacent normal hepatic tissues (at least 5 cm distant from the tumor edge) were also collected from HCC patients. Tumor stage was classified according to the American Joint Committee on Cancer tumor-node-metastasis (TNM) classification. The investigation project and its informed consent have been examined and certified by the Ethics Committee of Beijing Cancer Hospital.

### Tissue microarray immunohistochemistry

The hepatic tissue microarray was constructed using a tissue array instrument as previously described<sup>[7]</sup>. For immunohistochemistry studies, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubation in 3% H<sub>2</sub>O<sub>2</sub> solution for 10 min at room temperature. After blocking with 5% skim milk, sections were incubated with specific murine p42.3 mAb (1:1000, our lab) at 4 °C overnight, followed by the incubation with the peroxidase-based EnVision TM kit (Dako Cytomation, Cambridgeshire, United Kingdom) for 30 min at room temperature. The reaction product was visualized with diaminobenzidine (DAB, Dako, Glostrup, Denmark) for 5 min at room temperature. Sections were counterstained with hematoxylin.

Purified IgG from normal mouse sera was used as a negative control. The number of tumor cells or normal hepatic cells was evaluated by two independent pathologists. A specimen with more than 20% immunostained cells was classified as a positive case.

### Cell lines and cell culture

The 6 human HCC cell lines MHCC97L, MHCC97M3,

BEL7402, Huh7, HepG2, and SMMC7721 and the immortal human hepatocyte line HL7702 were routinely maintained as previously described<sup>[8]</sup>. HL7702 was cultured in Roswell Park Memorial Institute medium (RPMI 1640; Gibco, Grand Island, NY, United States), supplemented with 20% fetal bovine serum (FBS; Gibco). BEL7402 and SMMC7721 cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS. The remaining cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FBS. All media contained 100 units/mL penicillin and 100 µg/mL streptomycin. All cell lines were maintained at 37 °C in 5% CO<sub>2</sub>.

#### Reverse transcription-polymerase chain reaction and quantitative real-time reverse transcription-polymerase chain reaction

Total RNA was extracted from cell lines using TRIzol (Qiagen, United States). The prepared RNA (5 µg) was mixed with oligo-dT primers and reverse-transcribed with moloney murine leukemia virus reverse transcriptase (Promega, United States) for 60 min at 37 °C, followed by polymerase chain reaction (PCR) amplification with specific primers for p42.3 (forward: 5'-TGGACTGCG-GCCTGCTGAA-3'; reverse: 5'-ACTCCATCGCTGT-GTTTCAAT-3'). PCR amplification was performed in 20 µL using a thermocycler (Biometra, Germany) with the following PCR program: pre-denaturation for 5 min at 94 °C, denaturation for 45 s at 94 °C, annealing for 45 s at 61 °C, extension for 45 s at 72 °C, and a final elongation at 72 °C for 10 min. β-Actin served as an internal positive control (forward: 5'-TCACCCACACTGTGCCCATC-TACGA-3'; reverse: 5'-CAGCGGAACCGCTCATTTGC-CAATGG-3'). PCR was performed for 24 or 32 cycles (β-actin 24 cycles; p42.3 32 cycles). PCR products were separated by electrophoresis on a 1.5% agarose gel. Quantitative real-time reverse transcription-PCR (Q-RT-PCR) using SYBR-Green Master PCR mix (Applied Biosystems, Carlsbad, CA) was performed in triplicate (p42.3 forward: 5'-CCTGGCATCTTTACTGGACTGGA-3'; p42.3 reverse: 5'-GTGCCAGCCTGTCTCACATTTTC-3'). Quantification was normalized to the endogenous control β-actin (forward: 5'-TTAGTTGCGTTACACCCTTTC-3'; reverse: 5'-ACCTTCACCGTTCCAGTTT-3').

#### Western blotting

Cell lysates were prepared by incubating cells at 4 °C for 1 h in a buffer containing 50 mmol/L Tris-HCl, pH 8.0, 0.5% Nonidet P-40, 2 mmol/L dithiothreitol, 5 mmol/L ethylene diamine tetraacetic acid, 100 mmol/L NaCl, and 2 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of protein were electrophoresed on a 12% sodium dodecylsulfate polyacrylamide gel and transferred to a polyvinylidene difluoride membrane using standard techniques. We used four specific antibodies obtained from Santa Cruz Biotechnology: proliferating cell nuclear antigen (PCNA) (diluted 1:300; F-2), cyclin B1 (diluted 1:500; H-433), cell division cycle 25 A (Cdc25A) (diluted 1:500; DCS-122), and cell division cycle 25 homolog

C (Cdc25C) (diluted 1:500; C-20). The following specific antibodies were also used: mitotic arrest deficient 2 (MAD2) (diluted 1:1000; Ab70383; Abcam, United Kingdom), actin (diluted 1:10000, AC-15; Sigma, United States), and p42.3 (diluted 1:1000; our lab). Nonspecific binding was blocked using a 5% fat-free milk solution. Signals were detected using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

#### Plasmid construction and cell transfection

The whole coding region of p42.3 was cloned into the pIRES2-EGFP vector at the *Bam*HI and *Hind*III sites. Nucleotide sequences of the subcloned cDNAs were verified by sequencing. HepG2 were selected and cultured at 60%-70% confluence in 35 mm plates. Cells were transfected with recombinant p42.3 plasmids or an empty vector using Lipofectamine 2000 (Invitrogen, Carlsbad, United States). At 48 h post-transfection, cells were seeded for 21 d in selection medium containing 400 µg/mL G418 to screen for stable clones. To confirm the transfection efficiency, RT-PCR and Western blot analysis were performed.

#### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and soft agar colony formation assay

Stably transfected cells were seeded ( $2 \times 10^3$ ) in duplicates into each well of a 96-well culture plate and grown in 200 µL DMEM with 5% FBS; 10 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Genview, Florida, United States) (5 mg/mL) was added at 0, 24, 48, 72, 96 and 120 h. The MTT was removed after 4 h incubation; 100 µL of dimethylsulfoxide (Amresco, Cochran, United States) was added to each well, then incubated for 30 min. Absorbency was measured at 570 nm using an iMark Microplate Reader (Bio-Rad, CA, United States).

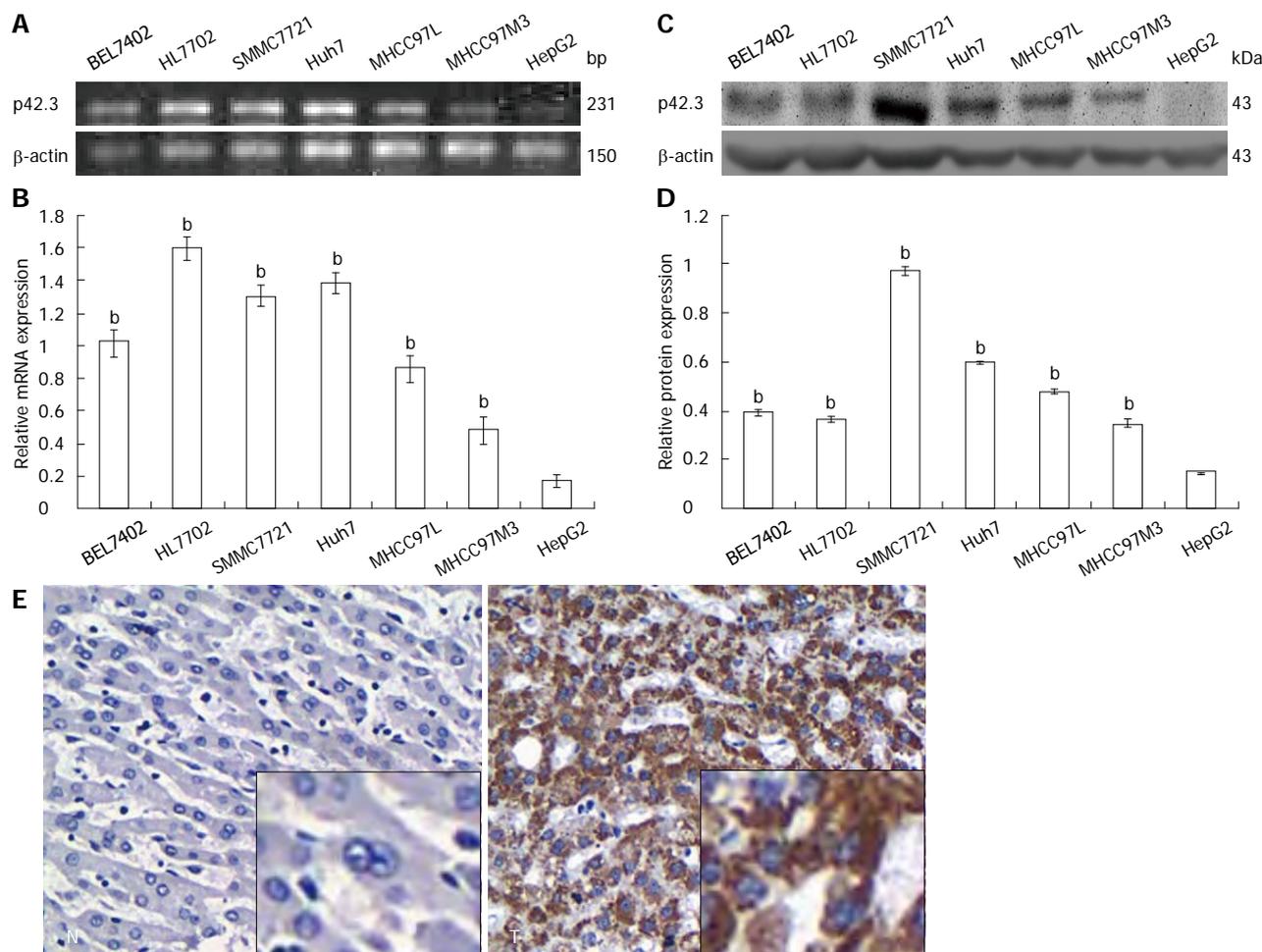
For the soft agar assay, cells ( $2 \times 10^3$ ) were trypsinized and resuspended in 4 mL of 0.3% agar in DMEM containing 10% FBS, and overlaid with 0.6% agar in 60-mm culture dishes. The dishes were incubated routinely for 21 d. Colonies were stained with 0.2% *p*-iodonitrotetrazolium violet, then photographed and counted.

#### Tumorigenicity assay in nude mice

Stably transfected cells were washed twice and resuspended in 1 × Hank's buffer at a concentration of  $1 \times 10^6$  cells/mL. A 100-µL cell suspension of HepG2-p42.3 was then injected subcutaneously into the left dorsal flank of 10, 4-wk-old female nude mice. As a control, the right side was inoculated with HepG2-vector. Tumor diameters were checked every 3 d, and tumor volume was calculated according to  $ab^2/2$  ( $a > b$ ). Tumor specimens were collected at 15 d after injections and split. Immunohistochemistry (IHC) analysis was used to detect p42.3 protein expression. Three independent experiments were performed and yielded similar results.

#### Statistical analysis

To evaluate the possible differences of p42.3 expression in different hepatic specimens, we performed Pearson's



**Figure 1** Detection of p42.3 in hepatic cell lines and hepatocellular carcinoma tissues. A: Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that p42.3 was detectable in all 7 cell lines, and the lowest expression was in HepG2 cells; B: Relative expression of p42.3 mRNA in seven hepatic cell lines using quantitative real-time RT-PCR. Data are shown as the mean  $\pm$  SD, endogenous references was  $\beta$ -actin ( $^*P < 0.01$  vs HepG2); C and D: Expression of p42.3 protein in hepatic cell lines analyzed by Western blotting (C) and shown as mean  $\pm$  SD (D) ( $^*P < 0.01$  vs HepG2); E: Negative staining of p42.3 in hepatocellular carcinoma-adjacent normal tissue (left), positive staining of p42.3 in tumor (right). Original magnification,  $\times 100$ ; the inset boxes are at original magnification  $\times 200$ .

$\chi^2$  test. The Student's two-sided *t*-test was used to compare test and control sample values in MTT assay, soft agar colony formation assay and tumorigenicity assay. All statistical analyses were carried out using the SPSS statistical software package 16.0 (SPSS Inc., United States). *P* values  $< 0.05$  were considered statistically significant.

## RESULTS

### p42.3 protein expression in human tumor cell lines

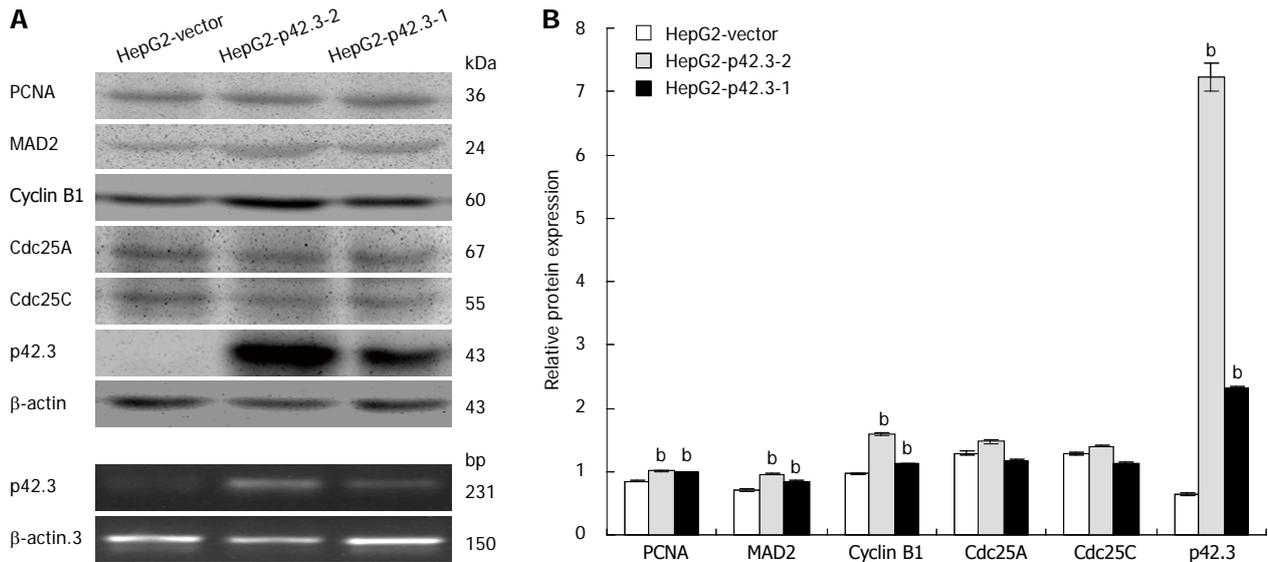
p42.3 mRNA and protein expression were examined in 6 human HCC cell lines and the immortal human hepatocyte HL7702. RT-PCR and Q-RT-PCR showed that p42.3 mRNA was expressed in all of 7 cell lines (7/7, 100%), and the lowest expression was found in HepG2 cells (Figure 1A and B). Consistent with mRNA expression levels, p42.3 protein was expressed at high levels in all cell lines except HepG2 cells (6/7, 85.7%) (Figure 1C and D). Thus, we confirmed that the HepG2 cell line is a p42.3-deficient line and could therefore be used as a model to investigate p42.3 protein function.

### p42.3 protein levels in human primary tumors

To characterize p42.3 expression in HCC specimens, IHC was performed on tumor tissues and tumor-adjacent normal tissues. We found p42.3 protein was detected in 69.6% (96/138) of hepatic tumor tissues. However, p42.3 expression was less apparent, with significantly less positive cells (30.7%, 35/114) in tumor-adjacent normal tissues ( $P < 0.001$ , Table 1 and Figure 1E). The results indicate that p42.3 protein is highly expressed in primary HCC tissues rather than tumor-adjacent normal tissues. Analysis of the clinicopathological characteristics of the 138 HCC specimens revealed a significant correlation between p42.3 expression and tumor differentiation ( $P = 0.031$ , Table 1). However, we found no relationship between p42.3 positivity and tumor TNM classification, hepatitis B virus status, or type of hepatoma.

### Overexpression of p42.3 induces PCNA, cyclin B1 and MAD2 expression in HepG2 p42.3-deficient cells

To examine the gene function of p42.3 overexpression on HCC cells, we stably transfected the pIRES2-EGFP-



**Figure 2** The effect on molecular by overexpression of p42.3 in HepG2 cells. A: Reverse transcription-polymerase chain reaction and Western blotting were performed to confirm p42.3 overexpression in a stable single colony of HepG2-p42.3-1 and HepG2-p42.3-2 cells. p42.3 expression was deficient in the HepG2-vector control cells.  $\beta$ -actin served as an internal control; B: Expression of proteins shown as mean  $\pm$  SD. Consistent with p42.3 protein expression, proliferating cell nuclear antigen (PCNA), mitotic arrest deficient 2 (MAD2) and cyclin B1 expression were significantly upregulated. The protein levels of cell division cycle 25 A (Cdc25A) and cell division cycle 25 homolog C (Cdc25C) hardly changed following p42.3 expression ( $^bP < 0.01$  vs HepG2-vector).

p42.3 expression vector into HepG2 cells. A cell line that stably expresses p42.3 (HepG2-p42.3) was generated and analyzed by western blotting. As shown in Figure 2, p42.3 protein was not detected in cells stably transfected with the empty vector. However, p42.3 protein was significantly increased in the p42.3 overexpressing cells, HepG2-p42.3-1 and HepG2-p42.3-2. These results indicated that the eukaryotic vector for p42.3 used in this study sufficiently upregulates p42.3 expression in HepG2 cells.

Since p42.3 is a novel cell cycle-dependent protein, we investigated cyclin B1 and other M phase-related proteins in p42.3-expressing HepG2 cells and control cells (HepG2-vector). We found that p42.3 expression resulted in a significant upregulation in PCNA, cyclin B1 and MAD2 protein levels. However, Cdc25A and Cdc25C protein levels only slightly changed with p42.3 expression (Figure 2).

#### Overexpression of p42.3 promotes growth and colony formation in HepG2 cells

The effects of p42.3 overexpression on the viability of HepG2 cells were measured using an MTT colorimetric assay. We found that transfection with pIRES2-EGFP-p42.3 promotes HepG2 cell growth. A stable single clone of HepG2-p42.3-1 and HepG2-p42.3-2 cells grew much faster over a 6-day period when compared to parental HepG2-vector cells, indicating that p42.3 may confer a strong growth capability in HepG2 cells ( $P < 0.01$ , Figure 3A).

The colony formation assay was used to evaluate the ability for anchorage-independent growth of cells in soft agar medium. Our data showed a significant increase in HepG2-p42.3-1 and HepG2-p42.3-2 colony formation in both number and size; however, the HepG2-vector

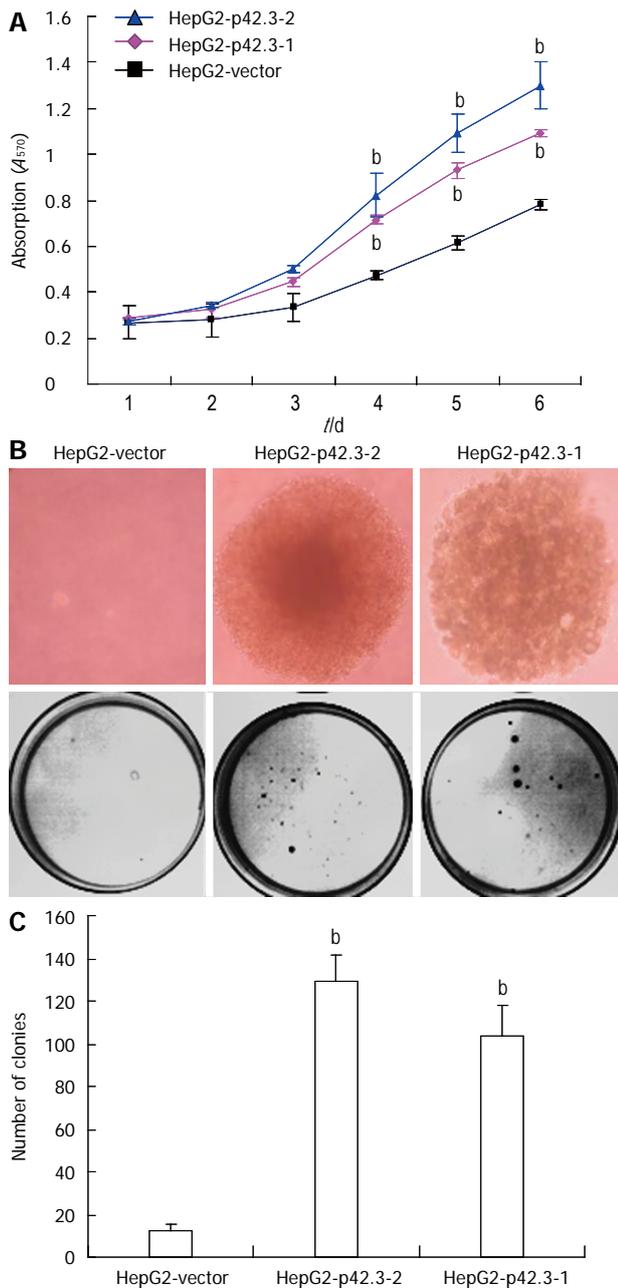
cells only formed a few small colonies ( $P < 0.01$ , Figure 3B and C). This suggests that p42.3 confers anchorage-independent growth to cells.

#### Overexpression of p42.3 promotes HepG2 cell tumorigenicity

We tested p42.3 tumorigenicity in nude mice. HepG2-p42.3 cells were injected subcutaneously into the left dorsal flank of female nude mice (BALB/c), the right side was injected with HepG2-vector cells as a control. Of the 5 animals injected with HepG2-p42.3-1 or HepG2-p42.3-2 cells, tumors appeared faster and were larger than in the controls (HepG2-vector) ( $P < 0.01$ , Figure 4A and B). After the animals were sacrificed, the xenografts were removed and collected for immunohistochemistry analysis. The results showed that p42.3 protein was found in all HepG2-p42.3 xenografts, but that p42.3 protein was not found in HepG2-vector xenografts (Figure 4C). These results further confirmed that the p42.3 overexpression promotes tumorigenicity of HepG2 cells.

## DISCUSSION

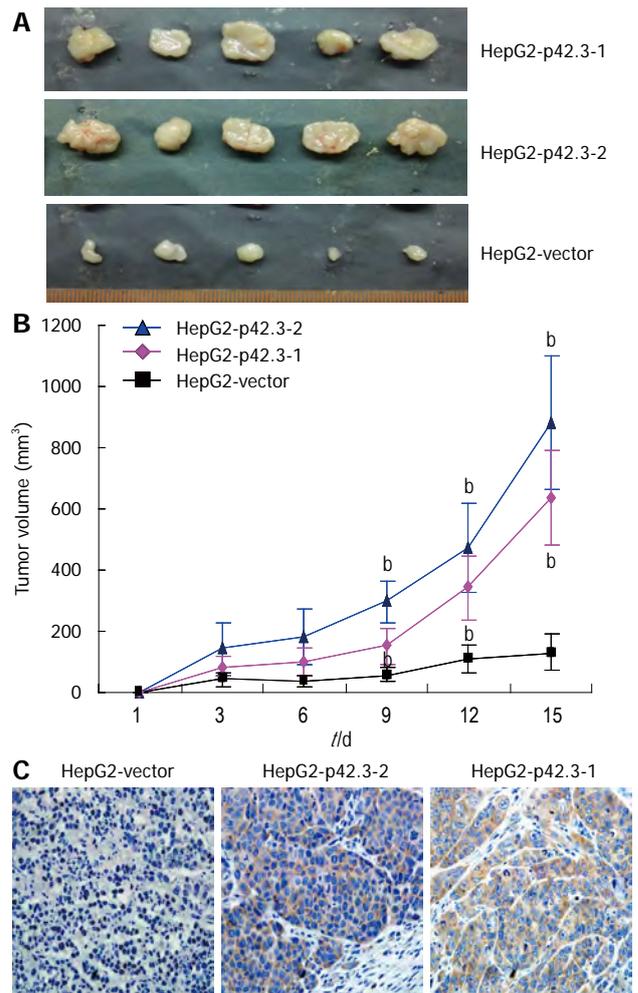
p42.3 is a highly conserved mammalian gene and strong G2 induction<sup>[5,9-11]</sup>. Moreover, p42.3 is involved in Chromosome segregation<sup>[12]</sup>, it may play a key role in tumorigenicity. Our previous studies have shown that p42.3 was overexpressed in GC tissue and its expression is cell cycle dependent in the BGC823 cell line, expression peaked at early G1 phase<sup>[5,13]</sup>. Additionally, reduced proliferation and tumorigenic properties were detected in the BGC823 cell line that lacked p42.3<sup>[5]</sup>, suggesting that p42.3 is involved in gastric carcinogenesis. While most studies have focused on the roles of p42.3 in GC development<sup>[5,11,14]</sup>,



**Figure 3** Promotion of cell growth and colony formation with p42.3 overexpression in HepG2 cells. A: Promotion of cell growth after overexpression of p42.3 in HepG2. Growth curve comparing HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector cells over a 6-d time course. Data are shown as the mean  $\pm$  SD of three independent experiments ( $^*P < 0.01$  vs HepG2-p42.3-1); B: The colonies of HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector formed on soft agar. The colonies grew faster and were larger in HepG2 cells that overexpressed p42.3-2 than in the HepG2-vector control cells; C: Raw value indicating colony number. Data revealed that the colony-forming activities of HepG2-p42.3-1 and HepG2-p42.3-2 were significantly promoted on soft agar. The data represent the mean  $\pm$  SD of three independent experiments ( $^*P < 0.01$  vs HepG2-vector).

the roles of p42.3 in other cancer remain to be elucidated. Therefore, we characterized p42.3 expression in HCC tissues from patients. Moreover, we investigated p42.3 functions and potential mechanisms of action in HepG2 cells.

In 7 human HCC cell lines, consistent with the mRNA



**Figure 4** Promotion of tumorigenesis by overexpression of p42.3 shows statistical significance compared with the control in HepG2 cells. A: The tumor induced by HepG2-p42.3-1 and HepG2-p42.3-2 was much larger than that in the control; B: Over the course of 15 d, the tumor growth curve comparing HepG2-p42.3-1, HepG2-p42.3-2, and HepG2-vector cells revealed that p42.3-overexpressing cells grew faster ( $^*P < 0.01$  vs HepG2-p42.3-1); C: Immunohistochemistry staining was performed in xenograft tissues from tumors. p42.3 protein was detectable in xenografts that were formed by injection of p42.3-overexpressing cells, but not in the tumors developed from HepG2-vector cells.

expression, p42.3 protein was highly expressed with the exception of the HepG2 line. In concert with our previous study in GC cells, we found that the p42.3 gene was highly expressed in the majority of the tested tumor cell lines. This suggests that the p42.3 gene is overexpressed in tumor cells. In previous research, data showed that the p42.3 gene is closely related to GC and CRC<sup>[5,6]</sup>. Similarly, our current data revealed that the p42.3 protein was expressed in 64.7% of hepatic tumor tissues compared to only 35.3% in tumor-adjacent normal tissues. The clinical p42.3 data in GC, CRC and HCC tissues suggest that upregulation of p42.3 may be a common feature in a variety of tumors.

Our results further support the hypothesis that p42.3 might stimulate cellular viability and malignant transformation since overexpression of p42.3, by stable transfection of the pIRES2-EGFP-p42.3 into HepG2 cells, significantly promotes cancer cell growth by MTT colo-

rimetry and colony formation *in vitro*, and significantly induced tumorigenicity in nude mice. Thus, these findings provide evidence that p42.3 plays an important role in tumorigenesis. Therefore, we investigated the molecular mechanism responsible for stimulating cell growth and malignant transformation. Since the expression of p42.3 is cell cycle dependent and G2/M checkpoint abrogated<sup>[5,11,13,15]</sup>, we analyzed the effects of elevated p42.3 levels on a series of cell cycle proteins. Our results indicate that p42.3 expression significantly upregulates PCNA, cyclin B1 and MAD2 protein levels. However, Cdc25A and Cdc25C protein levels hardly change with p42.3 expression. Cyclin B1 is one of the key genes involved in M-phase regulation<sup>[16-20]</sup>. Together with Cdc2, cyclin B1 forms a complex that controls M-phase entry and exit<sup>[17,21]</sup>. Cyclin B1 can promote the G2-M transition, and even leads to a loss cell proliferation control, thus leading to malignant transformation<sup>[22,23]</sup>. The alteration of cyclin B1 protein levels shown here is consistent with our previous study<sup>[5]</sup>, it shows p42.3 may a regulator of cyclin B1. Furthermore, MAD2 is a component of the mitotic spindle assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate<sup>[24,29]</sup>, and MAD2 is involved in mediating the upregulation of cyclin B1 proteins<sup>[30,31]</sup>. Our results showed that the significant increase in expression of cyclin B1 and Mad2 may correlation with G2/M checkpoint abrogation. On the other hand, though Cdc25 phosphatases involved in cell cycle checkpoints as key regulators of normal cell division and the cell's response to DNA damage<sup>[32-35]</sup>, our data did not reveal any obvious change in total Cdc25A and Cdc25C levels with p42.3 overexpression, Cdc25 phosphatases may have no role in the cell's response to induced p42.3 expression.

In summary, the data obtained in this study demonstrate that p42.3 protein is upregulated in primary HCC tissues but not tumor-adjacent normal tissues, and that cyclin B1 might be responsible for the cellular proliferation and malignant transformation induced by p42.3. These insights may help to identify p42.3 as a potential target for improved cancer therapies or as a diagnostic marker in clinical cancer treatment.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is a major world health problem due to its high incidence and fatality rate. Discovering novel biomarkers that correlate with HCC may present opportunities to reduce the severity of this disease. As a novel tumor-specific and mitosis phase-dependent expression gene, p42.3 is involved in cell proliferation and tumorigenesis in gastric cancer (GC). Previous data also indicate that p42.3 expression is significantly elevated in GC and colorectal cancer (CRC), thus raising the possibility that it may act as a potential tumor biomarker.

### Research frontiers

p42.3 is a novel tumor-specific and mitosis phase-dependent expression gene. It is involved in tumorigenesis in GC and CRC. However, the research concerning p42.3 in HCC is lacking. In this study, the authors investigate p42.3 expression and function in primary HCC. These results suggest that p42.3 may act as a novel disease biomarker and aid in the development of improved therapy strategies.

### Innovations and breakthroughs

Recent reports have highlighted that p42.3 is involved in GC and CRC. This is the first study to investigate the expression and function of p42.3 in HCC. The authors found that p42.3 promotes tumorigenicity and tumor growth in HepG2 cells and is overexpressed in HCC.

### Applications

In understanding the expression and function of p42.3 in HCC, this study may represent a future strategy as a therapeutic target and/or improve clinical cancer HCC treatment.

### Peer review

The authors examined the expression of p42.3 and its function in HCC. These data revealed that p42.3 was increased in HCC and in all HCC cells with the exception of HepG2 cells. Moreover, p42.3 expression was correlated with tumor differentiation. p42.3 promotes tumorigenicity and tumor growth in HCC; therefore, it may be used as a potential target to improve the clinical treatment of HCC.

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P- Reviewers Butterworth J, Grassi G, Yu DY  
S- Editor Wen LL L- Editor A E- Editor Xiong L



## Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation

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Supported by Grants from the National Natural Science Foundation of China, No. 30970886; The Science and Technology Project of Dalian, No. 2010E15SF179; and the Initial Doctoral funding of Liaoning Province, No. 20121110

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Received: January 13, 2013 Revised: March 15, 2013

Accepted: March 28, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To investigate the effects of the heme oxygenase (HO)-1/carbon monoxide system on iron deposition and portal pressure in rats with hepatic fibrosis induced by bile duct ligation (BDL).

**METHODS:** Male Sprague-Dawley rats were divided randomly into a Sham group, BDL group, Fe group, deferoxamine (DFX) group, zinc protoporphyrin (ZnPP) group and cobalt protoporphyrin (CoPP) group. The levels of HO-1 were detected using different methods. The serum carboxyhemoglobin (COHb), iron, and portal vein pressure (PVP) were also quantified. The plasma and mRNA levels of hepcidin were measured. Hepatic fibrosis and its main pathway were assessed using Van

Gieson's stain, hydroxyproline, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1), nuclear factor-E2-related factor 2 (Nrf2), matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1).

**RESULTS:** Serum COHb and protein and mRNA expression levels of HO-1 and Nrf2 were increased in the BDL group compared with the Sham group and were much higher in the CoPP group. The ZnPP group showed lower expression of HO-1 and Nrf2 and lower COHb. The levels of iron and PVP were enhanced in the BDL group but were lower in the ZnPP and DFX groups and were higher in the CoPP and Fe groups. Hepcidin levels were higher, whereas superoxide dismutase levels were increased and malonaldehyde levels were decreased in the ZnPP and DFX groups. The ZnPP group also showed inhibited TGF- $\beta$ 1 expression and regulated TIMP-1/MMP-2 expression, as well as obviously attenuated liver fibrosis.

**CONCLUSION:** Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity through the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and regulating PVP.

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**Key words:** Heme oxygenase-1; Hepcidin; Iron accumulation; Oxidative stress; Portal vein pressure; Carboxyhemoglobin; Bile duct ligation

**Core tip:** In this study, inhibiting heme oxygenase-1 (HO-1)/carbon monoxide (CO) system by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation, the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity through the nuclear factor-E2-related factor 2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining portal vein pressure.

Wang QM, Du JL, Duan ZJ, Guo SB, Sun XY, Liu Z. Inhibiting heme oxygenase-1 attenuates rat liver fibrosis by removing iron accumulation. *World J Gastroenterol* 2013; 19(19): 2921-2934 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2921.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2921>

## INTRODUCTION

Iron is an essential nutrient for growth and survival, but excessive iron accumulation in cells can result in cell injury<sup>[1,2]</sup>. Iron overload is not uncommon in many patients with end-stage liver cirrhosis, and it can also occur in patients with a history of multiple blood transfusions<sup>[3,4]</sup>.

Research has shown that in cultured hepatocytes, iron activates hepatic stellate cells and increases the secretion of latent transforming growth factor- $\beta$  (TGF- $\beta$ ) due to hepatocytes being injured by iron in the pathogenesis of iron-induced liver fibrosis<sup>[5]</sup>. In mice, iron overload enhanced the development of carbon tetrachloride-induced hepatic fibrosis<sup>[6]</sup>. In clinical studies, approximately half of patients with hereditary iron accumulation (hemochromatosis) developed liver fibrosis<sup>[7]</sup>. Moreover, a significant reduction of fibrosis in the liver was demonstrated in a number of thalassemia patients treated with deferasirox<sup>[8]</sup>.

Clinically, repeated large-volume blood transfusions are sometimes necessary for cirrhotic patients with massive upper gastrointestinal bleeding; in most cases, patients are transfused with packed red blood cells (RBCs), which results in iron overload as the human body cannot excrete iron. Each unit of RBCs contains approximately 250 mg of iron, and after 10-15 RBC transfusions, iron typically accumulates in the liver, heart, skin, and endocrine organs<sup>[9]</sup>. However, how iron overload affects the pathogenesis and treatment of patients with hepatic fibrosis is not yet well understood.

Heme oxygenase-1 (HO-1) is the primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme into free iron, carbon monoxide (CO), and biliverdin<sup>[10,11]</sup>.

Previous reports have recently shown HO-1 to be protective in liver cells in various liver diseases such as acute liver injury, alcoholic liver disease, liver fibrosis and ischemia/reperfusion injury through multiple pathways<sup>[12-15]</sup>. Other reports have indicated that this protection might be restricted to a narrow threshold of HO-1 over-expression<sup>[13,16]</sup>. Our previous studies showed that over-expression of HO-1 could be harmful to the liver functioning of rats with cirrhosis induced by bile duct ligation (BDL)<sup>[17,18]</sup>, which was also reported by Froh *et al*<sup>[19]</sup>, but whether this effect was related to iron accumulation and CO release was not clear.

In normal Sprague-Dawley (SD) rats, increased HO activity as a pro-oxidant mechanism resulted in iron accumulation in the liver; in contrast, decreased HO activity reduced intracellular iron levels and oxidative stress<sup>[20]</sup>.

In this study, we investigated the effect of HO-1 on iron accumulation and CO release by inhibiting or inducing HO-1 expression with zinc protoporphyrin (ZnPP) or cobalt protoporphyrin (CoPP) in fibrotic rat models induced by BDL, and we further studied whether regulating HO-1 expression could improve liver fibrosis by reducing hepatic iron accumulation and portal vein pressure (PVP).

## MATERIALS AND METHODS

### Animal care

The experimental protocols were approved by the Animal Care and Use Committee of Dalian Medical University (Liaoning Province, China), in accordance with the guidelines established by the Canadian Council on Animal Care.

### BDL and treatment in rat

Fifty-three healthy male SD rats, weighing 200-220 g, were obtained from the Laboratory Animal Center of Dalian Medical University and were randomly divided into six groups: a Sham group ( $n = 6$ ), BDL group ( $n = 10$ ), CoPP treatment group ( $n = 12$ ), ZnPP treatment group ( $n = 8$ ), Fe treatment group ( $n = 9$ ) and DFX treatment group ( $n = 8$ ). The rats were housed in a specific pathogen-free (SPF) center, at room temperature of 24-26 °C and relative humidity of 60%-65%. Water was provided ad libitum.

The rats were well fed and housed for 3 d before any experimental protocols. Biliary cirrhosis was induced by BDL<sup>[21,22]</sup>. Five groups underwent BDL together with Sham-operated animals as a healthy control. The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. Laparotomy was performed under anesthesia with ether. The bile duct was isolated and double-ligated with a 3-0 silk suture. The abdominal wall and skin were closed with 4-0 silk sutures, and the antibiotic gentamicin (0.3 mL) was injected intramuscularly. Rats in the Sham group underwent laparotomy with the bile duct isolated but not ligated. After surgery, the Sham and BDL groups received an intraperitoneal injection of saline. Other groups received an intraperitoneal injection consisting of CoPP, ZnPP, iron-dextran (ID) and deferoxamine (DFX) (5, 5, 50, 100 mg/kg body weight) three times per week, respectively. After the establishment of the rat models, the number of rats was reduced to 6 in each group because of deaths during the study process.

ZnPP and CoPP (Sigma, St Louis, MO, United States) were dissolved in 0.2 mol/L of NaOH, adjusted to a pH of 7.4, were diluted in 0.85% NaCl, with a final concentration of 1 mg/mL as previously described, and were used for inhibiting and inducing HO-1 expression, respectively<sup>[23]</sup>. DFX mesylate salt and ID (Sigma, St Louis, MO, United States) were diluted in 0.85% NaCl with final concentrations of 40 and 20 mg/mL, respectively. Histostain™ - Plus Kits (SP9001) (Zhongshan Goldenbridge

Biological Technology, Beijing, China); hydroxyproline (HYP), malonaldehyde (MDA) and superoxide dismutase (SOD) (Key GEN Biotech Nanjing, China); a hepcidin enzyme-linked immunosorbent assay (ELISA) Kit (EIAab Science, Wuhan, China); and a TaKaRa RNA polymerase chain reaction (PCR) kit (avian myeloblastosis virus), version 3.0 (TaKaRa Biotechnology, Dalian, China), were used in this study.

### Sample collection and examination

Four weeks after surgery, a catheter connected to a pressure transducer (BL-420F biological experimental system, Chengdu Technology and Market Co. Ltd., China), was placed in the portal vein to measure PVP. Subsequently, 1 mL of arterial blood was withdrawn to measure carboxy-hemoglobin (COHb), using a Rapid Lab 1245 Blood Gas Analyzer (Siemens, New York, NY, United States). Then, blood samples were collected from the abdominal aorta to measure serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), and serum iron, using a Hitachi 7600-110 automatic biochemical analyzer (Hitachi Co, Tokyo, Japan). The levels of liver SOD and MDA were determined with a UV-2100 spectrophotometer (Chemito Instruments Pvt. Ltd.).

### Liver iron content measurement

Liver iron content was determined by atomic absorption spectrometry with acetylene-air flame atomization. The analysis was performed with a Varian atomic absorption spectrometer (Mulgrave) with deuterium background correction. Measurements were obtained with a 248.3 nm analytical line in the spectral interval of 0.2 nm. Iron concentration was determined by the standard addition method. Sample digestion was accomplished with the MDS 2000 microwave sample preparation system (CEM) in Teflon cartridges, using a mixture of nitric acid (5 mL) and hydrogen peroxide (2 mL) (both from Merck, ultrapure grade) for 20 min at a pressure of 120 psi. The resulting product was analyzed directly in the Teflon cartridges.

### Histology and immunohistochemistry

Part of liver lobe was excised, and the tissue was fixed in 10% neutral formalin solution and embedded in paraffin. Hematoxylin and eosin staining and Van Gieson's (VG) staining were performed according to standard procedures. The severity and degree of lesions were graded according to methods previously described<sup>[24,25]</sup>. Briefly, tissue sections (4  $\mu$ m thick) were treated with HCl (5%) to liberate ferric ions. The samples were then treated with 5% potassium ferrocyanide to produce insoluble ferric ferrocyanide. The slides were counterstained with Neutral red. For immunohistochemical examination, deparaffinized sections were incubated with HO-1 antibodies (1:1000 dilution) and appropriate biotinylated secondary antibodies, followed by the avidin-biotin-peroxidase complex. The immunoreactive signal was developed by color

deposition, using diaminobenzidine as a substrate. Yellow material in the cytoplasm was considered to indicate positive cells. Cell staining was assigned 4 scores, as previously described<sup>[26]</sup>. The final score was defined as staining intensity  $\times$  percentage of positive cells. The mean score of five fields was used to compare the six groups.

### Hepatic HYP content

Liver tissue (100 mg) was prepared for HYP determination, according to a modification of the method previously described<sup>[27]</sup>. The HYP content of the liver, as an indirect measurement of tissue collagen content, was expressed in microgram per gram of wet weight ( $\mu$ g/g).

### Measurement of plasma hepcidin

Plasma hepcidin was measured by ELISA and was determined using 96-well microtiter plates coated with the recombinant peptide and a polyclonal antibody (Santa Cruz Biotechnology, INC, 1:3000 dilution). Assay procedures were performed according to the manufacturer's instructions, and absorbance of each well was determined at a 450 nm wavelength. The process was performed as described previously<sup>[28]</sup>.

### Western blot analysis

The resected hepatic tissues were extracted with lysis buffer (1% Triton X-100; 50 mmol/L Tris-HCl, pH 7.6; 150 mmol/L NaCl; and 1% protease inhibitor cocktail). The protocols for western blot analyses have been described previously<sup>[29]</sup>. Western blot analyses were performed with liver homogenates (30  $\mu$ g protein) using anti-nuclear factor-E2-related factor 2 (Nrf2) antibody (Boster Biological Technology, Wuhan, China, 1: 100 dilution), anti-TGF- $\beta$ 1 antibody (Boster Biological Technology, Wuhan, China, 1:100 dilution), anti-HO-1 antibody (Abcam, Cambridge, MA, United States, 1:2000 dilution), anti- $\beta$ -actin antibody (Zhongshan Goldenbridge Biological Technology, Beijing, China, 1:500 dilution), and secondary antibody anti-rabbit and anti-mouse immunoglobulin G (Biosynthesis Biotechnology, Beijing, China, 1:500 dilution). The intensity of each signal was corrected using the values obtained from the immunodetection of  $\beta$ -actin, and the relative protein intensity was expressed as multiples of the content in the normal group.

### RNA isolation and gene expression analysis

Total RNA was extracted from the livers following a standard guanidinium phenol-chloroform extraction protocol. The quantity of RNA was determined by measuring the optical density at 260 nm ( $A_{260} = 1$  for 40  $\mu$ g/mL RNA), and the purity of RNA was assessed by determining the ratio of the optical density obtained at 260 and 280 nm (pure RNA:  $A_{260}/A_{280} = 2.0$ ) using a UV-1206 spectrophotometer (Shimadzu, Japan). An aliquot of each mixture was used for reverse-transcription (RT)-PCR amplification, using reagents purchased from Takara Bio Inc. (Dalian, China). PCR products were separated by 2.5% agarose gel electrophoresis. The product bands were

**Table 1** Primers used for the reverse transcription-polymerase chain reaction and polymerase chain reaction analysis

| Gene           | Gene ID  | Forward/reverse | Sequences 5'-3'      | Product size (bp) |
|----------------|----------|-----------------|----------------------|-------------------|
| HO-1           | NM012580 | Forward         | ATATCTATACGGCCCTGGAA | 350               |
|                |          | Reverse         | GATGCTCGGGAAGGTGAA   |                   |
| Nrf2           | AF304364 | Forward         | GACGGCAACACTGATTCCA  | 345               |
|                |          | Reverse         | CATCCGCCACTCATTCT    |                   |
| TGF- $\beta$ 1 | NM021578 | Forward         | CCGCAACAACGCAATCTA   | 437               |
|                |          | Reverse         | TGAGGAGCAGGAAGGGTC   |                   |
| Hepcidin       | NM053469 | Forward         | GCTGCCTGTCTCCTGCTT   | 159               |
|                |          | Reverse         | GGTGTCTCGCTTCCTTCG   |                   |
| TIMP-1         | NM053819 | Forward         | CTCTGGCCTCTGGCCTCCT  | 300               |
|                |          | Reverse         | ACTCCTCGCTGCGGTCT    |                   |
| MMP-2          | NM031054 | Forward         | CTGGGCAACAAGTATGAGA  | 430               |
|                |          | Reverse         | CTGTCCGCCAAATAAAC    |                   |
| $\beta$ -actin | NM031144 | Forward         | GAGGGAAATCGTGCCTGAC  | 445               |
|                |          | Reverse         | CTGGAAGGTGGACAGTGAG  |                   |

HO-1: Heme oxygenase-1; Nrf2: Nuclear factor-E2-related factor 2; TGF- $\beta$ 1: transforming growth factor- $\beta$ 1; TIMP-1: Tissue inhibitor of metalloproteinase-1; MMP-2: Matrix metalloproteinase-2.

photographed, and the density of each product band was quantified. The results are expressed as the ratios of the band density for target mRNA to that of  $\beta$ -actin mRNA. The primers utilized for PCR and RT-PCR are listed in Table 1.

### Statistical analysis

All of the data are presented as the mean  $\pm$  SD. Statistical testing was performed with SPSS software, version 16.0. The groups were compared using one-way analysis of variance with Dunnett's multiple comparison test (where applicable). Correlative comparison of two non-hierarchical variances with normal distribution was evaluated by Pearson's test, whereas Spearman's test was used for non-normally distributed data.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Measurement of biochemical indicators in liver fibrosis induced by BDL

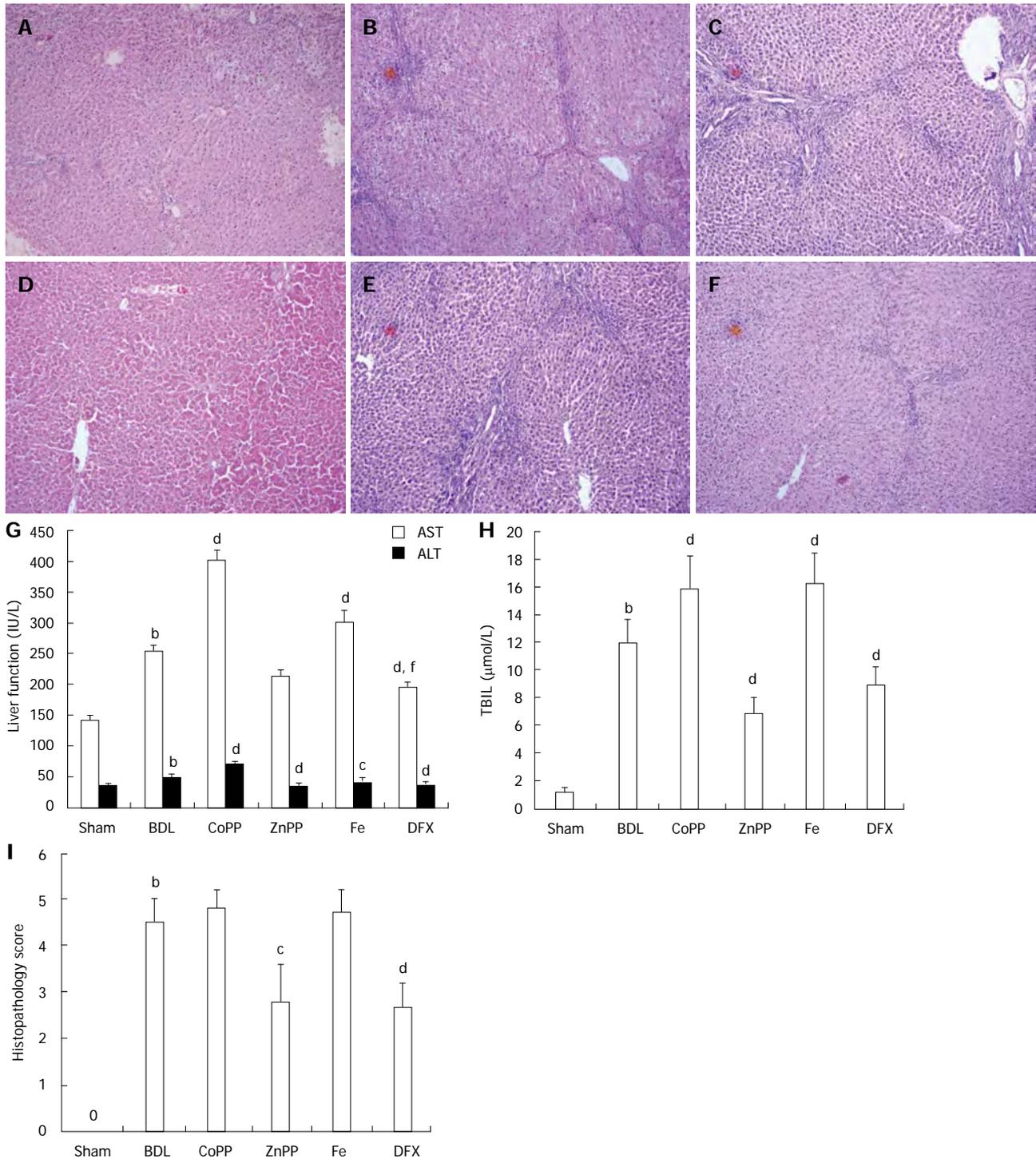
Four weeks postoperatively, common bile duct dilatation was observed in the BDL group, and ascites and jaundice also developed in the BDL group, suggesting that the BDL model was successfully established in our experiments.

The serum levels of AST, ALT and TBIL in the BDL group were much higher than those in the Sham group ( $P < 0.01$ ). These levels were much lower in the ZnPP group and DFX group, but the levels in the CoPP group and Fe group were significantly higher than those in the BDL group ( $P < 0.01$ ) (Figure 1G and H). The serum levels of AST were decreased in the DFX group compared with those in the ZnPP group ( $P < 0.05$ ) (Figure 1G). These data indicated that inhibiting HO-1 expression and further reducing iron accumulation could improve liver function; in contrast, inducing HO-1 expression aggravated liver injury.

### Inhibiting HO-1 expression reduced liver fibrosis and PVP induced by BDL

Liver damage was analyzed by hematoxylin and eosin staining. The livers in the Sham group showed normal lobular architecture with central veins and radiating hepatic cords (Figure 1A). Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation were found in the interlobular and central venous regions in the livers in the BDL and CoPP groups (Figure 1B and C). Compared with the BDL group, fibrous hyperplasia was significantly reduced around the central veins in the ZnPP group (Figure 1D). The histopathological scores for fibrosis in the livers of BDL rats were improved in the ZnPP group (Figure 1E). Collagen type I was observed with VG staining (Figure 2A-F). In the BDL group, collagen type I in the portal area and bile duct wall was much thicker than in the Sham group ( $P < 0.01$ ) (Figure 2A and B). Compared with the BDL group, there was a decrease in type I collagen in the ZnPP group. The extent of fibrosis was much higher in the CoPP group than in the BDL group ( $P < 0.01$ ) (Figure 2G). The change in HYP content in liver tissue was in accordance with type I collagen. It was observed that HYP was significantly decreased in the ZnPP group compared with the BDL group (Figure 2H). These data showed that inhibiting HO-1 expression reduced collagen deposition in liver fibrosis.

The COHb levels in arterial blood were significantly higher in the BDL group compared with the Sham group ( $P < 0.01$ ), and they were much lower following ZnPP and DFX treatment, while they were higher in the CoPP- and Fe-treated groups than in the BDL group ( $P < 0.01$ ) (Figure 3A). PVP was significantly higher in the BDL group compared with the Sham group ( $P < 0.01$ ). Compared with the BDL group, PVP decreased in the ZnPP and DFX groups ( $P < 0.01$ ) and was enhanced in CoPP and Fe rats ( $P < 0.01$ ). Moreover, PVP decreased following ZnPP treatment relative to DFX treatment ( $P < 0.05$ ) (Figure 3B).



**Figure 1** Pathological features of rat liver tissue detected by hematoxylin and eosin staining and serum index. A: Normal lobular architecture in the Sham group; B, C, E: Obvious fibrous hyperplasia and fibrosis extension with fibroblast proliferation in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D and F: Less fibrous hyperplasia and fibrosis in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; G, H: Levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (TBIL); I: Histopathological scores for fibrosis (magnification  $\times 100$ ). Values are expressed as mean  $\pm$  SE ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.01$  vs BDL group; <sup>f</sup> $P < 0.01$  vs ZnPP group.

Levels of TGF- $\beta 1$  were significantly enhanced in the BDL group compared with the Sham group ( $P < 0.01$ ). These levels were lower in the ZnPP group and higher in the CoPP group compared with the BDL group (Figure 4A and B). The mRNA expression levels of MMP-2 and TIMP-1 were much higher in the BDL group than in the

Sham group ( $P < 0.01$ ). These levels were significantly lower in the ZnPP group and higher in the CoPP group than in the BDL group ( $P < 0.01$ ) (Figure 4C). These results showed that down-regulated HO-1 expression reduced extracellular matrix (ECM) deposition and fibrosis.

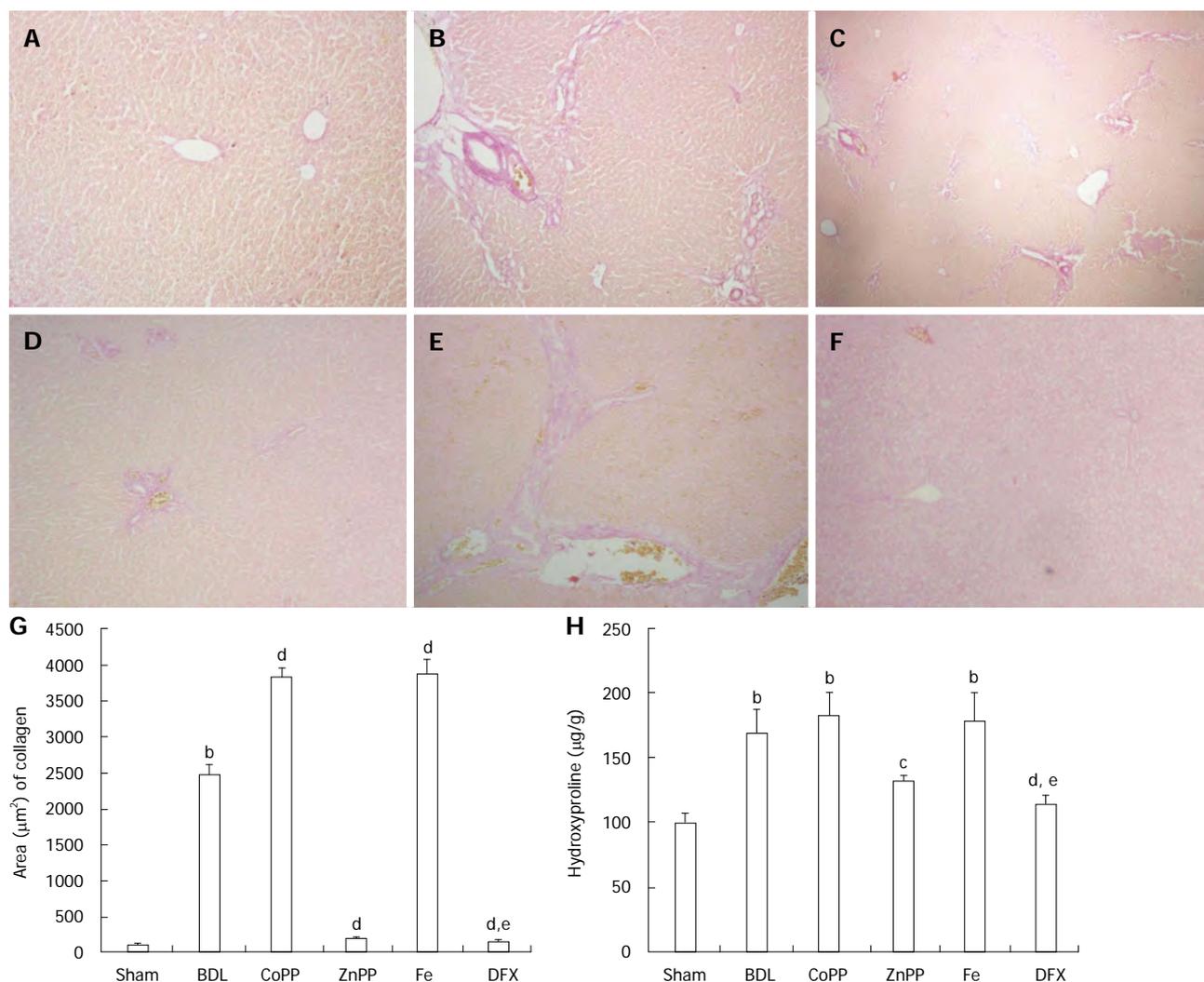


Figure 2 Van Gieson's staining of collagen I for liver sections and liver hydroxyproline content. A-F: Collagen type I was deposited in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group (B, C and E) and was rarely found in the Sham group, zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group (A, D and F); G: The area of collagen type I; H: Hydroxyproline content of liver tissue (magnification,  $\times 100$ ). Values are expressed as mean  $\pm$  SE ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs BDL group; <sup>e</sup> $P < 0.05$  vs ZnPP group.

### HO-1 mediated iron accumulation and oxidative stress in liver

The mRNA and protein expression levels of HO-1 were significantly higher in the BDL group than in the Sham group ( $P < 0.01$ ). These levels were obviously lower in the ZnPP group and higher in the CoPP group than in the BDL group ( $P < 0.01$ ) (Figure 4A and B). Hepatic immunostaining showed that HO-1 was mainly expressed in the liver cells and partly in the mesenchymal cells and Kupffer cells. Localization of HO-1 occurred mainly around the centrilobular veins (Figure 5A-F). The values of HO-1 expression were consistent with the above data (Figure 5G).

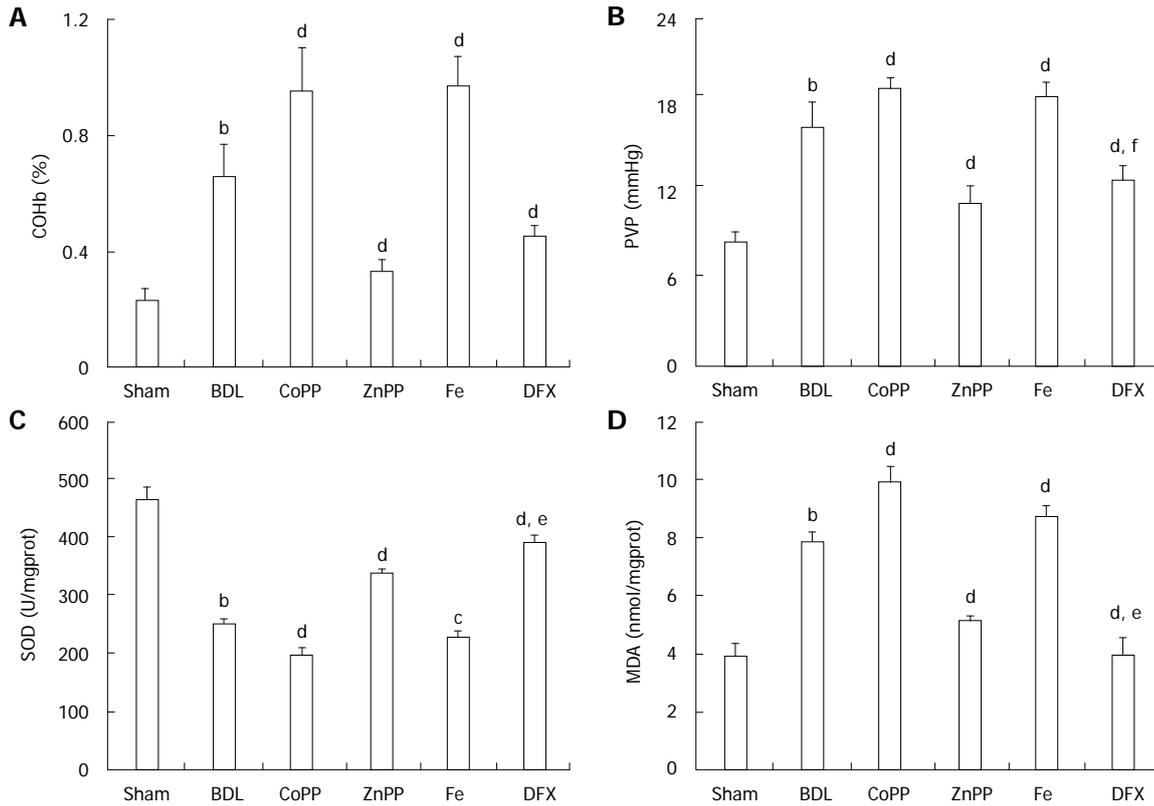
The serum levels of iron in the BDL group were significantly higher than in the Sham group ( $P < 0.01$ ). These levels were greatly lower in the ZnPP group than in the BDL group ( $P < 0.01$ ) (Figure 6G). The change in liver iron content was in accordance with serum iron levels (Figure 6H).

The mRNA and plasma levels of hepcidin were signi-

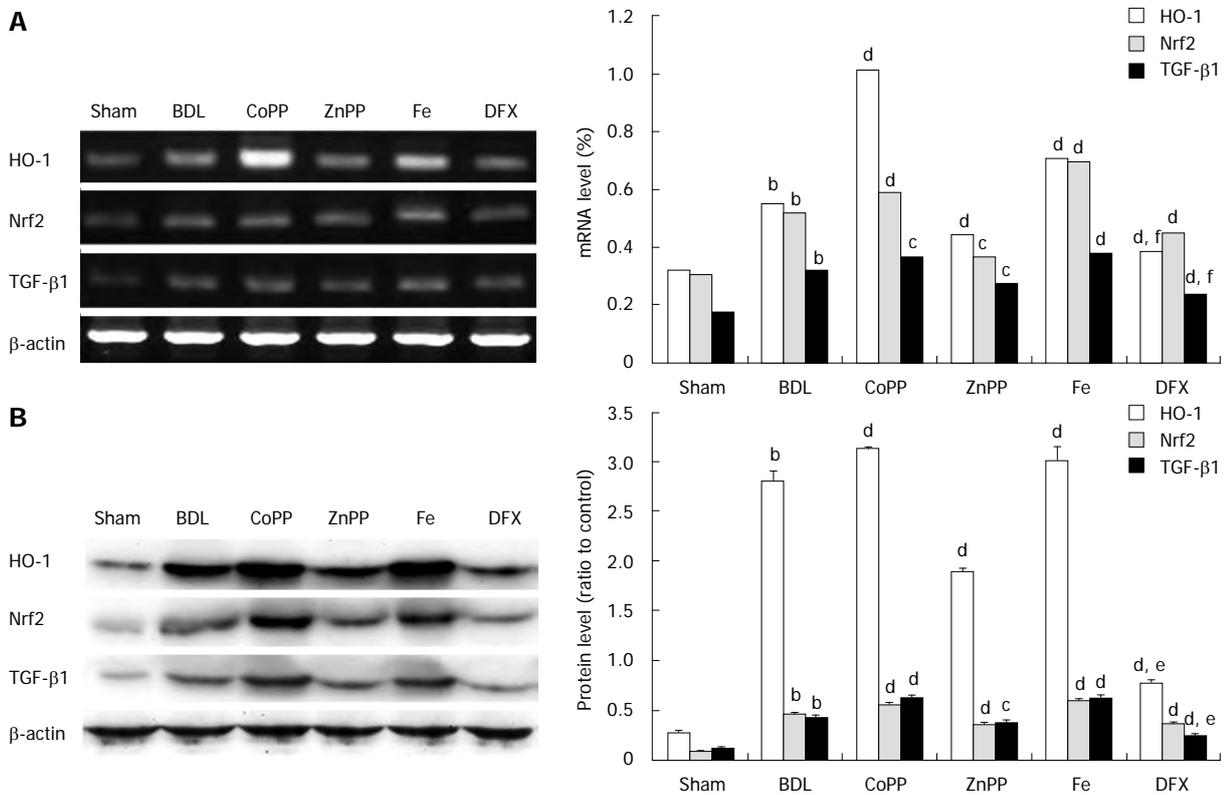
ficantly lower in the BDL group than in the Sham group ( $P < 0.01$ ). These levels were higher in the ZnPP group and lower in the CoPP group compared with the BDL group, and they were higher in the DFX group than in the ZnPP group (Figure 4C and 6I).

We used Prussian blue staining to localize iron accumulation in liver tissue and found that iron obviously accumulated in the BDL and CoPP groups. Iron was strongly stained mainly in Kupffer cells in these groups (Figure 6B and C). However, iron staining was rarely found in the Sham and ZnPP groups (Figure 6A and D). These results indicate that inhibiting HO-1 expression could reduce iron production, resulting in decreased iron accumulation in the liver. In contrast, enhanced HO-1 expression led to increased hepatic accumulation of iron.

Levels of SOD were obviously lower in the BDL group than in the Sham group ( $P < 0.01$ ), and they were significantly higher in the ZnPP group and lower in the CoPP group than in the BDL group ( $P < 0.01$ ) (Figure 3C). The MDA change tendency was opposite that of



**Figure 3** The levels of carboxyhemoglobin, portal vein pressure malonaldehyde and superoxide dismutase. A, B: The levels of carboxyhemoglobin (COHb) were accordance with heme oxygenase-1 expression, and portal vein pressure (PVP) levels were measured; C, D: The levels of superoxide dismutase (SOD) and malonaldehyde (MDA) was detected. Values are expressed as mean  $\pm$  SE ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.01$  vs BDL group; <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$  vs zinc protoporphyrin (ZnPP) group. BDL: Bile duct ligation; CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.



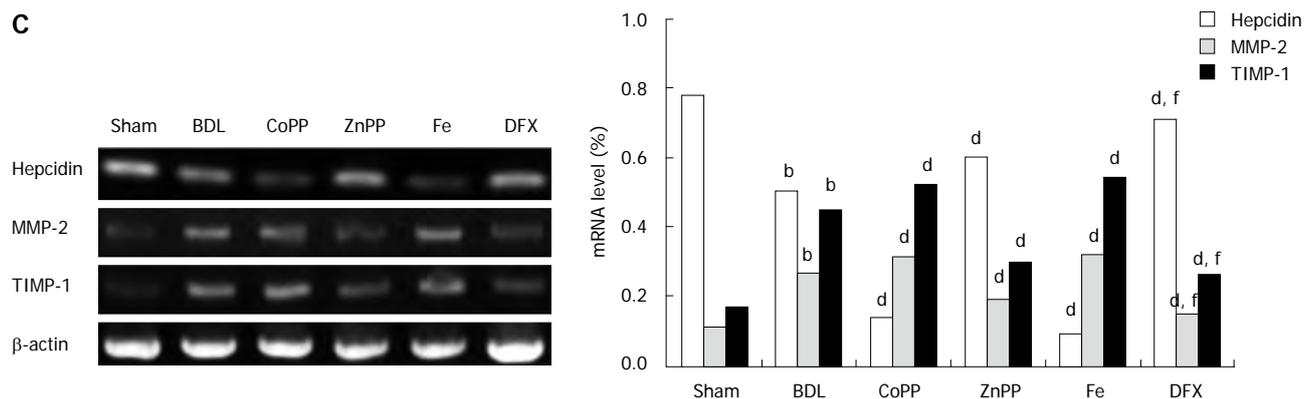


Figure 4 The heme oxygenase-1, transforming growth factor- $\beta$ 1, nuclear factor-E2-related factor 2, hepcidin, matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression were detected by Western blot and reverse transcription-polymerase chain reaction. A, B: The mRNA and protein levels of heme oxygenase-1 (HO-1), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) and nuclear factor-E2-related factor 2 (Nrf2); C: The mRNA levels of hepcidin, matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-1 (TIMP-1). Values are expressed as mean  $\pm$  SE. <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs bile duct ligation (BDL) group; <sup>e</sup> $P < 0.05$ , <sup>f</sup> $P < 0.01$  vs zinc protoporphyrin (ZnPP) group. CoPP: Cobalt protoporphyrin; DFX: Deferoxamine.

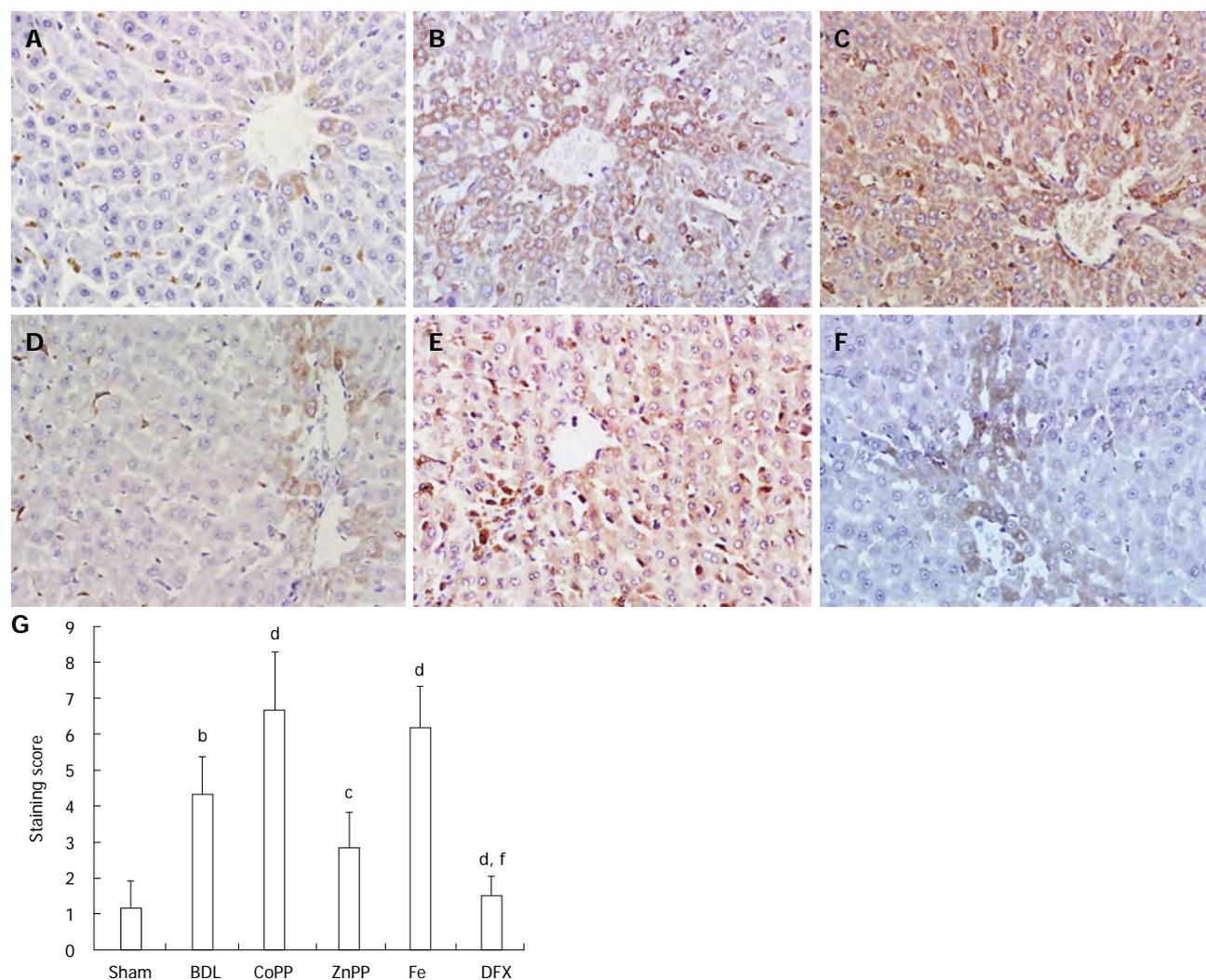
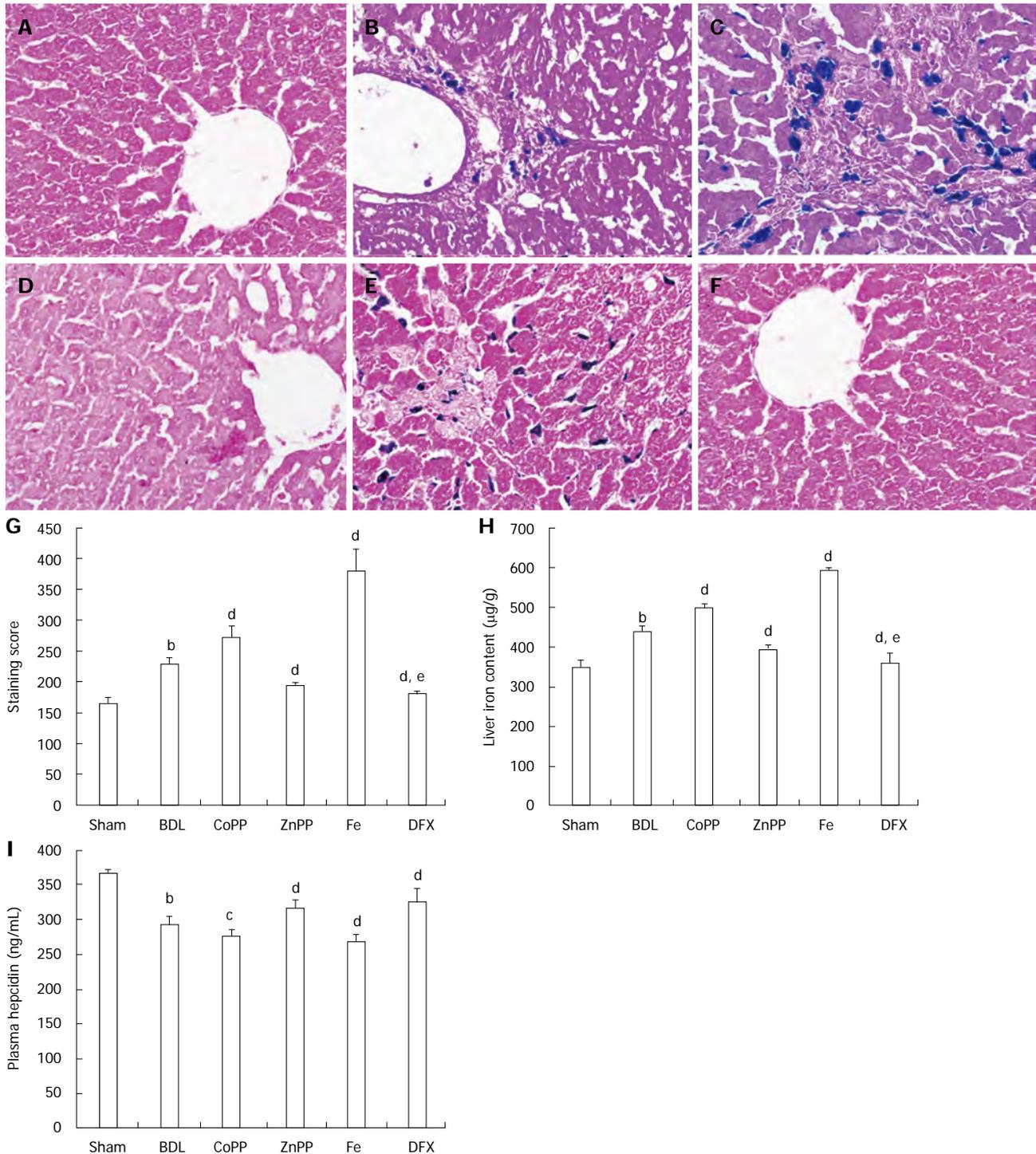


Figure 5 Liver sections were stained with heme oxygenase-1 antibody. A: Heme oxygenase-1 (HO-1) expression was less around the central veins in the Sham group; B, C, E: Much more HO-1 expression was found around the central veins in the bile duct ligation (BDL) group, cobalt protoporphyrin (CoPP) group and Fe group; D, F: Less staining was observed in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; H: Immunohistochemical staining scores (magnification  $\times 400$ ). Values are expressed as mean  $\pm$  SE ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs BDL group; <sup>f</sup> $P < 0.01$  vs ZnPP group.



**Figure 6** Perl's Prussian blue staining, levels of hepcidin, serum and liver iron. A: No iron accumulated in the Sham group; B: A small amount of iron mainly accumulated on Kupffer cells in the bile duct ligation (BDL) group; C: Much more iron accumulation was found in interlobular and macrophagocytes in the cobalt protoporphyrin (CoPP) group; D, F: Almost no iron accumulation was detected in the zinc protoporphyrin (ZnPP) group and deferoxamine (DFX) group; E: Massive iron accumulation was observed in the Fe group; G, H: There were no differences in the hepatic and serum iron content of these six groups; I: Plasma hepcidin also was measured by enzyme-linked immunosorbent assay (magnification  $\times 400$ ). Values are expressed as mean  $\pm$  SE ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs BDL group; <sup>e</sup> $P < 0.05$  vs ZnPP group.

SOD (Figure 3D). In the BDL model, inhibiting HO-1 expression reduced oxidative stress.

#### Iron induced oxidative stress and *Nrf2* expression

Both the serum iron and liver iron content were higher in the Fe group but lower in the DFX group compared

with the BDL group (Figure 6G and H). Prussian blue staining showed more iron accumulation in the Fe group and less in the DFX group (Figure 6E and F). The ZnPP group showed low iron levels (Figure 6D and F-H). The levels of plasma hepcidin were obviously lower in the Fe group but higher in the DFX group compared with the

BDL group (Figure 6I). The expression of HO-1 was significantly higher in the Fe group but was lower in the DFX group compared with the BDL group ( $P < 0.01$ ). It was also lower in the DFX group than in the ZnPP group (Figure 4A and B).

The mRNA and protein expression levels of Nrf2 were enhanced in the BDL group compared with the Sham group. Additionally, these levels were significantly higher in the CoPP and Fe groups than in the BDL group, and they were lower in the ZnPP and DFX groups ( $P < 0.01$ ) (Figure 4A and B). We found the levels of SOD in the Fe group to be slightly lower than in the BDL group ( $P < 0.05$ ); however, they were much higher than in the DFX group ( $P < 0.01$ ) (Figure 3C). The levels of MDA were significantly increased in the Fe group but were reduced in the DFX group compared with the BDL group ( $P < 0.01$ ) (Figure 3D). The levels of SOD were much higher, and MDA was lower in the DFX group compared with the ZnPP group ( $P < 0.01$ ) (Figure 6C and D). These data indicate that iron accumulation in the liver increased the oxidative stress reaction and caused further damage to the liver.

Hematoxylin and eosin staining showed more fibrous hyperplasia in the Fe group and less in the DFX group compared with the BDL group (Figure 1E and F). The content of HYP was significantly higher in the Fe group than in the Sham group ( $P < 0.01$ ), and it was lower in the DFX group than in the BDL group ( $P < 0.01$ ) (Figure 2H). Compared with the BDL group, collagen I was increased in the Fe group and decreased in the DFX group (Figure 2E-G). The mRNA and protein expression levels of  $\alpha$ -smooth muscle actin and TGF- $\beta$ 1 were significantly enhanced in the Fe group and decreased in the DFX group compared with the BDL group (Figure 4A and B). The levels of MMP-2 and TIMP-1 mRNA were much higher in the Fe group and were lower in the DFX group compared with the BDL group ( $P < 0.01$ ). Compared with the ZnPP group, TGF- $\beta$ 1 expression and ECM were lower in the DFX group (Figure 4C).

### Correlation between oxidative stress and liver fibrosis

Correlation analysis revealed that both SOD and MDA were significantly correlated with HYP levels ( $R = -0.912, 0.887$ , respectively,  $P < 0.01$ ). These data also showed that oxidative stress could result in ECM deposition in the liver and could further aggravate liver fibrosis.

## DISCUSSION

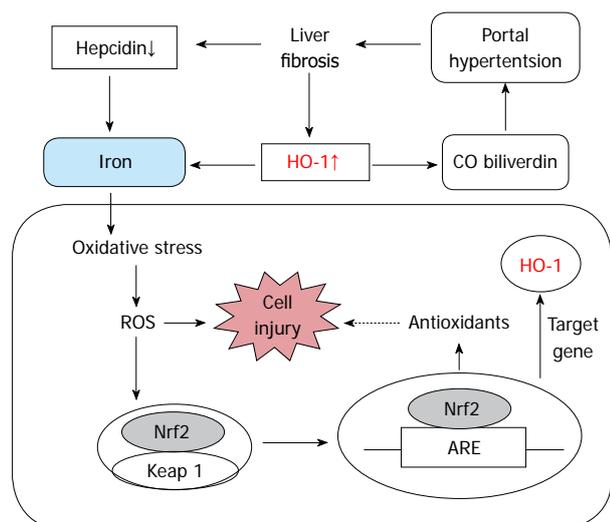
Many chronic liver diseases progress to hepatic fibrosis<sup>[30]</sup>. Iron overload in the liver increased the risk of developing fibrosis, as well as subsequent morbidity and mortality<sup>[31]</sup>. HO-1 catalyzes heme into iron, and it plays an important role in iron homeostasis. A previous study showed that HO-1 was associated with hepatocellular damage and had multiple mechanisms to influence liver fibrosis progression. In this study, we aimed to investigate how iron and CO, the product affected by HO-1 activity, affected hepatic fibrosis and PVP. We found that

lower HO-1 expression could reduce iron accumulation and PVP and improve fibrosis.

In several chronic liver diseases, HO-1 plays a protective effect in the liver against oxidative stress-dependent damage<sup>[32-34]</sup>. However, its protective effects in inflammation and fibrosis have been disputed. Some studies have shown that HO-1 over-expression increases liver injury in rats under conditions of experimental chronic cholestasis<sup>[19]</sup>. Low HO-1 induction was shown to be cytoprotective, and high levels of HO-1 could result in the accumulation of free divalent iron, thus increasing oxidative injury in fibroblast cell cultures<sup>[35]</sup>. We found that lower HO-1 expression could benefit end-stage liver cirrhosis by reducing iron accumulation, which is accordance with the findings of the above studies. Surprisingly, induction of HO-1 interfered with chronic inflammation and prevented progression of liver fibrosis in Mdr2-knockout mice, and it further might delay progression to hepatocellular carcinoma<sup>[33]</sup>. Our previous study indicated that induction of HO-1 could ameliorate immune liver fibrosis<sup>[36]</sup>. The reason why the above studies are different from this study could be that HO-1 plays a diverse role in different stages during the progression of liver fibrosis. In early stages of liver fibrosis, inducing HO-1 could have a protective effect, but it could increase liver injury in end stages *via* liver hypertension. Moreover, the different animal models for inducing fibrosis could constitute another explanation of these results.

The majority of endogenous CO is catalyzed by inducible expression of HO-1. CO can modulate blood flow and maintain the integrity of the vessel wall<sup>[37]</sup>. COHb levels can be used to estimate HO activity in experimental animals. Interestingly, we observed that up-regulated COHb resulted from increased HO-1, which aggravated PVP in BDL rats. Moreover, lower levels of COHb can decrease the PVP found in the ZnPP and DFX treatment groups. HO/CO plays a role in the pathophysiology of portal hypertension, and CO can regulate the intrahepatic vascular resistance of cirrhotic rats<sup>[38]</sup>. Tarquini *et al*<sup>[39]</sup> indicated that the HO/CO system is activated in patients with liver cirrhosis, and CO contributes to the hyperdynamic circulatory syndrome. CO might improve intrahepatic microcirculation in early stage hepatic fibrosis, and excessive CO could be harmful, leading to an unbalanced nitric oxide/CO system in end-stage hepatic fibrosis. It therefore seems best to reduce PVP by decreasing CO.

Normally, HO-1 is only slightly expressed in hepatocytes and Kupffer cells. In hepatic cirrhosis, the expression of HO-1 is increased. Khan *et al*<sup>[20]</sup> reported that an increase in HO-1 expression is associated with iron accumulation. The study of Kartikasari *et al*<sup>[40]</sup> showed that iron is derived from intracellular heme degradation, and HO-1 activity contributes to increased levels of intracellular labile iron. Other research has shown that non-heme iron increases are associated with the induction of HO-1 in neurons, microglia and capillary endothelial cells, whereas HO-2 levels remain unchanged, implying that the non-heme iron increases might be the result



**Figure 7** Iron is involved in the heme oxygenase-1 cycle *via* the nuclear factor-E2-related factor 2/Keap1 pathway, and heme oxygenase-1 regulates portal pressure in liver cirrhosis. Low hepcidin and heme oxygenase-1 (HO-1) over-expression could mediate iron accumulation, which accelerates oxidative stress, leading to cell injury, and it also increases HO-1 expression through the nuclear factor-E2-related factor 2 (Nrf2)/Keap1 pathway. Nrf2 protects cells against oxidative stress, but this effect is limited, and Nrf2 contributes to induction of HO-1 expression, which can produce iron. HO-1 can increase carbon monoxide (CO) production, and an unbalanced CO/nitric oxide system could play a role in portal pressure. ROS: Reactive oxygen species. ARE: Antioxidant response element.

of HO-1-mediated heme degradation<sup>[41]</sup>. These results showed that HO-1 played a central role in maintaining iron homeostasis *in vivo*. In this study, we found that serum iron and liver iron contents all increased in the CoPP group, and inhibiting HO-1 activity with ZnPP reduced iron accumulation in the liver and further attenuated liver fibrosis in liver fibrosis induced by BDL.

Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum. It was also noted that hepcidin was abnormally low in alcoholic patients with associated iron overload<sup>[42]</sup>. Iron was accumulated in the liver and pancreas of hepcidin-deficient mice<sup>[43]</sup>. It also was found that serum pro-hepcidin concentrations were lowered in liver cirrhosis, which could be the result of impaired liver functioning<sup>[44]</sup>. Hepcidin is down-regulated during progressive cholestasis in biliary atresia<sup>[45-47]</sup>. Furthermore, Huang *et al.*<sup>[48]</sup> showed that iron loading down-regulates hepcidin by inhibiting both inflammatory and iron-sensing pathways and inhibiting transducers and activators of transcription 3 and SMAD4 signaling *in vivo*. These findings are consistent with the results of our experiment. Under physiological conditions, hepcidin expression is stimulated by iron overload and inflammation and is suppressed by anemia and tissue hypoxia<sup>[49,50]</sup>. However, levels of hepcidin were decreased in the iron accumulation group and were increased in the ZnPP and DFX groups in our study. The reason for this finding might have been the various signals affecting hepcidin production. Up-regulation of hepcidin by inhibiting HO-1 expression could be benefi-

cial for cholestasis in cirrhosis.

It is now commonly accepted that HO-1 plays an important role in the control of inflammation and oxidative stress<sup>[51]</sup>. HO-1 protected primary human hepatocytes from ethanol-derived oxidative stress *via* the MAPK/Nrf2 pathway<sup>[52]</sup>. Surprisingly, however, in this study, we found that inducing HO-1 expression increased MDA and decreased SOD. Further, these results indicate that HO-1 could not reduce the oxidative stress reaction. Other studies have shown the pro-oxidant nature of the released cellular low-molecular-mass iron and the antioxidant effect of the released bilirubin<sup>[53]</sup>. In this study, we demonstrated that the pro-oxidant activities of iron accumulation were much stronger than the antioxidant effects of bilirubin.

Iron primarily accumulates in the reticuloendothelial cells. Previously, it was shown that increased deposition of iron in the liver often triggered oxidative stress and inflammation and induced liver cell damage<sup>[54]</sup>. It can participate in Fenton and Haber-Weiss chemistry, and excessive redox-active iron might lead to oxidative stress, with damage to membranes, proteins and DNA<sup>[55]</sup>. It was also shown that increased deposition of iron in the liver induced liver cell damage and cirrhosis by triggering oxidative stress and inflammation<sup>[56]</sup>. In fact, signs of iron-catalyzed lipid peroxidation and oxidative stress have been found by many investigators during chronic iron overload in rodents<sup>[57,58]</sup>. In this study, iron intoxication dramatically enhanced MDA adducts, decreased antioxidant enzyme SOD and aggravated liver injury in the BDL, CoPP and Fe groups. Our results revealed that iron accumulation exacerbated the oxidative stress reaction, leading to the aggravation of liver cirrhosis.

Previous reports have shown that elevated hepatic iron can activate Nrf2 in 3,5,5-trimethyl-hexanoyl-ferrocene-treated mouse models<sup>[59]</sup>. In our study, Nrf2 was up-regulated in the BDL, CoPP and Fe groups, in which iron accumulation in the liver was found. Nuclear translocation of activated Nrf2 is an important upstream contributor to the induction of HO-1 expression<sup>[60]</sup>. Up-regulation of Nrf2 increased HO-1 gene transcription in the CoPP and Fe groups. The pathway of iron-dependent HO-1 induction involves Nrf2/Keap. Nrf2 also plays a key role in the protection of cells against oxidative stress<sup>[61]</sup>. However, in our study, Nrf2's protective effect was limited, and HO-1, which is its target gene, increased iron production, resulted in oxidative stress.

Previous studies have demonstrated that oxidative stress significantly contributes to hepatic fibrogenesis from various liver injuries<sup>[62]</sup>. Reactive oxygen species (ROS) can stimulate the production of type I collagen and could act as intracellular signaling mediators of TGF- $\beta$ <sup>[63,64]</sup>. Our study showed that preventing oxidative stress by inhibiting HO-1 expression could attenuate liver fibrosis through regulation of the TGF- $\beta$ 1 pathway and reducing collagen deposition.

In conclusion, HO-1 played a pivotal role in iron accumulation and portal pressure in the livers in our study

(Figure 7). In the clinic, many end-stage cirrhosis patients with upper gastrointestinal bleeding treated with multiple massive transfusions run the risk of iron overload and further liver injury. Today, iron chelation therapy is often utilized to remove excess stored iron in some diseases<sup>[65,66]</sup>. In our study, iron accumulation induced hepatic fibrogenesis, indicating that cirrhotic patients with massive stored RBC transfusions would benefit from iron removal therapy. Our research provided a new way to reduce liver iron and portal pressure by inhibiting HO-1 expression. However, we must find a proper model for the simulation of upper gastrointestinal bleeding and transfusion. In clinical experiments, we will attempt to include cirrhotic patients with bleeding complications, who would eventually receive transfusions, to investigate the effects of iron transfusions on liver cirrhosis.

Removing iron and reducing portal pressure by inhibition of HO-1 improves liver fibrosis in bile duct-ligated rats. In addition, iron is also closely related to another complication of cirrhosis: hepatocellular carcinoma<sup>[67]</sup>. Regulation of iron homeostasis, by interfering with HO-1, could effectively treat hepatic cirrhosis and also prevent hepatocellular carcinoma.

## ACKNOWLEDGMENTS

The authors thank Li Lv for providing the clinical and experimental equipment. The authors also thank Qin Zhou for technical support.

## COMMENTS

### Background

Iron overload in the liver is a very common phenomenon in many chronic liver diseases. Heme oxygenase-1 (HO-1) and its by-products, iron and carbon monoxide (CO), play crucial roles in hepatic fibrosis. The underlying molecular mechanisms of HO-1, regarding iron deposition and portal vein pressure (PVP) in hepatic fibrosis, remain unknown.

### Research frontiers

HO-1 and degradation products are important to cytoprotection in many types of liver injury, but protection can be restricted to a narrow threshold. Iron overload often triggers oxidative stress and inflammation and induces liver cell damage, and the CO/nitric oxide system could be harmful to portal pressure. Iron can activate nuclear factor-E2-related factor 2 (Nrf2) and increase HO-1 expression. Inhibiting HO-1 activity is necessary for reducing iron and PVP.

### Innovations and breakthroughs

In this study, by inhibiting HO-1 expression by zinc protoporphyrin in rat liver fibrosis induced by bile duct ligation (BDL), the author aimed to affect the HO-1/CO system by iron deposition and portal pressure. Reducing hepatic iron deposition and CO levels by inhibiting HO-1 activity through the Nrf2/Keap pathway could be helpful in improving hepatic fibrosis and maintaining PVP.

### Applications

Removing iron and reducing CO by inhibiting HO-1 activity provides a new strategy for treating liver fibrosis, and further, it could help prevent liver carcinoma.

### Terminology

HO-1 is a primary rate-limiting enzyme in heme catabolism. It catalyzes the oxidative degradation of heme to free iron, CO, and biliverdin. Nrf2 is an important upstream contributor to the induction of HO-1 expression, and it has a protective effect on cells against oxidative stress. Hepcidin is expressed mainly in the liver, and it functions as a negative regulator of iron absorption from the duodenum.

## Peer review

This study analyzed the role of HO-1 inhibition in rat liver fibrosis using an experimental model of BDL. The results are interesting, and they suggest that regulation of iron homeostasis and CO production could effectively treat liver cirrhosis and PVP and even prevent hepatocellular carcinoma.

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**P- Reviewers** Bustos M, Boscá L, Montecucco F, Okoshi S  
**S- Editor** Huang XZ **L- Editor** A **E- Editor** Xiong L



## Long-term follow-up study of gastroduodenal lesions after radioembolization of hepatic tumors

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Received: November 6, 2012 Revised: January 18, 2013

Accepted: February 2, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To evaluate the long-term natural history of the gastroduodenal lesions secondary to extrahepatic embolization with Yttrium 90 ( $^{90}\text{Y}$ ) spheres.

**METHODS:** From September 2003 to January 2012, 379 procedures of liver radioembolization (RE) using resin microspheres loaded with  $^{90}\text{Y}$  were performed in

our center. We have retrospectively compiled the data from 379 RE procedures performed in our center. We report a comprehensive clinical, analytical, endoscopic and histologic long-term follow-up of a series of patients who developed gastroduodenal lesions after the treatment.

**RESULTS:** Six patients (1.5%) developed gastrointestinal symptoms and had gastrointestinal lesions as shown by upper endoscopy in the next 12 wk after RE. The mean time between RE and the appearance of symptoms was 5 wk. Only one patient required endoscopic and surgical treatment. The incidence of gastrointestinal ulcerations was 3.75% (3/80) when only planar images were used for the pre-treatment evaluation. It was reduced to 1% (3/299) when single-photon emission computed tomography (SPECT) images were also performed. The symptoms that lasted for a longer time were nausea and vomiting, until 25 mo after the treatment.

**CONCLUSION:** All patients were free from severe symptoms at the end of follow-up. The routine use of SPECT has decreased the incidence of gastrointestinal lesions due to unintended deployment of  $^{90}\text{Y}$  particles.

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**Key words:** Radioembolization; Liver neoplasms; Gastroduodenal ulcer; Single-photon emission computed tomography; Liver

Rodríguez-Lago I, Carretero C, Herráiz M, Subtil JC, Betés M, Rodríguez-Fraile M, Sola JJ, Bilbao JI, Muñoz-Navas M, Sangro B. Long-term follow-up study of gastroduodenal lesions after radioembolization of hepatic tumors. *World J Gastroenterol* 2013; 19(19): 2935-2940 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2935.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2935>

## INTRODUCTION

Radioembolization (RE) with microspheres embedded with Yttrium 90 ( $^{90}\text{Y}$ ) is a therapeutic option for primary and secondary liver tumors. Prior to treatment, an angiography is performed to identify the hepatic vasculature and to mimic the actual procedure by injecting  $^{99}\text{Tc}$ -radiolabeled macroaggregated albumin ( $^{99}\text{Tcm}$ -MAA)<sup>[1]</sup>. During this procedure, guidelines recommend the embolization of the gastroduodenal artery, right gastric, and other extrahepatic arteries to isolate the hepatic circulation<sup>[2]</sup>. Unintended extrahepatic deployment of particles is a rare but well known complication of this procedure, that may result in cholecystitis<sup>[3]</sup>, pancreatitis<sup>[4]</sup>, radiation pneumonitis<sup>[5]</sup> and gastrointestinal ulcerations<sup>[6,7]</sup>. Radiation injury is likely to be the main pathogenical factor of these lesions<sup>[6]</sup>. However, although the small diameter of the particles (20–30  $\mu\text{m}$ ) does not induce a significant macroembolic effect, it has been shown in animal models that microspheres may sometimes aggregate and occlude small size vessels<sup>[8]</sup>.

Radiation-induced gastrointestinal ulcerations may have a significant negative impact in quality of life due to decreased oral intake, pain or anemia, and the even need to perform gastric surgery has been described<sup>[9]</sup>. However, most case reports or series only describe the appearance and immediate consequences and very little is known about the long-term outcome of this complication. We describe for the first time a case series of six patients with gastrointestinal lesions secondary to RE and their clinical, endoscopic and histologic long-term follow-up.

## MATERIALS AND METHODS

From September 2003 to January 2012, 379 procedures of liver RE using resin microspheres loaded with  $^{90}\text{Y}$  (SIR-Spheres, Sirtex Medical, Sidney, Australia) were performed in our center. Six patients (1.5%) developed gastrointestinal symptoms and had an upper endoscopy performed in the next 12 wk after the procedure that showed gastrointestinal lesions. Findings in the initial and the subsequent gastroduodenoscopies and their histopathologic studies were compiled retrospectively. Although the follow-up was not structured, at least two endoscopies were available from all but one patient. The maximum endoscopic follow-up time was 78 mo. We have gathered from the medical reports and thoroughly analyzed all clinical, analytical, endoscopic and histopathological findings.

## RESULTS

### *Patient characteristics*

The characteristics of the six patients included in the study are shown in Table 1. Mean age was 53.1 years and a range of primary tumors were treated including cholangiocarcinoma and liver metastases from different primary tumors. None of the patients had any significant gastrointestinal disease before RE and only two patients were

receiving concomitant anticancer treatments, namely the combination of 5-fluorouracil, leucovorin and oxaliplatin (FOLFOX, patient 1), and the combination of gemcitabine and oxaliplatin (GEMOX, patient 4).

### *RE procedure*

From the day of RE and for the following 2 mo, all patients received pantoprazole (40 mg daily) and a tapered regimen of corticosteroids.

A selective embolization of any extrahepatic vessel that could be included in the treated volume was performed prior to microsphere injection. Regarding variant anatomies, patient 2 presented a complete occlusion of the celiac axis and reversed blood flow of the pancreatoduodenal artery and in patient 4 the right hepatic artery originated from the superior mesenteric artery. The mean activity of  $^{90}\text{Y}$  administered was 1.02 GBq. In 4 patients (66%) the spheres were infused in the common or proper hepatic artery. In one case a bilobar treatment was performed by injecting the microspheres into the left and right branches of the hepatic artery (patient 4). In this patient only half the prescribed dose could be injected into the left hepatic artery due to spasm, while the entire prescribed dose was injected into the right hepatic artery uneventfully. This was the only case in which premature occlusion of the vessel occurred. In the remaining case the site of injection was the left hepatic artery (lobar infusion, patient 2).

$^{99}\text{Tcm}$ -MAA scans obtained prior to the procedures consisted in planar scintigraphic images in 3 patients and planar plus single-photon emission computed tomography (SPECT) images in the 3 other patients. When considered relative to the number of RE procedures, the incidence of gastrointestinal ulcerations was 3.75% (3 out of 80 patients) in the early period when planar images were used, and only 1% (3 out of 299 patients) in the late period when SPECT was also performed. We have retrospectively reviewed the  $^{99}\text{Tcm}$ -MAA scans of these 6 patients and no activity was observed in the gastrointestinal area prior to RE.

### *Endoscopic and histological findings*

Patients had their first upper endoscopy performed 1 to 12 wk after RE (Table 2) but none of them required an urgent procedure. At diagnosis, all of them showed ulcers and erosions in the stomach and/or duodenum (Figure 1). Gastrointestinal lesions were found only in the stomach in 3 cases, while the duodenum and the pyloric channel were also affected in 1 and 2 patients, respectively. Mucositis (gastritis or duodenitis) was present in 5 patients (83%) and a friable mucosa was described in 2 patients (33%). The mean endoscopic follow-up was 29.6 mo and the end of follow-up was usually due to clinical improvement (5 patients, 83%). One patient (case 6) died 2.5 mo after the procedure due to tumor progression. No patient showed neoangiogenesis, scars or antral deformities at diagnosis but these findings were lately seen in 4 patients (67%) after a mean period of 7.5 mo.

Table 1 Patient and treatment characteristics

| Patient | Age (yr) | Primary tumor      | Concomitant treatment | Gastroduodenal artery embolization | Site of injection             | Gastrointestinal activity on <sup>99m</sup> Tc-MAA scan | Dose administrated | Pain during infusion |
|---------|----------|--------------------|-----------------------|------------------------------------|-------------------------------|---|--------------------|----------------------|
| 1       | 60       | Colorectal         | FOLFOX                | Yes                                | Common hepatic artery         | No  | 75%                | Yes                  |
| 2       | 39       | Pancreatic NET     | No                    | No                                 | Left hepatic artery           | No  | 100%               | No                   |
| 3       | 53       | Ileal carcinoid    | No                    | Yes                                | Proper hepatic artery         | No  | 100%               | Yes                  |
| 4       | 65       | Cholangiocarcinoma | GEMOX                 | No                                 | Right and left hepatic artery | No  | 75%                | Yes                  |
| 5       | 54       | Renal              | No                    | Yes                                | Proper hepatic artery         | No  | 100%               | Yes                  |
| 6       | 48       | Colorectal         | No                    | Yes                                | Proper hepatic artery         | No  | 60%                | Yes                  |

<sup>99m</sup>Tc-MAA scan: <sup>99m</sup>Tc-radiolabeled macroaggregated albumin; FOLFOX: Folinic acid, fluorouracil and oxaliplatin; GEMOX: Gemcitabine and oxaliplatin; NET: Neuroendocrine tumour.

Table 2 Endoscopic and histologic findings

| Patient | Time from RE to first endoscopy (wk) | Findings in first endoscopy (CTCAE 4.02 grading scale)   | Histology   | Endoscopic follow-up time (mo) | Total number of endoscopies | Endoscopic treatment                                      |
|---------|--------------------------------------|--|---|--------------------------------|-----------------------------|---|
| 1       | 6                                    | Ulcers in cisura angularis and gastric antrum (20 mm);   | Microspheres in lamina propria;   | No                             | 1                           | No  |
| 2       | 5                                    | Multiple erosions in duodenal bulb (2) Severe mucositis in gastric fundus, body and antrum, with mucosal friability and superficial ulcers (2) | No <i>H. pylori</i> bacilli<br>Microspheres;<br>No <i>H. pylori</i> bacilli | 15                             | 3                           | No  |
| 3       | 8                                    | Severe gastritis (cisura angularis, antrum and pylorus) and duodenitis with ulcers (3)   | Microspheres;<br>No <i>H. pylori</i> bacilli                                | 53                             | 8                           | Argon plasma coagulation (after 13 mo of RE) <sup>1</sup> |
| 4       | 7                                    | Mucositis in gastric body; Extense ulcer in antrum (2)   | Microspheres;<br>No <i>H. pylori</i> bacilli                                | 1                              | 2                           | No  |
| 5       | 12                                   | Deep ulcer in pyloric channel and severe mucositis in gastric antrum; Superficial ulcer in duodenal bulb (2)                                   | Microspheres in lamina propria;<br>No <i>H. pylori</i> bacilli              | 78                             | 11                          | No  |
| 6       | 4                                    | Severe mucositis in gastric antrum, with mucosal friability; Ulcer in pyloric canal (2)  | Microspheres in lamina propria;<br>No <i>H. pylori</i> bacilli              | 1                              | 2                           | No  |

<sup>1</sup>A gastroenteroanastomosis was later performed (25 mo after the diagnosis of gastrointestinal ulcers). RE: Radioembolization; CTCAE: Common Terminology Criteria for Adverse Events.

Microspheres were detected in all the biopsy specimens, mainly located in the vessels of the *lamina propria*. Granulation tissue was frequently present next to the ulcers, along with a variable grade of lymphoplasmocytic and eosinophilic inflammatory infiltrate. In some samples, focal atypia (patient 1) or anaplasia (patient 3) were present. None of the samples from the gastric mucosa showed bacilli suggestive of *Helicobacter pylori* (*H. pylori*) infection and none of the patients had a previous documented *H. pylori* infection.

### Treatment

Only one patient (patient 3) required endoscopic treatment because of late anemia and duodenal stenosis. Thirteen mo after RE, argon plasma coagulation was successfully used to treat the antral and duodenal mucosa. He required this endoscopic treatment because of significant and progressive anemia (hemoglobin dropped from 13.7 g/dL at baseline to 8 g/dL prior to endoscopic treatment and the patient had already received transfusion of 6 units of packed red blood cells) and weight loss. Anemia resolved but his nutritional status worsened over the next mo due to a duodenal stenosis. Finally a gastroentero-

anastomosis to the first jejunal loop was performed 25 mo after the diagnosis of gastrointestinal ulcers with antropyloric deformity.

All patients referred abdominal pain as the initial symptom, mainly located in the epigastric region (Table 3). Other symptoms were nausea and vomiting (5 patients, 83%) and anorexia (3 patients, 50%). The mean time between RE and the appearance of symptoms was 5 wk (range 2 to 12 wk). Patients were treated with multiple combinations of proton pump inhibitors, Sucralfate, Almagate, Domperidone, Misoprostole, Pentoxifylline, Cinitapride and Metoclopramide. A slow but progressive improvement was seen in all but one of the patients described above (83%). The majority of these symptoms were mild and graded 1 or 2 in the Common Terminology Criteria for Adverse Events grading scale. Abdominal pain had a maximum grade of 1 or 2, which means a mild pain that only in some patients limited activities of daily living. Nausea and vomiting was graded with a maximum of 1 in all but one patient. This correlates with one or two episodes of emesis a day in the worst period with symptoms during the follow-up.

Table 3 Clinical follow-up and treatment

| Patient | Time from RE to symptoms (wk) | Clinical follow-up time (mo) | Symptoms (CTCAE v4.02 grading scale) |                     |          | Treatment  |                        |                   | Weight loss <sup>1</sup> (kg) | Hemoglobin <sup>2</sup> (g/dL) | Reason for end of endoscopic follow up |
|---------|-------------------------------|------------------------------|--------------------------------------|---------------------|----------|--|------------------------|-------------------|-------------------------------|--------------------------------|--|
|         |                               |                              | Pain                                 | Nausea and vomiting | Anorexia | Treatment used   | Time on treatment (mo) | Clinical response |                               |                                |  |
| 1       | 4                             | 6                            | Epigastric pain (1)                  | No                  | No       | Pantoprazole, domperidone and almagate                   | 4                      | Yes               | 6                             | -1.5                           | Improvement                            |
| 2       | 4                             | 29                           | Epigastric pain (1)                  | Yes (1)             | No       | Esomeprazole, cinitapride sucralfate and ranitidine      | 8                      | Yes               | 7                             | -0.6                           | Improvement                            |
| 3       | 4                             | 88                           | Epigastric pain (2)                  | Yes (1)             | Yes (3)  | Pantoprazole, metoclopramide, sucralfate and cinitapride | 10                     | Yes               | 17                            | -6.9                           | Improvement                            |
| 4       | 2                             | 9                            | Left subcostal pain (2)              | Yes (1)             | No       | Pantoprazole, sucralfate and almagate                    | 5                      | Yes               | 4                             | -3.1                           | Improvement                            |
| 5       | 12                            | 88                           | Epigastric pain (1)                  | Yes (1)             | Yes (2)  | Pantoprazole, pentoxifylline, esomeprazole and almagate  | 5                      | Yes               | 4                             | -1.6                           | Improvement; still on follow-up        |
| 6       | 4                             | 3                            | Epigastric pain (2)                  | Yes (2)             | Yes (2)  | Esomeprazole, pentoxifylline and misoprostole            | 1                      | No                | 4.1                           | -2.4                           | Exitus                                 |

<sup>1</sup>Maximal loss of weight over 4 mo; <sup>2</sup>Maximal change in hemoglobin over 4 mo. CTCAE: Common Terminology Criteria for Adverse Events; RE: Radioembolization.

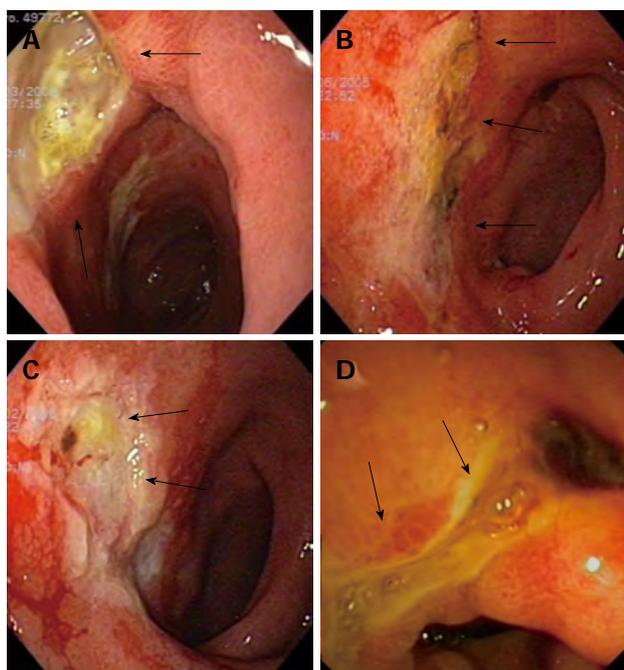


Figure 1 Figures of patient 5. A: A big and deep ulcer was seen in the pyloric channel and duodenal bulb at diagnosis (arrows); B: The same ulcer as in (A) in the same location seen 3 mo later, partially healed (arrows); C: Ulcer located in the duodenal bulb (arrows) with an irregular and friable mucosa after 11 mo; D: Endoscopic view of antral, pyloric and duodenal bulb deformity (arrows) seen with endoscopic ultrasound scope 78 mo after diagnosis.

## DISCUSSION

RE is an accepted therapeutic technique for advanced

primary and secondary liver tumors. It seems to be better tolerated than transarterial chemoembolization in terms of abdominal pain<sup>[10]</sup>, length of hospital stay<sup>[11]</sup> and post-embolization symptoms<sup>[12]</sup>. Main complications do not result from the microembolic effect of the spheres, even in patients with portal vein occlusion, but rather from an excessive irradiation of non-target tissues, including the liver<sup>[13]</sup>.

We describe in this case series an incidence of symptomatic gastroduodenal lesions after RE of 1.5% (6 out of 379 patients). Other authors have reported an incidence between 0% and 28%, with an average 4.8% calculated from the data previously published in 23 studies<sup>[7]</sup>. The two largest series of patients with secondary liver tumors from colorectal cancer or other malignancies reported rates of grade 3 gastrointestinal ulceration of 1% to 2%<sup>[14,15]</sup>. A higher incidence of 3.7% was found among 325 patients with hepatocellular carcinoma treated in 8 different European institutions<sup>[16]</sup>.

A decrease in the incidence of gastroduodenal lesions was observed when SPECT images were used for evaluation the RE procedures compared with planar images. These data suggest that SPECT imaging is an important tool to minimize the risk of gastrointestinal adverse events secondary to RE treatment. Our results arise from a retrospective single center study. If they are further confirmed by other series, a possible change in the current guidelines could be considered in favor of the use of SPECT imaging.

In most patients (4 out of 6 patients, 66%) the spheres were injected from the common or proper hepatic artery.

This site of injection leads to a greater chance of non-target embolization of the spheres in extrahepatic vessels. However, we have retrospectively reviewed all the angiographies and none of them showed collaterals or had extrahepatic deposits of MAA in the subsequent scans. In one of the patients treated by bilobar injection, a spasm in the left hepatic artery could have contributed to this complication.

In our case series, two patients were receiving combined concomitant treatment with the combination of oxaliplatin and 5-fluorouracil (FOLFOX) or gemcitabine (GEMOX). None of these drugs produce gastrointestinal lesions that could act as a confounding factor. In a literature search, we have found only one case of gastroduodenal ulceration with FOLFOX therapy and the patient was concurrently treated with Bevacizumab<sup>[17]</sup>. Saif *et al*<sup>[18]</sup> described a case of a patient treated with gemcitabine and warfarine who presented with gastrointestinal haemorrhage secondary to gastric and duodenal ulcers (with an international normalized ratio of 8.0). The lesions in our series are not likely to have been caused by these drugs, but we can not exclude a certain contribution.

The most common presenting symptoms in our series were abdominal pain, nausea/vomiting and anorexia. The intensity was usually mild and did not limit daily activities. Many drugs have been used, alone or in combination, demonstrating a slow but effective relieve of symptoms of this complication. However, more studies are needed to determine the best medical treatment. Only one patient required elective surgical treatment due to a late (> 1 year) duodenal stenosis. No urgent endoscopic or surgical treatment was performed. All but one patient are free of symptoms at the end of follow-up. All these data suggest that unintended gastrointestinal deployment of particles should be considered as a potential but transient cause of abdominal pain after the procedure. As shown in our series, the largest so far reported, a conservative approach must be considered as the primary treatment option. Nevertheless, the long-standing duration of symptoms (maximum 25 mo) in association with other factors (anemia, tumor progression and other comorbidities) certainly impacts the quality of life of affected patients. A common approach between oncologists, hepatologists and endoscopists would help improving the management and prognosis of the RE procedure and its complications.

## COMMENTS

### Background

Primary and secondary liver neoplasms are an important cause of morbidity and mortality worldwide. Radioembolization is a treatment indicated in those patients not eligible for curative resection or liver transplantation but still have their disease confined to the liver. It is considered a liver-directed therapy in which implanted radioactive microspheres are delivered into the arteries that feed the tumors. Secondary to this procedure some of the spheres can get into the arteries that feed some extrahepatic territories as the gastrointestinal tract.

### Research frontiers

As the unintended extrahepatic deployment of Yttrium 90 spheres is a rare complication of radioembolization little is known about its management and long-term prognosis. The authors have reviewed the clinical, endoscopic, analytical

and histologic long-term follow-up of patients treated with this liver-directed therapy. They also describe how the introduction of new image techniques has decreased the incidence of this complication.

### Innovations and breakthroughs

In this retrospective single-center study, the authors have found an incidence of 1.5% of gastrointestinal lesions secondary to the inadvertent deployment of radioactive microspheres. The incidence of this complication decreased from 3.75% to 1% when single photon emission computed tomography images were included in the routine planification of the treatment. The most frequent symptoms referred at diagnosis were abdominal pain and nausea and vomiting.

### Applications

The authors have shown the good long-term prognosis of this rare complication. It should be considered as a potential but transient cause of abdominal pain after the procedure. As it may be associated with long-standing symptoms the authors should take into account this complication to increase the patients' quality of life. It seems like the inclusion of new image techniques have decreased the incidence of this complication, but more research is needed to confirm these promising results.

### Terminology

Radioembolization: Treatment procedure in which radioactive microspheres loaded with Yttrium 90 are delivered into the hepatic artery.

### Peer review

This paper is well written and adds to understanding of outcomes of patients with ulcers following radioembolization.

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**P-Reviewers** Çil BE, Noshier J **S-Editor** Jiang L  
**L-Editor** A **E-Editor** Li JY



## Predominant mucosal *IL-8* mRNA expression in non-*cagA* Thais is risk for gastric cancer

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Supported by JSPS Ronpaku (Dissertation PhD) program (No. NRCT 10726) award by Japan Society for the Promotion of Science and in collaboration with Kobe University School of Medicine, Kobe, Japan; JSPS Asian CORE Program 2012, Nippon Medical School, and the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (in part)

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Received: December 28, 2012 Revised: April 1, 2013

Accepted: April 9, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To study gastric mucosal interleukine-8 (*IL-8*) mRNA expression, the cytotoxin-associated gene A (*cagA*) mutation, and serum pepsinogen (PG) I / II ratio related risk in Thai gastric cancer.

**METHODS:** There were consent 134 Thai non-cancer volunteers who underwent endoscopic narrow band imaging examination, and 86 Thais advance gastric cancer patients who underwent endoscopic mucosal biopsies and gastric surgery. Tissue samples were taken by endoscopy with 3 points biopsies. The serum PG I, II, and *Helicobacter pylori* (*H. pylori*) immunoglobulin G (IgG) antibody for *H. pylori* were tested by enzyme-linked immunosorbent assay technique. The histopathology description of gastric cancer and non-cancer with *H. pylori* detection was defined with modified Sydney Score System. Gastric mucosal tissue *H. pylori* DNA was extracted and genotyped for *cagA* mutation. Tissue *IL-8* and cyclooxygenase-2 (COX-2) mRNA expression were conducted by real time relative quantitation polymerase chain reaction. From 17 Japanese advance gastric cancer and 12 benign gastric tissue samples, all were tested for genetic expression with same methods as well as Thai gastric mucosal tissue samples. The multivariate analysis was used for the risk study. Correlation and standardized *t*-test were done for quantitative data, *P* value < 0.05 was considered as a statistically significant.

**RESULTS:** There is a high non *cagA* gene of 86.8 per cent in Thai gastric cancer although there are high yields of the East Asian type in the positive *cagA*. The *H. pylori* infection prevalence in this study is reported by combined histopathology and *H. pylori* IgG antibody test with 77.1% and 97.4% of sensitivity and specificity, respectively. The serum PG I / II ratio in gastric cancer is significantly lower than in the non-cancer group, *P* = 0.045. The serum PG I / II ratio of less

than 3.0 and *IL-8* mRNA expression  $\geq 100$  or  $\log_{10} \geq 2$  are significant cut off risk differences between Thai cancer and non-cancer,  $P = 0.03$  and  $P < 0.001$ , respectively. There is a significantly lower PGI/II ratio in Japanese than that in Thai gastric cancer,  $P = 0.026$ . Serum PG I / II ratio at cut off less than 3.0 and *IL-8* mRNA expression Raw RQ  $> 100$  or  $\log_{10} > 2$  are significantly difference between Thai cancer group when compared to non-cancer group,  $P = 0.013$  and  $P < 0.001$ , respectively. In the correlation study, low PG I / II ratio does not associate with chronic atrophic gastritis severity score in Thais non-cancer cases. However, there is a trend, but not significant convert correlation between *IL-8* mRNA expression level and low PG I / II ratio in Thai positive *H. pylori* infection. The high expression of *IL-8* gene demonstrates a poorer prognosis by stage and histology.

**CONCLUSION:** Predominant gastric mucosal *IL-8* mRNA expression level, *H. pylori* infection, and low PG I / II ratio are relative risks for Thai gastric cancer without correlation with *cagA* mutation.

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**Key words:** Gastric cancer; *CagA* mutation; Interleukine-8 mRNA expression; *Helicobacter pylori*; Pepsinogen I / II ratio

**Core tip:** A high level of interleukine-8 (*IL-8*) mRNA expression was detected in more than eighty percent of Thai gastric cancer patients and nearly two fold in the normal Thai population. The majority of northern Thai gastric cancer patients who had negative *cagA* gene *Helicobacter pylori* infection even with or without its mutation, still have a high *IL-8* mRNA expression level. The pathogenesis of Thai gastric cancer may primarily involve another gate-way besides the bacterial factor. The results show that there is a predominantly cancer inflammation state regulated by *IL-8* mRNA expression level that can be detected in Thai gastric cancer patients.

Yamada S, Kato S, Matsuhisa T, Makonkawkeyoon L, Yoshida M, Chakrabandhu T, Lertprasertsuk N, Suttharat P, Chakrabandhu B, Nishiumi S, Chongrakсут W, Azuma T. Predominant mucosal *IL-8* mRNA expression in non-*cagA* Thais is risk for gastric cancer. *World J Gastroenterol* 2013; 19(19): 2941-2949 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2941.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2941>

## INTRODUCTION

Gastric cancer pathogenesis is a well-known worldwide multifactorial condition. The gastric cancer incidence rate in Thailand ranks ninth by 4.1:100000 in males and 2.1:100000 in females. Despite being a low incidence country, northern Thailand has a higher gastric cancer in-

cidence rate with 6.6:100000 in males that ranks fifth of overall cancer in the northern Thai region, and 4.5:100000 in females<sup>[1]</sup>. The author was interested in the carcinogenesis of gastric cancer in Thais, and why the incidence in Thais is much different from other East Asian countries. The interleukin-8 (*IL-8*) gene is one of the principal mediators for the inflammatory response gate way that was first reported in 1970s, and it is one of factors that are possible to affect gastric cancer carcinogenesis<sup>[2]</sup>. A recent case-controlled surveillance study in northern Thailand on cytokine gene *IL-1b-511* mutations in three East Asian populations showed no predominantly correlated specific causative factor responsible for differences among ethnics and histologic types<sup>[3,4]</sup>. Therefore, the author proposed the study on other gate-ways of cytokine expression in the human gastric mucosal cell.

Recently, an *in vitro* study showed the association of the mucosal tissue *IL-8* mRNA expression related to the *Helicobacter pylori* (*H. pylori*), positivity cytotoxin-associated gene A (*cagA*) gene. The East Asian genotype was reported in Japanese gastric cancer in about 85% of the cases. Many *in vitro* studies showed this toxicity gene related to gastric mucosal cell injury, inflammation, and oncogenic potential<sup>[5-7]</sup>. The *cagA*, East Asian genotype is commonly detected in chronic gastritis and gastric cancer of the Japanese<sup>[8,9]</sup>. There is reported data that a low serum pepsinogen (PG) I / II ratio of less than 3.0 with a PG I level of less than 70 ng/dL was considered as a high risk factor for Japanese gastric cancer<sup>[10,11]</sup>. There is no recent *in vivo* study reporting a correlation among these above factors, especially *IL-8* and cyclooxygenase-2 (COX-2) mRNA expression level in Thais.

The author hypothesized that gastric mucosal tissue *IL-8* mRNA expression may be different among ethnicities, and it may correlate to other reference pathogenesis factors. This study aimed to look for the risk and correlation of these factors in Thai gastric cancer. The level of *IL-8*, COX-2 mRNA expression, and *cagA* gene mutation distribution were also to be the first report in Thai gastric cancer.

## MATERIALS AND METHODS

Research methodology was considered and permitted by Thai and Japanese local ethical committees, the NRCT and Japan Society for the Promotion of Science code ID-NRCT 10726.

### Patient characteristics and volunteer selection

An experimental based cross-sectional study was conducted in the Gastrointestinal Surgery and Endoscopy Unit, Chiang Mai University Hospital from 2007 through 2010. Informed consents were obtained from 86 Thai gastric cancer patients who underwent narrow band imaging (NBI) endoscopy and gastric surgery during year 2007-2010, and 134 Thai non-cancer volunteers who underwent NBI endoscopic examination from 2006 to 2008. All gastric cancer patients in this study had locally

advanced gastric cancer, and underwent examinations by endoscopy before curative gastric resection. Seventeen advanced stage Japanese gastric cancer and 12 non-cancer surveillance patients were recruited. Peptic ulcer disease was excluded in this study. Gastric mucosal tissue samples were taken by endoscopy with three biopsy sites for pathology and bimolecular genetic tests before surgical treatment. In cancer cases, biopsy points were specified from non-necrotic areas of the tumor. The histopathology description of the tumor and histologic type were defined. For pathological examination in both groups, chronic gastritis and metaplasia with *H. pylori* detection were classified with a modified Sydney Score System.

### Serum PG I and II level, and *H. pylori* immunoglobulin G antibody test

A 5 cc sample of venous blood was collected from each study participant. The red blood cell and serum separation was done, and preserved at -20 °C. The serum PG I, II, and immunoglobulin G (IgG) antibody for *H. pylori* were tested by the standard enzyme-linked immunosorbent assay technique. The standard cut off value used was a PG I level of more than 70 ng/mL or PG I / II ratio more than 3.0 for no atrophy or positive Grade 1, PG I < 70 ng/mL and PG I / II ratio < 3.0 excluding severe atrophy for moderate atrophy or positive Grade 2, and PG I < 30 ng/mL and PG I / II ratio < 2.0 for severe atrophy or positive Grade 3, respectively<sup>[10,11]</sup>. All samples were tested twice for reliability confirmation (Toyobo, co, Ltd., Japan)

### Tissue *H. pylori* DNA extraction and *cagA* genotyping method

The tissue *H. pylori* DNA extracted from the lower antral position in the stomach was examined by the polymerase chain reaction method, and genotyped for *cagA* mutation in all samples by the author (Samples were also examined by double blinded test by Toyobo, co, Ltd). The *H. pylori* positive control of *cagA* positive strain number 11638 (Western), 26695 (Western), and F57 (East Asian) were provided by the collaborative institute. The bacterial tissue DNA and genotyping method with primers used in this study were conducted as recently described. The specific oligonucleotide primers forward (5'-AAAAGC-GACCTTGAAAATTC-3'; nucleotides 2299-2319), reverse-1 (5'-CTTCATTTTTTGAGCTTGTTGAGC-3'; nucleotides 2488-2463) and reverse-2 (5'-ATTAAT-GCGTATGTGGCTGTTAGTAGC-3'; nucleotides 3222-3195, were originally described by Azuma *et al.*<sup>[12]</sup>.

### Cell line culture and gastric mucosal total mRNA extraction with reverse transcriptase reaction for cDNA synthesis

The AGS cell line was grown before cell collection for mRNA extraction at a cell count of  $2 \times 10^6$ - $4 \times 10^6$ . They underwent a total mRNA extraction protocol. The technique followed was a reverse transcriptase reaction using a commercial high capacity RNA-to-cDNA kit (Applied Biosystems)<sup>[13]</sup>.

### Gastric mucosal *IL-8* and *COX-2* mRNA expression by relative quantification real time reverse transcription-polymerase chain reaction

We conducted the experiment from three positions of gastric mucosal biopsies in all Thai and Japanese study participants. All of gastric mucosal tissue samples were transformed to cDNA after total mRNA extraction. The analysis was substantially correctable by analysis both in raw relative quantitation (RQ) and log<sub>10</sub> value for adjusted normal distribution curve. All Human TaqMan probe primer express that was used in this study had 81-base pairs (bp) *IL-8* specific human primer assay ID number Hs99999034\_m1, 111- base pairs (bp) *COX-2* assay ID number Hs01573471\_m1, and 121- base pairs (bp) specific human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Hs99999905\_m1 those designed and supplied by Applied Biosystems, United States. The internal control was performed by GAPDH of a matched number template. The real-time relative quantitation value was measured by comparing to the base line value of AGS cell line subject control before making the analysis.

### Statistical analysis

A student *t*-test was used for quantitative data, *IL-8* and *COX-2* mRNA expression level, and PG level. The  $\chi^2$  test was used for qualitative data. The correlation study for pair factors was done in subgroup analysis for defined groups of ethnic, cancer and non-cancer populations. The multivariate analysis was used for risk study for both non-normal distribution and normal distribution curve data bases. STATA 11.0, United States and SPSS 16, United States were used for statistical analysis, and the *P* value of less than 0.05 was considered statistically significant.

## RESULTS

There were 86 cases of advanced gastric cancer and 45 (33.8%) normal control cases, 46 (34.6%) non-peptic disease benign lesions without recent history of any treatment, and 42 (31.6%) chronic gastritis cases among 134 non-cancer control cases who were included in the genetic expression experiment. Thai male and female cancer incidences are 60.5% (52/86) and 34.0% (39/86), respectively. Males are also the predominant gender in Japanese. Both nations have significantly high incidence of gastric cancer at age 40 years old or above.

The *H. pylori* infection prevalence is reported by combined histopathology, *H. pylori* IgG antibody level, and 23S rDNA results that have 77.1% and 97.4% of sensitivity and specificity, respectively. Among Thai cancer patients and non-cancer volunteers, *H. pylori* prevalence was 72.1% and 71.6%, respectively. Meanwhile, Thai gastric cancer cases had a *cagA* genotype demonstrated in only 7/62 (12.3%) in positive *H. pylori* infection cases by 23S rDNA that yields six cases of East Asian type and one case of Western type. In non-cancer volunteers, there were 62/98 (63.9%) of positive *cagA* and 34/98 (36.1%) of negative *cagA* genotyping in positive *H. pylori* infec-

**Table 1** Characteristics of 220 Thais examined for interleukine-8 mRNA expression *n* (%)

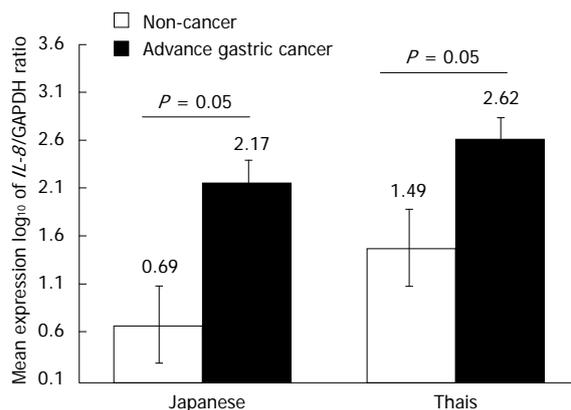
| Variable   | Cancer<br>( <i>n</i> = 86) | Benign<br>( <i>n</i> = 134) |
|--|----------------------------|-----------------------------|
| Sex  |                            |                             |
| Male   | 52 (60.5)                  | 41 (30.6)                   |
| Female   | 34 (39.5)                  | 93 (69.4)                   |
| Age (yr)   |                            |                             |
| < 40   | 5 (5.8)                    | 28 (20.9)                   |
| ≥ 40   | 81 (94.2)                  | 106 (79.1)                  |
| mean ± SD  | 56 ± 11.3                  | 48.5 ± 11.2                 |
| Alcohol drinking   |                            |                             |
| No   | 50 (58.1)                  | 80 (59.7)                   |
| Yes  | 36 (41.9)                  | 54 (40.3)                   |
| Smoking  |                            |                             |
| No   | 62 (72.1)                  | 122 (91.0)                  |
| Yes  | 24 (27.9)                  | 12 (9.0)                    |
| Diseases   |                            |                             |
| Normal   | -                          | 45 (33.8)                   |
| Benign lesion<br>(polyps, erosion, mild superficial gastritis) | -                          | 46 (34.6)                   |
| Chronic active gastritis                                       | -                          | 42 (31.6)                   |
| Cancer   | 86 (100.0)                 | -                           |

tion cases that yielded 47.7% of East Asian, 27.4% of Western, and 24.9% of Mixed genotype. For the six year follow up of 18 cases of high grade chronic atrophic gastritis (CAG group) in non-cancer Thais who had long term *H. pylori cagA* East Asian type infection, no one has developed gastric cancer.

The enzyme PG results, showed a significantly lower PG I / II ratio with a mean of 3.3 ± 1.7 in gastric cancer patients than one in non-cancer volunteers, *P* = 0.045, and of other CAG, *P* = 0.002. There is a significantly lower PG I / II ratio in Japanese gastric cancer than in Thai gastric cancer, *P* = 0.026.

For *IL-8* and *COX-2* mRNA expression results, 86 Thai gastric cancers were tested successfully in comparison with 134 Thai non-cancer volunteers. The detection rates of *IL-8* mRNA expression were 77/86 (89.5%) in Thai gastric cancer and 102/134 (74.6%) in Thai non-cancer volunteers. Thai population characteristic data that was examined for *IL-8* mRNA expression is demonstrated in Table 1. Serum enzyme PG I, II level, and *H. pylori* infection status are demonstrated in the cancer population and non-cancer volunteers in Thais is demonstrated in Table 2. We found a remarkable number of Thai gastric cancers with a negative *cagA*; therefore, *IL-8* mRNA expression was examined and the cut-off point of expression value difference is demonstrated in Table 3. Serum PG I / II ratio at cut-off point of less than 3.0 and raw RQ ≥ 100 or log<sub>10</sub> ≥ 2 of *IL-8* mRNA expression level showed the significantly different between the Thai gastric cancer group and the non-cancer group, *P* = 0.045 and *P* < 0.001, respectively. In the multivariate analysis application, the four co-factors related to gastric cancer risk including *IL-8* mRNA expression in Thais are shown in Table 4.

At the same stage of advanced gastric cancer, the mean levels of *IL-8* mRNA expression in Thai cancer



**Figure 1** Mean interleukine-8 mRNA expression level measurement of relative quantitation by real-time reverse transcription-polymerase chain reaction study in gastric cancer comparing with non-cancer population both Japanese and Thais. In Japanese and Thais, gastric mucosal interleukine-8 (*IL-8*) mRNA expression in cancer is higher than in non-cancer with *P* = 0.05. The mean level of *IL-8* mRNA expressions in Thai cancer and Japanese cancer were 9615.65 (log<sub>10</sub> = 2.62) and 1509.11 (log<sub>10</sub> = 2.17), respectively, *P* = 0.014. The mean level of *IL-8* mRNA expression in non-cancer Thais is 2262 (log<sub>10</sub> = 1.49) while that in non-cancer Japanese is 10.79 (log<sub>10</sub> = 0.69), *P* < 0.001.

and Japanese cancer were 9615.65 (log<sub>10</sub> = 2.62) and 1509.11 (log<sub>10</sub> = 2.17), respectively, *P* = 0.014. For gastric cancer risk at cut-off *IL-8* expression level by log<sub>10</sub> greater than two in Thais and Japanese, odds ratio (OR) = 7.97 (95%CI: 3.75-16.97, *P* < 0.001) and OR = 4 (95%CI: 1.29-12.40), respectively. In the non-cancer group, we found that the *IL-8* mRNA expression level was lower than cancer population with a significant difference, *P* < 0.001. The total mean *IL-8* mRNA expression in non-cancer Thais was 2262 (log<sub>10</sub> = 1.49) while that in Japanese non-cancer was 10.79 (log<sub>10</sub> = 0.69), *P* < 0.001. In comparison within the same ethnic group, the mean levels of *IL-8* mRNA expression in Thai and Japanese cancer were higher than those in non-cancer, *P* = 0.05 as showed in Figure 1.

The *COX-2* mRNA expression did not indicate significant rising level with detection rate of 65% in Thai and Japanese gastric cancer. In comparison with *IL-8* mRNA expression, although the level of *COX-2* mRNA expression was slightly higher in gastric cancer than normal gastric mucosal tissue, there were much lower levels than those of *IL-8* mRNA expression.

In the correlation study, low PG I / II ratio was not associated with the CAG severity score in Thai non-cancer cases because of a few number of CAG in both Thai gastric cancer and non-cancer populations in this study. There was no significant difference for the *IL-8* mRNA expression level in cancer between positive and negative *H. pylori* infection. There was no direct correlation of *IL-8* mRNA expression level and serum *IgG* levels. In subgroup analysis, there was a significant difference of higher levels in groups of poorly differentiated histopathology in comparing both nations. For the diffuse histologic type, the *IL-8* mRNA expression level is about 1.5 times higher than that of intestinal histologic type with a statistically significant difference in Japanese.

**Table 2** Serum enzyme pepsinogen I, II level and *Helicobacter pylori* infection detection results in Thais *n* (%)

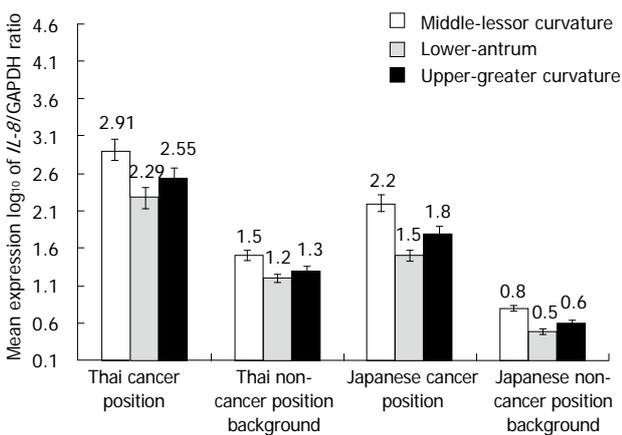
| Variable                             | Cancer<br>( <i>n</i> = 86) | Benign<br>( <i>n</i> = 134) | <i>P</i> value       |
|--------------------------------------|----------------------------|-----------------------------|----------------------|
| PG I / II, (ng/μL), mean ± SD        |                            |                             |                      |
| I                                    | 57.39 ± 46                 | 54.86 ± 68.5                | 0.780                |
| II                                   | 19.42 ± 21                 | 15.42 ± 11.7                | 0.090                |
| PG I / II ratio                      |                            |                             |                      |
| ≤ 3                                  | 28 (39.4)                  | 34 (27.9)                   | 0.045 <sup>1</sup>   |
| > 3                                  | 43 (60.6)                  | 88 (70.1)                   |                      |
| <i>H. pylori</i> pathology           |                            |                             |                      |
| Negative                             | 31 (36.8)                  | 60 (44.8)                   | 0.001                |
| Positive                             | 55 (63.2)                  | 74 (55.2)                   |                      |
| Serum IgG                            |                            |                             |                      |
| Negative                             | 31 (46.3)                  | 57 (44.2)                   | 0.820 <sup>1</sup>   |
| Positive                             | 36 (53.7)                  | 72 (55.8)                   |                      |
| CagA genotyping in positive 23S rDNA |                            |                             |                      |
| Negative                             | 55 (88.7)                  | 62 (64.6)                   | < 0.001 <sup>1</sup> |
| Positive                             | 7 (12.3)                   | 34 (35.4)                   |                      |
| <i>H. pylori</i> Infection status    |                            |                             |                      |
| True negative                        | 20 (24.4)                  | 37 (36.0)                   | 0.120 <sup>1</sup>   |
| True positive                        | 62 (75.6)                  | 96 (64.0)                   |                      |

<sup>1</sup>Some numbers are not included in the analysis due to missing laboratory entries. The statistical analysis was performed by  $\chi^2$  of each factor. IgG: Immunoglobulin G; CagA: Cytotoxin-associated gene A; PG: Pepsinogen; *H. pylori*: *Helicobacter pylori*.

**Table 4** Multivariate risk analysis for Thai gastric cancer

| Variable                          | OR   | 95%CI      | <i>P</i> value |
|-----------------------------------|------|------------|----------------|
| Male                              | 4.32 | 2.06-9.04  | < 0.001        |
| <i>H. pylori</i> infection status | 0.98 | 0.96-0.99  | 0.020          |
| PG II / I ratio ≤ 3               | 2.06 | 0.94-4.47  | 0.060          |
| <i>IL-8</i> mRNA expression       | 7.97 | 3.75-16.97 | < 0.001        |

OR: Odds ratio; *H. pylori*: *Helicobacter pylori*; PG: Pepsinogen; *IL-8*: Interleukin-8.



**Figure 2** The mean interleukine-8 mRNA expression in Thais divided by histology and cancer position. Middle is the lessor curvature and non-cancer position, lower is the antrum, and Upper is the greater curvature. *IL-8*: Interleukine-8.

High *IL-8* mRNA expression was primarily found in the non-*cagA* Thai gastric cancer population. There was a significantly different mean *IL-8 mRNA* expression level between groups of negative *cagA* by  $\log_{10} = 2.46 (\pm 1.04)$

**Table 3** Molecular genetic results of cyclooxygenase-2 and interleukine-8 mRNA expression in Thais *n* (%)

| Variable                      | Cancer<br>( <i>n</i> = 86) | Benign<br>( <i>n</i> = 134) | <i>P</i> value       |
|-------------------------------|----------------------------|-----------------------------|----------------------|
| COX2 raw RQ                   |                            |                             |                      |
| No expression detection       | 30 (34.90)                 | 43 (53.10)                  | < 0.001 <sup>1</sup> |
| Expression detection          | 56 (65.10)                 | 38 (46.90)                  |                      |
| COX2 raw RQ mean ± SD         | 41.69 ± 4.90               | 5.37 ± 4.20                 | < 0.001 <sup>1</sup> |
| COX2 log <sub>10</sub> (N, %) |                            |                             |                      |
| mean ± SD                     | 1.62 ± 0.96                | 0.73 ± 0.62                 | < 0.001 <sup>1</sup> |
| <i>IL-8</i> raw RQ            |                            |                             |                      |
| No expression detection       | 9 (10.47)                  | 32 (25.37)                  | < 0.001              |
| Expression detection          | 77 (89.53)                 | 102 (74.63)                 |                      |
| <i>IL-8</i> raw RQ            |                            |                             |                      |
| ≤ 100 or undetected           | 33 (38.37)                 | 105 (78.36)                 | < 0.001              |
| > 100                         | 53 (61.63)                 | 29 (21.64)                  |                      |
| mean ± SD                     | 9615.64 ± 49715.00         | 2262.29 ± 10454.60          | < 0.010              |
| <i>IL-8</i> log <sub>10</sub> |                            |                             |                      |
| ≤ 2 or undetected             | 32 (37.21)                 | 105 (78.36)                 | < 0.001              |
| > 2                           | 54 (62.79)                 | 29 (21.64)                  |                      |
| mean ± SD                     | 2.62 ± 1.10                | 1.49 ± 1.20                 | < 0.010              |

<sup>1</sup>Some numbers are not included in the analysis due to missing laboratory data. RQ: Relative quantity; COX2: Cyclooxygenase-2; *IL-8*: Interleukin-8.

**Table 5** Comparative means interleukine-8 mRNA expression level detection between Thai and Japanese Cancer populations divided by histopathology *n* (%)

| Histopathology  | Thai<br>( <i>n</i> = 77) | Japanese<br>( <i>n</i> = 17) | <i>P</i> value |
|-----------------|--------------------------|------------------------------|----------------|
| Diffuse type    | 55 (71.4)                | 4 (23.5)                     | 0.01           |
| mean ± SD       | 2.85 ± 1.10              | 2.55 ± 0.52                  | 0.95           |
| Intestinal type | 22 (28.6)                | 13 (76.5)                    |                |
| mean ± SD       | 2.52 ± 1.11              | 1.56 ± 1.06                  | 0.01           |

Statistical difference between groups (*P* = 0.04). *IL-8*: Interleukin-8.

and positive *cagA* by  $\log_{10} = 3.29 (\pm 1.68)$  in the Thai gastric cancer group. However, there were few numbers of Thai gastric cancers with positive *cagA*. In other subgroup analysis of 18 Thais who had high grade CAG, some level of *IL-8* mRNA expression in the 12 Japanese non-cancer patients appeared which an equivalently lower level.

Gastric cancer mucosal tissue *IL-8* mRNA expression in the cancer position had a significantly higher mean level than its level at the non-cancer background position in both Thai and Japanese shown in Figure 2. There was significantly different *IL-8* mRNA expression level between intestinal (favorable) and diffuse (unfavorable) histologic cell types. In Thai gastric cancer, the poorly differentiated gastric adenocarcinoma and signet ring cell were predominantly found in this study. The  $\log_{10}$  *IL-8* mRNA mean expressions in unfavorable cell type were 2.55 and 2.85 in Japanese and Thais, respectively, as shown in Table 5. There is a significantly higher level of *IL-8* mRNA expression in diffuse cell type than that in a differentiated histologic cell type, *P* = 0.04. The differentiated histologic cell type in Thais has higher expression level than that in Japanese with a statistically significant difference, *P* = 0.013. The RT-PCR results of gastric mucosal tissue and AGS

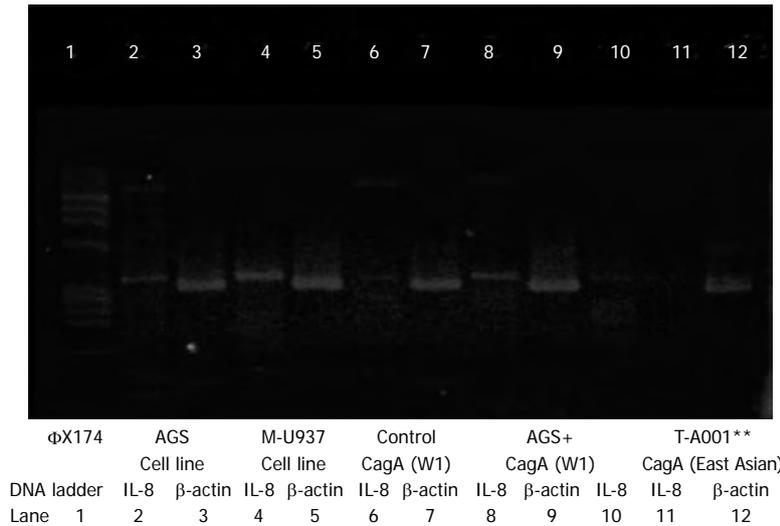


Figure 3 Basic experiment result on real-time reverse transcription-polymerase chain reaction of interleukine-8 mRNA expression with AGS, macrophage cell line, normal gastric mucosal cell, AGS cancer cell line co-culture with two strains of *cagA Helicobacter pylori*, and positive *cagA*, Thai non-cancer samples sequences showed on 12 lanes. M: Marker.

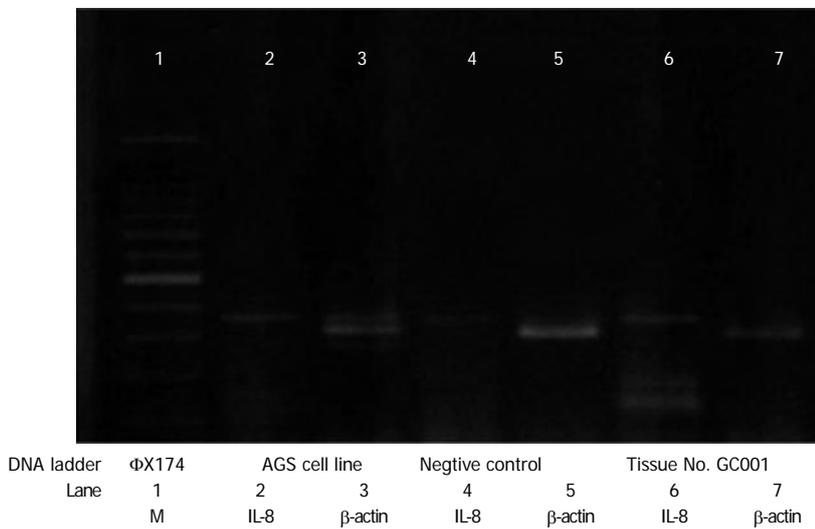


Figure 4 Real-time reverse transcription-polymerase chain reaction result of interleukine-8 mRNA expression from AGS, negative control, and Thai gastric cancer mucosal tissues. The positive results of interleukine-8 (*IL-8*) mRNA expression appeared at 320 bp band comparing with 300 bp band of marker (M) in lane 1.

cell line *IL-8* mRNA expression were demonstrated in Figure 3. and Figure 4. However, there was no significant difference of *IL-8* mRNA expression level between positive and negative *H. pylori* infection in subgroup analysis of non-cancer background positions.

In summary, *IL-8* mRNA expression level is predominantly found, and trend toward an inverted correlation to PG I / II low ratio in Thai gastric cancer patients. There is no direct correlation of *IL-8* mRNA expression level with the *cagA* gene mutation in Thai gastric cancer.

## DISCUSSION

In the present *in vivo* study, there is a significant risk of *IL-8* mRNA expression level predominantly found rising up more than 80% of northern-Thai gastric cancer. There is significantly higher level of *IL-8* mRNA expression in poorly differentiated than in well differentiated carcinoma in both Thailand and Japan. There is a trend of converted independent correlation with the very low PG I / II ratio in gastric cancer as well as a few numbers of Thai severe CAG, but no direct correlation with positive *H. pylori* infection or *cagA* genotypes.

Higher level *IL-8* mRNA expression in non-cancer Thais comparing with non-cancer Japanese demonstrated the difference of gastric mucosal defense and genetic expression between the two nations. Thai gastric cancer has less background of CAG. In this study, there is no evidence that showed a direct correlation of *IL-8* mRNA expression with *cagA* mutation genotype in *H. pylori* positive cases. Predominant *IL-8* mRNA expression level resulted in non-atrophic mucosa of both gastric cancer and non-cancer Thais. The result is different from previous *in vitro* or some *in vivo* studies in high incidence gastric cancer countries, such as Japan, China, and South Korea.

In Thai gastric cancer patients, *IL-8* mRNA expression level at the lesser curvature is the most represented location. Its predominantly high level may represent relatively vascular invasion as well as major gastric mucosal inflammation. However, the area of gastric antrum in Thai gastric cancer patients has less activity than the lesser curvature because atrophy occurs more frequently.

Also, a high level of *IL-8* mRNA expression is matched with the poor prognosis by histopathology cell type and tumor stage. Long-term bacterial infection has less effect to change the gastric mucosa into CAG. In our

recent six year followed up study in non-cancer cases controlled with positive *H. pylori* infection and without eradication of 500 new non-cancer cases, a few people developed severe gastritis and still have a high PG I / II ratio. In this study, the number of high PG I / II ratio in Thai cancer is still about 45% which is nearly the same percentage in our recently published study<sup>[4]</sup>.

A few gastric cancer preventive models on natural Thai products were reported<sup>[14]</sup>. There is one study on diet consumption in Thais showing a linkage of gastric cancer risk. The factors which were found to be a higher risk but not statistically significant were low intake of vegetables and fruits (OR = 1.2, 95%CI: 0.74-1.96) and Jeaw prik (mainly chilly with Plara broth or pickled fish), a kind of preserved food in North and North-eastern regions of Thailand (OR = 1.2, 95%CI: 0.76-2.01)<sup>[15]</sup>. The consensus of the Asian Pacific guideline on gastric cancer prevention is still debated in some experts' opinions<sup>[16]</sup>. *H. pylori* infection screening in a low risk gastric cancer population is not recommended, but serum PG may be helpful to screen the high risk population in northern Thailand. We reported its different characteristics that rely on *H. pylori* IgG antibody and PG I / II ratio in our cancer populations comparing to the data in a Japanese report<sup>[17]</sup>.

The Japanese study primarily reported the relation of *cagA* genotype and a low PG I / II ratio. Nevertheless, approximately half of the Thai cancer population demonstrates a low ratio of PG I / II similar to the Japanese. There are still a small number of Thais who had a severe atrophy score related to *H. pylori* infection though in the positive *cagA* Thai population.

In this study, the negative *cagA* gene is found in the majority of Thai gastric cancer unlike the Japanese. In Thais, the poorly differentiated cell type gastric adenocarcinoma occurred mainly in the negative *cagA* gene *H. pylori* infection, not in Western type *cagA*.

There is a small number of the Thai population who had a severe atrophy score of gastric mucosa found in our recent and present collaborative study<sup>[18]</sup>. Reduction of the fundic gland in chronic gastritis was also related to the low level of PG I / II ratio in Japanese<sup>[19,20]</sup>. In our recent study of subgroup analysis on the PG I / II ratios, there was no significant difference between CAG and gastric cancer group,  $P = 0.12$ . However, the low PG I / II ratio was significantly related to gastric cancer when compared to normal population in a recent match-case control study by OR of 2.3 (95%CI: 1.10-4.80),  $P = 0.025$ <sup>[4]</sup>. The tumor location demonstrated locations mainly at the upper portion and corpus in both of our studies. In the present study, the author found risk for cancer by OR 2.06 (95%CI: 0.94-4.47),  $P = 0.059$  that seems to be close to the result of our recent study. The low PG I / II ratio was not found to be a high percentage in Thais unlike in Japanese gastric cancer<sup>[9]</sup>.

For other genetic host factors, in one interesting study, two of the four gastric carcinoma cell lines expressed vascular endothelial growth factor (VEGFR-3) mRNA. In 17 of 36 gastric carcinoma specimens, VEGFR-3-specific

immune activity was detected in tumor cells. These angiogenesis and lymphangiogenesis were also detected in VEGF-C-transfected tumors than in control tumors<sup>[21]</sup>. *IL-8* mRNA expression is found to be the gate way mechanism of the vascular epithelial growth factor. For *IL-1* gene, it is a pro-inflammatory cytokine, and the T/T genotype of *IL-1β-511* is suspected as the risk factor of both hypochlorhydria related *H. pylori* infection and gastric cancer in a case-control population in the United States<sup>[22]</sup>. The author reported that *c/c* genotype was a risk in Japanese, and a lower number of *c/c* genotype was found as a minor risk related to Thai gastric cancer<sup>[4]</sup>.

Recent *in vivo* animal models studies showed the expressions of *IL-8* and COX-2 had linkage to the epithelial cell which was co-infected with *H. pylori*. However, our preliminary study reported that there was no difference of *IL-8* mRNA expression level between a cell line which was co-infected with *H. pylori* and the Thai gastric mucosa tissues which had positive or negative *H. pylori*<sup>[23,24]</sup>. The toleration, remarkable host response to cancer inflammatory process, healing of stomach mucosal turnover rate, and re-healing process of ulcer in Thais may be different and caused by host susceptible differences to the virulence bacteria.

The theory regarding inflammatory cytokine's influence on cancer development was first contributed by Rudolph Virchow around 150 years ago<sup>[25]</sup>. Many studies regarding *IL-8* gene expression remarkably found significant relation with *H. pylori* infection and many *cag* pathogenicity island both *in vitro*<sup>[26,27]</sup> and in some number in *in vivo* study of Japanese cancer<sup>[28,29]</sup>. However, no study has demonstrated differences of expression level in the individualized host<sup>[30]</sup>.

In this study, Japanese gastric cancer has a lower *IL-8* mRNA expression on average than that in Thai gastric cancer patients at the same stage of disease and *H. pylori* infection status. However, Japanese gastric adenocarcinoma cases are mostly an intestinal type and infected by positive *cagA* strain *H. pylori*. In contrast with northern Thai gastric adenocarcinoma cases, they are mostly diffuse histologic cell type, and infected by negative *cagA* strain *H. pylori*. Therefore, the authors speculate that the predominant level of *IL-8* mRNA expression found in non-*cagA* gene *H. pylori* infection is not directly related to atrophic gastritis mucosa in Thai gastric cancer.

Some results in this study are unexpected outcomes and different from our recent knowledge of *in vitro* and *in vivo* study in Japanese atrophic gastric mucosa which almost easily infected by positive *cagA H. pylori* infection. The *cagA* was also the suspected cause of the *IL-8* gene expression rising.

This result in Thais showed that gastric mucosal tissue *IL-8* mRNA expression has a higher level in the advanced stage and poorer differentiated cell type than in favorable histology or differentiated cell type. The author was suspicious that the less atrophic background of Thai stomach cancer and non-cancer gastric mucosa may be caused by non-*cagA H. pylori* infection. However, the high level of mRNA *IL-8* gene expression in Thai gastric can-

cer cases may be explained by the cancer inflammation carcinogenesis that may not be directly related to only *H. pylori* infection in Thais.

The level of *IL-8* mRNA expression in Thai gastric cancer or poorly differentiated gastric carcinoma may be regulated by other factors besides of *H. pylori* infection. Also, unknowns remain regarding how long *IL-8* mRNA expression has been high before the occurrence of gastric cancer or after becoming a more advanced stage. Although the author analyzed the level of *IL-8* and *COX-2* mRNA expression level in normal mucosa and of advanced gastric cancer, this occurrence could not be shown in early gastric cancer. The environmental factors and bacterial virulence effect cannot be excluded.

In this study, the signet ring cell type is predominantly found in Thai gastric cancer population. The poor prognostic histological cell type may have different disease carcinogenesis related to gastric mucosal tissue *IL-8* mRNA high expression level and severity of the disease. The *COX-2* mRNA expression level is directly correlated with only the *H. pylori* infection, and tended to be suppressed unlike *IL-8* mRNA expression. The author supposed that extremely high gastric mucosal *IL-8* expression level may relate to other factors, such as VEGF that could not be demonstrated in this study and should be explored further.

In conclusion, this present *in vivo* study shows results of new factual data on predominant gastric mucosal *IL-8* mRNA expression level in Thai gastric mucosal biopsy tissues in both non-cancer and gastric cancer volunteers. In the present study, the northern Thai gastric cancer population has a high incidence of signet ring cell by the nature of histologic type. The positive *H. pylori* may be one of the co-factors, though the host is infected with non-*cagA* gene and still has an extremely high *IL-8* mRNA expression level. This cytokine expression may represent the individual host defense in both high and low incidence gastric cancer ethnics. The factual results in an experimental based study demonstrated how prevalence of the northern Thai gastric cancer host had active co-infection or had been recently infected by non-*cagA* gene *H. pylori* infection. The *IL-8* mRNA expression level does not directly correlate to non-*cagA* *H. pylori* infection in Thai gastric cancer. However, there is a trend of converted correlation between *IL-8* mRNA expression and low ratio PG I / II without statistical significance, and it seems to be an independent correlation. By these preliminary results, the author expected to do further study on *IL-8* mRNA expression that may act as one of prognostic genetic biomarkers in clinical practice and for chemotherapy application in the nearby future. A study on the current cancer chemotherapy with northern Thai gastric cancer population is on-going.

## ACKNOWLEDGMENTS

This study with preliminary results was presented in a free oral paper symposium session of Oncology and Stomach session, International Surgical Week 2011, in Yokohama during August 28-September 1.

## COMMENTS

### Background

The incidence of gastric carcinoma is very low although the incidence of *Helicobacter pylori* (*H. pylori*) seemed not to be low in Thais. However, the northern Thai population still has the highest gastric cancer incidence in Thailand. Currently, the disease incidence is rising faster than in the past and still the second cause of cancer death worldwide. The cause and risk factors for gastric cancer carcinogenesis in Thais is unclear especially the risk related with *H. pylori* infection or other cofactors.

### Research frontiers

Interleukine-8 (*IL-8*) mRNA expression is a common event found in some epithelial malignancies and in gastric adenocarcinoma either due to *H. pylori* caused chronic inflammation or other causes by unrelated carcinogens. It is not clear how the level of this expression related to gastric adenocarcinoma. The authors demonstrated that the predominant overexpression of *IL-8* mRNA could be a potential relative risk for gastric adenocarcinoma in Thais and demonstrated the difference of its level in relationship with the histologic type of gastric cancer.

### Innovations and breakthroughs

Recent reports have highlighted the importance of cytotoxin-associated gene A (*cagA*) *H. pylori* infection and its mutation type that is shown predominantly in Japanese gastric cancer carcinogenesis. Particularly in the well differentiated histologic type, the *IL-8* mRNA expression level in the Japanese seems to be much lower than in Thais. However, its level in poorly differentiated cell type of Thai gastric adenocarcinoma has less atrophic background and a higher level of expression than in well differentiated gastric adenocarcinoma. This is the first study to report how measurement of *IL-8* mRNA expression level demonstrates risk in non-*cagA* *H. pylori* infection of Thai gastric cancer and the trend of differences in carcinogenesis related to *H. pylori* infection between high and low incidence ethnics. Furthermore, their *in vivo* studies would suggest that the *IL-8* mRNA expression level yields high prevalence detection in gastric adenocarcinoma and may be a useful tool for gastric cancer prognostic or therapeutic study.

### Applications

By understanding different *IL-8* mRNA expression levels, this study may represent a future study with a tissue molecular biomarker for gastric cancer.

### Terminology

*IL-8* mRNA expression is pro-inflammatory cytokines that is detected in gastric epithelial mucosa and gastric cancer cell lines, such as Kato III and AGS cells.

### Peer review

The authors examined the expression of *IL-8* and cyclooxygenase-2 in AGS cell line, normal gastric mucosa, gastritis, and gastric adenocarcinoma tissues. It revealed that *IL-8* mRNA expression predominantly increased in poorly differentiated or signet ring cell gastric adenocarcinoma that showed the trend of a poorer prognosis. The expression was not directly correlated to *cagA* *H. pylori* infection and its mutation type. The results are interesting and may represent a different carcinogenesis of Thai gastric cancer in comparison to recent Japanese studies.

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P- Reviewer Aoyagi K S- Editor Wen LL  
L- Editor A E- Editor Xiong L



## Current application situation of gastrointestinal endoscopy in China

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Received: December 24, 2012 Revised: February 18, 2013  
Accepted: March 22, 2013  
Published online: May 21, 2013

### Abstract

**AIM:** To study the current application situation of gastrointestinal (GI) endoscopy in mainland China.

**METHODS:** From 12 August, 2011 to 15 February, 2012, draft questionnaires were sent by e-mail to 289 hospital-based GI endoscopy units, including units with three levels (provincial, prefecture and county level) in mainland China. All the surveyed GI endoscopy units were state-owned and hospital-based. Proportions were compared using  $\chi^2$  tests. Comparisons between groups were performed using the Mann-Whitney *U* test. A probability of  $P < 0.05$  was considered to represent a statistically significant difference.

**RESULTS:** Based on satisfactory replies, 169/279 (60.6%) of units were enrolled in the survey, which covered 28 provinces (90.3%, 28/31) in mainland China. Compared with published survey data, the number of GI endoscopes per unit has increased by nearly three times (from 2.9 to 9.3) in the past decade. About

33 of 169 (19.5%) endoscopy units possessed an X-ray machine, which was mainly owned by provincial endoscopy units (43.2%, 19/44). Video capsule endoscopes, which were almost unavailable ten years ago, were owned by 20.7% (35/169) of GI endoscopy units. Endoscopic submucosal dissection could be performed by 36.4% (19/44) of the provincial units, which was significantly higher than the prefecture level (9.9%,  $P < 0.01$ ) and county level (0.0%,  $P < 0.01$ ) units, respectively.

**CONCLUSION:** Rapid development in GI endoscopy has been made in mainland China, and major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly used in large endoscopy units.

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**Key words:** Application situation; Gastrointestinal endoscopy; Video capsule endoscopy; Endoscopic submucosal dissection

**Core tip:** Rapid developments in gastrointestinal (GI) endoscopy have taken place in China in the past decade. Major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly confined to large endoscopy units, whereas small and medium units, often perform fewer endoscopic procedures and have less equipment, and are mostly restricted to diagnostic endoscopy. In addition to improvement in GI endoscopy equipment, standard procedures including the standard reprocessing for endoscopy will be the focus in the future in China.

Zhang XL, Lu ZS, Tang P, Kong JY, Yang YS. Current application situation of gastrointestinal endoscopy in China. *World J Gastroenterol* 2013; 19(19): 2950-2955 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2950.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2950>

## INTRODUCTION

Endoscopy is a universally popular, minimally invasive intervention for gastrointestinal (GI) and pancreato-biliary disorders<sup>[1]</sup>. It is reported that > 10 million GI endoscopies are performed every year in the United States<sup>[2]</sup>, and the number of procedures worldwide, although there are no exact figures, is believed to be increasing yearly due to the rapid increase in popularity of GI endoscopy. Although GI endoscopy services have become a routine procedure in western countries<sup>[3]</sup>, in most developing countries GI endoscopy services are sometimes available in so-called centres of excellence<sup>[4]</sup>.

In the past decade, the emergence and application of a variety of novel endoscopic techniques and equipment, *e.g.*, video capsule endoscopy (VCE) and double/single balloon enteroscopy (DBE/SBE) have substantially promoted the diagnostic value for GI tract lesions<sup>[5-7]</sup>. Additionally, also in the past decade, GI endoscopy has experienced rapid evolution from a diagnostic medical procedure to a minimally invasive therapeutic procedure, *e.g.*, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) for removal of mucosal lesions<sup>[8-10]</sup>. All these advances in GI endoscopy have ushered in a new era in digestive medicine. However, the systemic data concerning the current status and development of GI endoscopy in China is still lacking. Here, we conducted a survey of GI endoscopy procedures and equipment in hospital-based GI endoscopy units, in order to demonstrate the rapid development of GI endoscopy in mainland China in the past decade.

## MATERIALS AND METHODS

### Survey design

According to the scale (bed number) and location, hospitals in mainland China are traditionally divided into three levels: provincial (bed numbers > 1000/hospital, usually located in the provincial capital city), prefecture (bed numbers 500-1000/hospital, usually located in the prefecture capital city) and county (bed numbers < 500/hospital, usually located in county city) level; or large scale (*i.e.*, provincial) and small-to-medium scale (*i.e.*, prefecture and county level). The endoscopy units from three levels of hospitals are regarded as provincial, prefecture and county level endoscopy units, respectively.

From 12 August, 2011 to 15 February, 2012, 289 GI endoscopy units, which are official members of the Chinese Society of Digestive Endoscopy, were included in the present study. All the surveyed GI endoscopy units were state-owned and hospital-based. They were required to fill in a questionnaire. The questionnaires were sent by e-mail to the physician in charge of the unit, and if an e-mail address was unavailable or invalid, a phone call was made to complete the questionnaire. The draft comprised 21 questions about the endoscopy equipment and procedures performed in the units. The queries pertained to: the number of GI endoscopy procedures performed per year; the number and brands of all kinds of GI endo-

scopes; the number of separate purpose-designed rooms for endoscopy procedures; the number of the full-time GI endoscopy physicians and nurses; the major auxiliary endoscopy equipment (*e.g.*, X-ray machine); which endoscopy procedure they could perform [*e.g.*, endoscopic retrograde cholangiopancreatography (ERCP), EMR and ESD].

### Ethical considerations

The study was approved by the Ethics Committee of Chinese PLA General Hospital.

### Statistical analysis

Proportions were compared using  $\chi^2$  tests. Comparisons between groups were performed using the Mann-Whitney *U* test. A probability of  $P < 0.05$  was considered to represent a statistically significant difference. Statistical analysis was performed using SPSS version 13.0 (Chicago, IL, United States).

## RESULTS

### Responding GI endoscopy units and their locations

Based on provision of a satisfactory reply, 169/289 (58.5%) GI endoscopy units from three levels of hospital were enrolled in our study, which covered 28 provinces (90.3%, 28/31) in mainland China. Of these, 44 (26.0%, 44/169) units were from provincial hospitals, 91 (53.8%, 91/169) from prefecture level hospitals, and 34 (20.1%, 34/169) from county level hospitals.

### Number of endoscopy procedures per year

All 44 provincial endoscopy units performed  $\geq 5000$  procedures, which was significantly higher than the prefecture-level (16.5%,  $P < 0.05$ ) and county-level (0.0%,  $P < 0.05$ ) endoscopy units (Table 1).

### Number of endoscopes per unit

The average number of gastrointestinal endoscopes (including all types of GI endoscope) for the 169 endoscopy units was 9.3/unit (1568/169). The average number of endoscopes in the provincial endoscopy units was  $22.4 \pm 5.5$ , which was significantly higher than that in the prefecture level ( $5.4 \pm 1.4$ ,  $P < 0.05$ ) and county level ( $2.7 \pm 1.2$ ,  $P < 0.05$ ) units. Moreover, 59.1% (26/44) of the provincial endoscopy units had at least 10 endoscopes, which was also significantly higher than that of the prefecture level (6.6%,  $P < 0.05$ ) and county level (0.0%,  $P < 0.05$ ) units (Table 1).

All of the 169 endoscopy units possessed gastroscopies. The possession rate of colonoscopies in provincial and prefecture level units was 100% and 97.8%, respectively, which was significantly higher than that of county level units (88.2%,  $P < 0.05$ ). Enteroscopies (DBE/SBE) were available only in provincial units (40.9%, 18/44) and a VCE was possessed by 20.7% (35/169) of all GI units (Table 1).

### Endoscope manufacturers

The most frequently used GI endoscopes were manufac-

**Table 1 Comparison of endoscopy items at three levels of endoscopy units *n* (%) / (mean  $\pm$  SD)**

| Endoscopy unit                         | Provincial<br>( <i>n</i> = 44) | Prefecture-level<br>( <i>n</i> = 91) | County-level<br>( <i>n</i> = 34) | Total<br>( <i>n</i> = 169) |
|--|--------------------------------|--------------------------------------|----------------------------------|----------------------------|
| Procedures per year                    |                                |                                      |                                  |                            |
| > 5000                                 | 44 (100.0)                     | 15 (16.5)                            | 0 (0.0)                          | 59 (34.9)                  |
| 3000-5000                              | 0 (0.0)                        | 65 (71.4)                            | 3 (8.8)                          | 69 (40.2)                  |
| < 3000                                 | 0 (0.0)                        | 11 (12.1)                            | 31 (91.2)                        | 42 (24.9)                  |
| Average number of endoscopes           | 22.4 $\pm$ 5.5 <sup>a</sup>    | 5.4 $\pm$ 1.4                        | 2.7 $\pm$ 1.2                    | 9.3 $\pm$ 3.2              |
| $\geq 10$                              | 26 (59.1) <sup>a</sup>         | 6 (6.6)                              | 0 (0.0)                          | 32 (18.9)                  |
| endoscopes/unit                        |                                |                                      |                                  |                            |
| Gastroscope                            | 44 (100.0)                     | 91 (100.0)                           | 34 (100.0)                       | 169 (100.0)                |
| Colonoscope                            | 44 (100.0) <sup>a</sup>        | 89 (97.8) <sup>c</sup>               | 30 (88.2)                        | 163 (96.4)                 |
| DBE/SBE                                | 18 (40.9) <sup>a</sup>         | 0 (0.0)                              | 0 (0.0)                          | 18 (10.7)                  |
| EUS                                    | 31 (70.5) <sup>a</sup>         | 7 (7.7)                              | 0 (0.0)                          | 38 (22.5)                  |
| VCE                                    | 28 (63.6) <sup>a</sup>         | 7 (7.7)                              | 0 (0.0)                          | 35 (20.7)                  |
| Average number of procedure rooms      | 4.9 $\pm$ 1.4 <sup>a</sup>     | 2.6 $\pm$ 1.3 <sup>c</sup>           | 1.6 $\pm$ 0.8                    | 3.6 $\pm$ 1.8              |
| Average number of full-time physicians | 3.0 $\pm$ 1.3 <sup>a</sup>     | 1.6 $\pm$ 1.2                        | 1.2 $\pm$ 0.9                    | 1.9 $\pm$ 1.4              |
| Average number of full-time nurses     | 5.4 $\pm$ 1.3 <sup>a</sup>     | 2.2 $\pm$ 1.1                        | 1.3 $\pm$ 0.8                    | 2.8 $\pm$ 1.3              |
| X-ray machine                          | 19 (43.2) <sup>a</sup>         | 14 (15.4) <sup>c</sup>               | 0 (0.0)                          | 33 (19.5)                  |
| Polypectomy                            | 44 (100.0) <sup>a</sup>        | 71 (78.0) <sup>c</sup>               | 14 (41.2)                        | 129 (76.3)                 |
| ERCP                                   | 34 (77.3) <sup>a</sup>         | 39 (42.9) <sup>c</sup>               | 4 (11.8)                         | 77 (45.6)                  |
| EMR                                    | 30 (68.2) <sup>a</sup>         | 20 (22.0) <sup>c</sup>               | 2 (5.9)                          | 52 (30.8)                  |
| ESD                                    | 16 (36.4) <sup>a</sup>         | 9 (9.9)                              | 0 (0.0)                          | 25 (14.8)                  |
| EVS/EVL                                | 33 (75.0) <sup>a</sup>         | 37 (40.7) <sup>c</sup>               | 3 (8.8)                          | 73 (43.2)                  |

<sup>a</sup>*P* < 0.05 vs prefecture-level or county-level endoscopy units; <sup>c</sup>*P* < 0.05 vs county-level endoscopy units. EUS: Endoscopic ultrasonography; VCE: Video capsule endoscope; DBE/SBE: Double balloon/single balloon enteroscopy; ERCP: Endoscopic retrograde cholangiopancreatography; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection; EVS: Endoscopic variceal sclerotherapy; EVL: Endoscopic variceal ligation.

tured by Olympus (140/169, 82.8%), followed by Fujinon (52/169, 30.8%) and Pentax (28/169, 16.6%).

#### Number of full-time staff in GI endoscopy units

The average number of full-time physicians in each provincial endoscopy unit was 3.0  $\pm$  1.3, which was significantly higher than that in prefecture level (1.6  $\pm$  1.2, *P* < 0.05) and county level (1.2  $\pm$  0.9, *P* < 0.05) endoscopy units. A similar trend was found for the numbers of full-time nurses in these three levels of endoscopy units (Table 1).

#### Possession of X-ray machine in GI endoscopy units

Thirty three of the 169 (19.5%, 33/169) endoscopy units possessed an X-ray machine. Furthermore, 43.2% of the provincial endoscopy units owned an X-ray machine, which was significantly higher than that of prefecture level (15.4%, *P* < 0.01) and county level (0.0%, *P* < 0.01) endoscopy units (Table 1).

#### Endoscopy procedures performed

Polypectomy could be performed by all the provincial units (100%), 78.0% of the prefecture level units and 41.2% of county level units. ERCP could be performed by 77.3% of the provincial units, which was significantly higher than

**Table 2 Comparison of main endoscopy items in three independent surveys**

| Endoscopy items                   | Shanghai survey<br>(2001) | Qinghai survey<br>(2003) | Present survey<br>(2011) |
|-----------------------------------|---------------------------|--------------------------|--------------------------|
| Number of units                   | 138.0                     | 37.0                     | 169.0                    |
| Average number of endoscopes/unit | 3.3                       | 1.4                      | 9.3                      |
| Possession rate                   |                           |                          |                          |
| Gastroscope                       | 100.00%                   | 100.00%                  | 100%                     |
| Colonoscope                       | 70.30%                    | -                        | 97.60%                   |
| Enteroscope                       | 4.30%                     | 0.00%                    | 10.60%                   |
| EUS                               | 7.20%                     | 0.00%                    | 22.50%                   |
| VCE                               | -                         | 2.70%                    | 20.70%                   |
| X-ray machine                     | 5.70%                     | -                        | 19.50%                   |
| Procedures                        |                           |                          |                          |
| Polypectomy                       | 54.30%                    | -                        | 76.30%                   |
| EVS/EVL                           | 40.20%                    | -                        | 43.20%                   |
| ERCP                              | 38.40%                    | -                        | 45.60%                   |

EUS: Endoscopic ultrasonography; VCE: Video capsule endoscope; ERCP: Endoscopic retrograde cholangiopancreatography; EVS: Endoscopic variceal sclerotherapy; EVL: Endoscopic variceal ligation.

in the prefecture level (42.9%, *P* < 0.05) and county level (11.7%, *P* < 0.01) units. A similar trend was also found for EMR, ESD and endoscopic variceal sclerotherapy (EVS)/endoscopic variceal ligation (EVL) (Table 1).

#### Comparisons of gastrointestinal endoscopy between current survey and previously published data

From January 2000 to November 2010, only two regional endoscopy surveys (*i.e.*, Shanghai survey in 2001 and Qinghai provincial survey in 2003) in mainland China were available by searching the Chinese national (Wanfang bases) and international databases (Medline), and they were both published in Chinese<sup>[11,12]</sup>. The major GI endoscopy items mentioned by these two regional surveys were compared with the current survey results, and a rapid development of GI endoscopy items, including the average number of GI endoscopes per unit, was shown in Table 2.

## DISCUSSION

In 1973, GI endoscopy was first introduced from Japan into China. Since then, much progress in GI endoscopy has been made by Chinese endoscopists, and the efforts and achievements of Chinese endoscopists in GI endoscopy have gradually gained international recognition<sup>[13-16]</sup>. For example, with regard to the latest endoscopy techniques, EUS, ERCP, VCE and DBE have been the focus for Chinese endoscopists, and they have accounted for 66% of all the international publications<sup>[17]</sup>.

Although the Shanghai survey in 2001 and the Qinghai provincial survey in 2003 were from single and different areas in China, which means lower comparability with the present survey here we use them to demonstrate the progress of GI endoscopy facilities in the past decade in mainland China because these are the only journal publications available<sup>[11,12]</sup>.

The total number of GI endoscopes per unit is an important and determinant factor for what kinds of and how many endoscopy procedures they can perform. In the present survey, the average number of GI endoscopes per unit increased nearly threefold (9.3/unit) compared with that of previously published data ten years ago (the average number for Shanghai and Qinghai surveys was 2.9/unit). This is major progress in mainland China in the past decade.

VCE was first reported in 2000 by an Israeli company, Given Imaging<sup>[5]</sup>, and it is predicted that major developments in endoscopy over the next 10-20 years will centre on this technique<sup>[18]</sup>. In the past eight years, the possession rate of VCE has markedly increased from 2.7% (Qinghai survey, 2003) to 20.7% in the present survey.

EUS and small-intestine enteroscopy have been used in clinical practice for many years<sup>[19,20]</sup>, but in the past decade, these two types of endoscopy have developed rapidly. For example, DBE, which has evolved from conventional push enteroscopy, was first introduced into clinical practice in 2001 by Yamamoto *et al*<sup>[21]</sup>. DBE is a completely new technique that allows complete visualization, biopsy and treatment of small-bowel diseases. In the Shanghai survey, the rates for EUS and push-type enteroscopy were 4.3% and 7.2%, respectively, but in the present survey, the possession rates had increased to 10.6% and 22.4%, respectively. However, these two types of endoscope were still mainly owned by provincial hospitals in mainland China, which was largely due to their high cost and fewer indications for such procedures in small-to-medium endoscopy units (*i.e.*, prefecture and county level units).

Another important piece of endoscopy equipment, especially for large scale units, is a dedicated X-ray machine, which is necessary for a variety of GI interventions, including ERCP, percutaneous trans-hepatic catheter drainage, luminal stent placements and dilation<sup>[22]</sup>. In the Shanghai survey, the rate of possession of a dedicated X-ray machine (not mentioned in the Qinghai survey) was 5.7% (8/138), and the rate has increased to 19.5% in the present survey. Our data also demonstrated that Olympus was the leading manufacturer of GI endoscopes in mainland China, which is in accordance with the fact that Tokyo-based Olympus is the world's largest manufacturer and provider of conventional endoscopes<sup>[23]</sup>.

Staffing requirements for GI procedures should be based on what is needed to ensure safe and proficient performance of the individual procedure<sup>[24]</sup>. As the number of procedures carried out and the complexity of the procedures and equipment have increased, the need for specialised staff has become apparent. Our survey indicated that most full-time endoscopy staff were found in provincial hospitals in mainland China.

Compared with the Shanghai survey in 2001, easily performed therapeutic procedures, such as polypectomy, have become more popular in the past 10 years; even in small-to-medium endoscopy units in China. Another conventional procedure is ERCP, which was first performed

in 1968 and is usually regarded as the representative endoscopic intervention in pancreaticobiliary disorders<sup>[25]</sup>. In 1973, ERCP was introduced to China, and since then it has been extensively used. It is estimated that Chinese physicians perform nearly 60000 ERCP procedures annually, including therapeutic ERCP<sup>[17]</sup>. Our survey showed that, in China, ERCP (diagnostic and/or therapeutic) was a frequently performed procedure not only in provincial hospitals (77.3%), but also in prefecture level hospitals (42.9%). In the Shanghai survey in 2001, the overall rate of capability of performing ERCP was 38.4%, but now the nationwide rate has increased to 45.6%.

EVS or EVL is an effective endoscopic procedure for treatment of oesophageal varices<sup>[26]</sup>, and it has been frequently performed in China because of the large number of patients with oesophagogastric variceal bleeding<sup>[27]</sup>. The Shanghai survey showed that 40.2% of the surveyed endoscopy units can perform an EVS procedure. According to the present survey, EVS/EVL is now frequently carried out in provincial (75.0%) and prefecture level (40.7%) units (Table 2); but in county level units (9.0%), there is still room to increase the popularity of this procedure, with regard to its simple operation without special devices. EMR and ESD were developed in the past decade as a novel therapeutic endoscopic procedure to remove the mucosal lesions, including early malignant lesion<sup>[8,9]</sup>. Our survey demonstrated that EMR or ESD is also predominantly confined to the large units (provincial level) in mainland China, which may be due to the shortage of special devices in small-to-medium endoscopy units, and the procedure itself is technically demanding.

In conclusion, rapid developments have taken place in GI endoscopy in China in the past decade. Major diagnostic endoscopes and therapeutic endoscopy procedures are predominantly confined to large endoscopy units (*i.e.*, provincial hospitals), whereas small-to-medium units, often perform fewer endoscopic procedures and have less equipment, and are mostly restricted to diagnostic endoscopy. In addition to improvements in GI endoscopy equipment, standard procedures including the standard reprocessing for endoscopy will be the focus in the future in China<sup>[28]</sup>. Therefore, there is still much room for improvement in GI endoscopy in China, and our results may provide crucial information needed for the national level GI endoscopy planning.

## ACKNOWLEDGMENTS

We thank Professor Yan-Qing Li (Department of Gastroenterology, Qilu Hospital, Shandong University, Jinan, Shandong Province), Professor Ming-Jun Sun (Department of Gastrointestinal Endoscopy, the First Affiliated Hospital, China Medical University, Shenyang, Liaoning Province), Professor Xiao-Ping Wu (Department of Gastroenterology, the Second Hospital, Central South University, Changsha, Hunan Province), Professor Xue-Hong Wang (Department of Gastroenterology, the Affiliated Hospital, Qinghai University, Xining, Qinghai

Province), Hai Xing Jiang (Department of Gastroenterology, the First Affiliated Hospital of Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region), Yu-Xiu Yang (Department of Gastroenterology, the People's Hospital of Henan Province, Zhengzhou, Henan Province) and Professor Yang Li (Department of Gastroenterology, Ningxia Medical University, Yinchuan, Ningxia Hui Autonomous Region) for their commitment to the survey, and we are indebted to all our colleagues who have taken the time to answer this survey.

## COMMENTS

### Background

Endoscopy is a universally popular, minimally invasive intervention for gastrointestinal (GI) and pancreatico-biliary disorders. Although GI endoscopy services have become a routine procedure in western countries, in most developing countries GI endoscopy services are sometimes available in so-called centres of excellence.

### Research frontiers

In the past decade, the emergence of a variety of novel endoscopic equipment and techniques, *e.g.*, video capsule endoscopy (VCE), double/single balloon enteroscopy, endoscopic mucosal resection and endoscopic submucosal dissection (ESD) have substantially promoted the application value for GI tract lesions worldwide. GI endoscopy is experiencing rapid evolution from a diagnostic medical procedure to a minimally invasive therapeutic procedure.

### Innovations and breakthroughs

The authors conducted a survey of GI endoscopy procedures and equipment in hospital-based GI endoscopy units, in order to demonstrate the current status of GI endoscopy in mainland China in the past decade. Based on provision of a satisfactory reply, 169/289 (58.5%) GI endoscopy units from three levels of hospital were enrolled in their study, which covered 28 provinces (90.3%, 28/31) in mainland China.

### Applications

The authors found that the number of GI endoscopes per unit increased by nearly three times (from 2.9 to 9.3) in the past decade. The VCE, which was almost completely unavailable ten years ago, was possessed by 20.7% (35/169) of GI endoscopy units. ESD could be performed by 36.4% (19/44) of the provincial units, which was significantly higher than the prefecture level (9.9%) and county level (0.0%) units, respectively.

### Terminology

The survey of GI endoscopy included equipment and procedures performed in each GI endoscopy unit. The questionnaires comprised 21 questions which were sent by e-mail to the physician in charge of the unit.

### Peer review

This is a very important survey reflecting the development of GI endoscopy in mainland China in the past decade.

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**P- Reviewers** Dinis-Ribeiro M, Dumonceau JM  
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## Intraperitoneal perfusion of cytokine-induced killer cells with local hyperthermia for advanced hepatocellular carcinoma

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Author contributions: Wang XP and Xu M contributed equally to this work; Xu M and Xu KC designed research; Wang XP and Xu M performed research; Zhao JF provided new reagents or analytic tools; Gao HF analyzed data; Wang XP and Xu M wrote the paper.

Supported by The National Natural Science Foundation of China, No.81273814

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Received: January 23, 2013 Revised: March 24, 2013

Accepted: April 10, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To study the effect and tolerance of intraperitoneal perfusion of cytokine-induced killer (CIK) cells in combination with local radio frequency (RF) hyperthermia in patients with advanced primary hepatocellular carcinoma (HCC).

**METHODS:** Patients with advanced primary HCC were included in this study. CIK cells were perfused intraperitoneal twice a week, using  $3.2 \times 10^9$  to  $3.6 \times 10^9$  cells each session. Local RF hyperthermia was performed 2 h after intraperitoneal perfusion. Following an interval of one month, the next course of treatment was administered. Patients received treatment until disease progression. Tumor size, immune indices (CD3<sup>+</sup>, CD4<sup>+</sup>,

CD3<sup>+</sup>CD8<sup>+</sup>, CD3<sup>+</sup>CD56<sup>+</sup>), alpha-fetoprotein (AFP) level, abdominal circumference and adverse events were recorded. Time to progression and overall survival (OS) were calculated.

**RESULTS:** From June 2010 to July 2011, 31 patients diagnosed with advanced primary HCC received intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia in our study. Patients received an average of  $4.2 \pm 0.6$  treatment courses (range, 1-8 courses). Patients were followed up for  $8.3 \pm 0.7$  mo (range, 2-12 mo). Following combination treatment, CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD56<sup>+</sup> cells increased from  $35.78\% \pm 3.51\%$ ,  $24.61\% \pm 4.19\%$  and  $5.94\% \pm 0.87\%$  to  $45.83\% \pm 2.48\%$  ( $P = 0.016$ ),  $39.67\% \pm 3.38\%$  ( $P = 0.008$ ) and  $10.72\% \pm 0.67\%$  ( $P = 0.001$ ), respectively. AFP decreased from  $167.67 \pm 22.44$  to  $99.89 \pm 22.05$  ng/mL ( $P = 0.001$ ) and abdominal circumference decreased from  $97.50 \pm 3.45$  cm to  $87.17 \pm 4.40$  cm ( $P = 0.002$ ). The disease control rate was 67.7%. The most common adverse events were low fever and slight abdominal erubescence, which resolved without treatment. The median time to progression was 6.1 mo. The 3-, 6- and 9-mo and 1-year survival rates were 93.5%, 77.4%, 41.9% and 17.4%, respectively. The median OS was 8.5 mo.

**CONCLUSION:** Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia is safe, can efficiently improve immunological status, and may prolong survival in HCC patients.

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**Key words:** Cytokine-induced killer cell; Radio frequency hyperthermia; Primary hepatocellular carcinoma; Intraperitoneal perfusion; Clinical observation

**Core tip:** Intraperitoneal perfusion of cytokine-induced killer (CIK) cells in combination with local radio frequency hyperthermia can result in a high concentration of

CIK cells. This treatment can efficiently improve immunological status, and attack small lesions in the abdominal wall, which can reduce ascites and relieve abdominal distention. This comprehensive treatment may prolong survival time and improve quality of life in patients with advanced hepatocellular carcinoma.

Wang XP, Xu M, Gao HF, Zhao JF, Xu KC. Intraperitoneal perfusion of cytokine-induced killer cells with local hyperthermia for advanced hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(19): 2956-2962 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2956.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2956>

## INTRODUCTION

Primary hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide and has a poor prognosis. Surgical resection at an early stage is still the best remedy<sup>[1]</sup>. As the onset of HCC is occult, it is typically diagnosed in stage III-IV when patients present with clinical symptoms. However, the surgical resection rate is only 10%-30%. Currently, there is still no standard treatment for advanced HCC, but minimally invasive therapy and targeted therapy are favored. Patients with HCC may have poor therapeutic outcomes due to multiple local lesions or excessive tumor load. Consequently, integrative therapy is now a useful treatment for patients with advanced HCC<sup>[2]</sup>.

Adoptive cellular immunotherapy has been applied in the clinical treatment of advanced HCC due to the close relationship between the pathogenesis of HCC and the autoimmune system, and the persistence of pathogenic factors such as hepatitis and cirrhosis<sup>[3-5]</sup>. Cellular immunotherapy includes several types of immunological cells, such as lymphokine-activated killer cells, tumor infiltrating lymphocytes and cytokine-induced killer (CIK) cells. CIK cells have been confirmed to have potential for immunotherapy against residual tumor cells. In recent years, several authors have reported that CIK cells were a heterogeneous population, and the major population to express both T cell marker monoclonal antibody and natural killer cell marker monoclonal antibody<sup>[6]</sup>. Cells with this phenotype are rare (1%-5%) in natural peripheral blood mononuclear cells. CIK cells are able to expand nearly 1000-fold when cultured in a cytokine cocktail, comprising interleukin (IL)-1 $\alpha$ , interferon- $\gamma$  (IFN- $\gamma$ ), IL-2 and mAbs against CD3, and have characteristics which are more effective in the treatment of tumors with a non-major histocompatibility complex (MHC)-restricted mechanism<sup>[7]</sup>. Thus, CIK cells may have some benefit in potential immunotherapeutic treatments in patients with HCC.

Hyperthermia treatment of tumors refers to tumor tissue which is treated with a continuous direct current through two or more electrodes placed outside the tumor. Some researchers have suggested that hyperthermia can

normalize cell growth, and accelerate cell division after inhibiting cell division when it becomes abnormally accelerated<sup>[8,9]</sup>. Hyperthermia is an alternative treatment for HCC patients and has a positive effect on tumors. This study aimed to evaluate the safety and efficacy of the combination of CIK cell therapy and local radio frequency (RF) hyperthermia in patients with advanced HCC.

## MATERIALS AND METHODS

### General information

From June 2010 to July 2011, 31 patients with advanced HCC in the Oncology Center of Clifford Hospital were included in this study. Seven patients were identified by pathological diagnosis and the remaining patients all met the clinical diagnostic criteria<sup>[10]</sup>. Staging of disease was by tumor-node-metastasis staging according to the American Joint Committee on Cancer staging criteria<sup>[11]</sup>. All patients were stage III-IV without metastases outside the abdomen. Reasons for being unable to undergo surgery and transcatheter arterial chemoembolization (TACE) were as follows: multiple tumors with obscure boundaries, extremely small remnant liver, lesions close to or involving great vessels, extreme damage to the cardiovascular system or other organs, liver functional lesion and rejection of surgery or TACE. Written informed consent was obtained before treatment.

### Instrument and reagents

The instrument used was the FACS Calibur Flow cytometer (manufactured by BD Biosciences, United States). The reagents required for incubation of CIK cells were as follows: IL-1 $\alpha$  (10  $\mu$ g/vial, PeproTech Inc., Rocky Hill, NJ, United States), monoclonal mouse anti-human CD3 (500  $\mu$ g/vial, PeproTech Inc.), IFN- $\gamma$  (1000000 U/vial, PeproTech Inc.), IL-2 (PeproTech Inc.), complete Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, RPMI 1640 + gentamicin + 5% human type AB serum), lymphocyte separation medium (Tianjin Biological Products Company, Tianjin, China), human type AB serum (Tianjin Biological Products Company) and normal saline.

### Preparation of CIK cells

CIK cells were prepared by the Cell Laboratory as follows: mononuclear cells were isolated from 50 mL peripheral blood from each patient using a blood cell separator, and then incubated in medium containing 1000 U/mL IFN- $\gamma$  at 37 °C; 24 h later 100 ng/mL monoclonal mouse anti-human CD3, 1000 U/mL IL-2 and 1000 U/mL IL-1 $\alpha$  were added; the culture medium was renewed every 3 d and rhIL-2 was replenished. After 7-10 d, CIK cells were collected when the cell count reached  $3.2 \times 10^9$  to  $3.6 \times 10^9$ , centrifuged, washed with normal saline, dispersed in 100 mL normal saline (containing 5 mL 20% human serum albumin) and retained as a sample.

### Abdominocentesis

Routine abdominocentesis was performed before intraperitoneal perfusion of CIK cells and a tube was left

**Table 1** Clinical characteristics of the patients included in this study (*n* = 31)

| Characteristics               | <i>n</i> (%)       |
|-------------------------------|--------------------|
| Patient                       |                    |
| Gender (M/F)                  | 22/9               |
| Age, yr (range)               | 47.6 ± 8.8 (28-61) |
| Stage status                  |                    |
| III                           | 18 (58.06)         |
| IV                            | 13 (41.93)         |
| Complications                 |                    |
| Seroperitoneum                | 14 (45.16)         |
| Hypoproteinemia               | 17 (54.83)         |
| Choloplania                   | 8 (25.80)          |
| ECOG performance status       |                    |
| 0                             | 3 (9.67)           |
| 1                             | 18 (58.06)         |
| 2                             | 10 (32.25)         |
| Past history before treatment |                    |
| Surgery                       | 4 (12.90)          |
| TACE                          | 7 (22.58)          |
| Chemotherapy                  | 1 (3.22)           |
| Surgery + TACE                | 8 (25.80)          |
| Surgery + chemotherapy        | 2 (6.45)           |
| TACE + chemotherapy           | 3 (9.67)           |
| Initial treatment             | 6 (19.35)          |

M: Male; F: Female; ECOG: Eastern Cooperative Oncology Group; TACE: Transcatheter arterial chemoembolization.

in the abdomen for perfusion. After abdominocentesis, 100-200 mL physiologic saline was perfused into the abdomen and observations on defecation sensation, discomfort, and whether the tube was smooth and in the correct position for the preparation of intraperitoneal perfusion of CIK cells were carried out. Patients with a recent history of surgery or suspicious ankyloenteron required ultrasonic guidance during abdominocentesis.

### Local RF hyperthermia

The machine used was an EHY-2000 local RF Hyperthermia Machine, made in Hungary. Health education and psychological preparation were carried out prior to treatment. Patients were evaluated for dysmetabolism and disturbed perception of temperature. Patients were asked to lie on the treatment water bed in the correct body position according to treatment requirements. An appropriate electrode plate size was chosen to cover the body surface projection area of the tumor. Treatment time was 60 min per session and power was 100-150 W (according to the tolerance level of patients).

### Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia

Intraperitoneal perfusion of CIK cells was performed twice a week (Monday and Thursday), with  $3.2 \times 10^9$  to  $3.6 \times 10^9$  cells each session. Local RF hyperthermia was carried out 2 h after intraperitoneal perfusion. In one treatment course consisting of 4 sessions,  $1.2 \times 10^{10}$  to  $1.5 \times 10^{10}$  cells were perfused. After an interval of one month, the next treatment course was administered, resulting in a time period of 1.5 mo for one cycle. Patients received

treatment until disease progression or intolerant adverse effects.

### Follow-up

Patients were followed up for 1 year. The treatment process, adverse reactions, and lost cases were recorded. A computed tomography (CT) or magnetic resonance imaging scan of the liver was performed every 2 mos. The size and number of tumors before and after treatment were compared. The therapeutic effect was evaluated and recorded. Levels of peripheral blood T lymphocyte subgroups ( $CD3^+$ ,  $CD4^+$ ,  $CD3^+CD8^+$  and  $CD3^+CD56^+$ ), abdominal circumference (patients with seroperitoneum) and AFP level were determined every 2 wk. Time to progression (TTP) and overall survival (OS) were calculated.

### Statistical analysis

Results were analyzed using SPSS 17.0 software. The data were expressed as mean ± SD. The survival curves were calculated using the Kaplan-Meier method. *P* < 0.05 was considered statistically significant.

## RESULTS

### Patient profile

From June 2010 to July 2011, a total of 31 patients aged 28-61 years (mean  $47.6 \pm 8.8$  years) were included in this study. The characteristics of the patients are shown in Table 1.

### Efficacy evaluation

According to the Response Evaluation Criteria In Solid Tumors, none (0%) of the patients had a complete response, 10 (32.25%) patients had a partial response (PR), 11 (35.48%) patients had no change (SD), and 10 (32.25%) patients had progressive disease. After treatment, AFP level decreased from  $167.67 \pm 22.44$  to  $99.89 \pm 22.05$  ng/mL (*P* = 0.001) and seroperitoneum in 14 patients significantly decreased, with abdominal circumference decreasing from  $97.50 \pm 3.45$  to  $87.17 \pm 4.40$  cm (*P* = 0.002).

### Changes in cell immunity indices

The immunologic markers examined included the serum levels of  $CD3^+$ ,  $CD4^+$ ,  $CD3^+CD8^+$  and  $CD3^+CD56^+$  T cells. After CIK cells were transfused back into the patients, all of these immune parameters increased, but not all of them increased significantly (Table 2).

### Adverse events

No serious adverse events were observed in this study. Several mild adverse events were observed, which rapidly resolved without treatment (Table 3).

### TTP and OS

TTP and OS were assessed in all 31 eligible patients. The median follow-up was  $8.3 \pm 0.7$  mo (range, 2-12 mo). At the time of the analysis, 25 patients were dead and 6 patients were alive. The median TTP was 6.1 mo (95%CI:

**Table 2** Immunity indices before and after treatment

| Group                              | Pre-treatment  | Post-treatment | P value |
|------------------------------------|----------------|----------------|---------|
| CD3 <sup>+</sup>                   | 70.44% ± 6.68% | 72.67% ± 6.22% | 0.55    |
| CD4 <sup>+</sup>                   | 35.78% ± 3.51% | 45.83% ± 2.48% | 0.016   |
| CD3 <sup>+</sup> CD8 <sup>+</sup>  | 24.61% ± 4.19% | 39.67% ± 3.38% | 0.008   |
| CD3 <sup>+</sup> CD56 <sup>+</sup> | 5.94% ± 0.87%  | 10.72% ± 0.67% | 0.001   |

**Table 3** Treatment-related adverse events (*n* = 31)

| Side effects              | <i>n</i> (%) |
|---------------------------|--------------|
| Abdominal side effects    |              |
| Abdominal pain            | 3 (9.67)     |
| Abdominal erubescence     | 5 (16.12)    |
| Abdominal lesser tubercle | 1 (3.22)     |
| Systemic side effects     |              |
| Slight fever              | 4 (12.90)    |
| Dizziness                 | 3 (9.67)     |
| Debilitation              | 6 (19.35)    |
| Nausea or vomiting        | 1 (3.22)     |
| Diarrhea                  | 2 (6.44)     |
| Tachycardia               | 1 (3.22)     |

4.8-7.2). The 3-, 6- and 9-mo and 1-year survival rates were 93.5%, 77.4%, 41.9% and 17.4%, respectively. The median OS was 8.5 mo (Figure 1).

### Typical case 1

A 57-year-old male was diagnosed with primary HCC by CT-guided aspiration biopsy of the liver in July 2010. Diffuse lesions in the liver with portal vein encroachment were observed on the CT scan. The patient also had complications of abdominal distention, debilitation, lack of appetite and emaciation. Eastern Cooperative Oncology Group (ECOG) was 2. AFP level was 201 ng/mL, alanine aminotransferase (ALT) 70 U/L, and aspartate aminotransferase (AST) 120 U/L. T lymphocyte subgroups were CD3<sup>+</sup> 76%, CD4<sup>+</sup> 22%, CD3<sup>+</sup>CD8<sup>+</sup> 17%, and CD3<sup>+</sup>CD56<sup>+</sup> 5.6%, respectively. The patient received 3 cycles of intraperitoneal CIK cells in combination with local RF hyperthermia. The patient achieved a PR. AFP fell to 98 ng/mL and ECOG increased to 1. ALT and AST fell to normal levels. T lymphocyte subgroups were CD3<sup>+</sup> 77%, CD4<sup>+</sup> 34%, CD3<sup>+</sup>CD8<sup>+</sup> 27% and CD3<sup>+</sup>CD56<sup>+</sup> 9.1%, respectively (Figure 2A and B).

### Typical case 2

A 51-year-old male was admitted to hospital due to abdominal distention and pain. The patient was diagnosed with primary hepatic carcinoma in December 2012, and underwent surgery in a local hospital with postoperative Sorafenib therapy. The patient had been experiencing obvious abdominal distention with abdominal pain, poor appetite, nausea, and emesis for 1 mo. Enhanced CT scanning showed multiple metastases in the liver, metastases in the portal lymph nodes and significant seroperitoneum. Due to the advanced stage of hepatic carcinoma, further surgical treatment and interventional therapies were inappropriate. Considering the failure

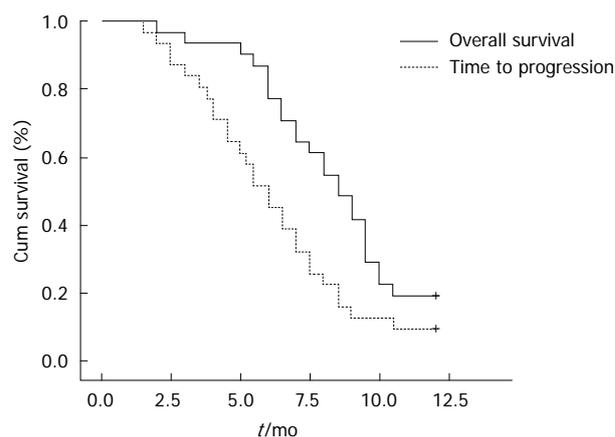


Figure 1 Time to progression and overall survival after treatment.

of targeted drug treatment, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia was administered to the patient. Before treatment, abdominal circumference was 97.5 cm. Ultrasound examination showed that the deepest portion of seroperitoneum was 8.16 cm. After 1 cycle of treatment with intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia, the patient noted that abdominal distention was significantly reduced, with a decrease in abdominal circumference to 93.0 cm and seroperitoneum decreased to 5.03 cm under ultrasound examination (Figure 2C and D).

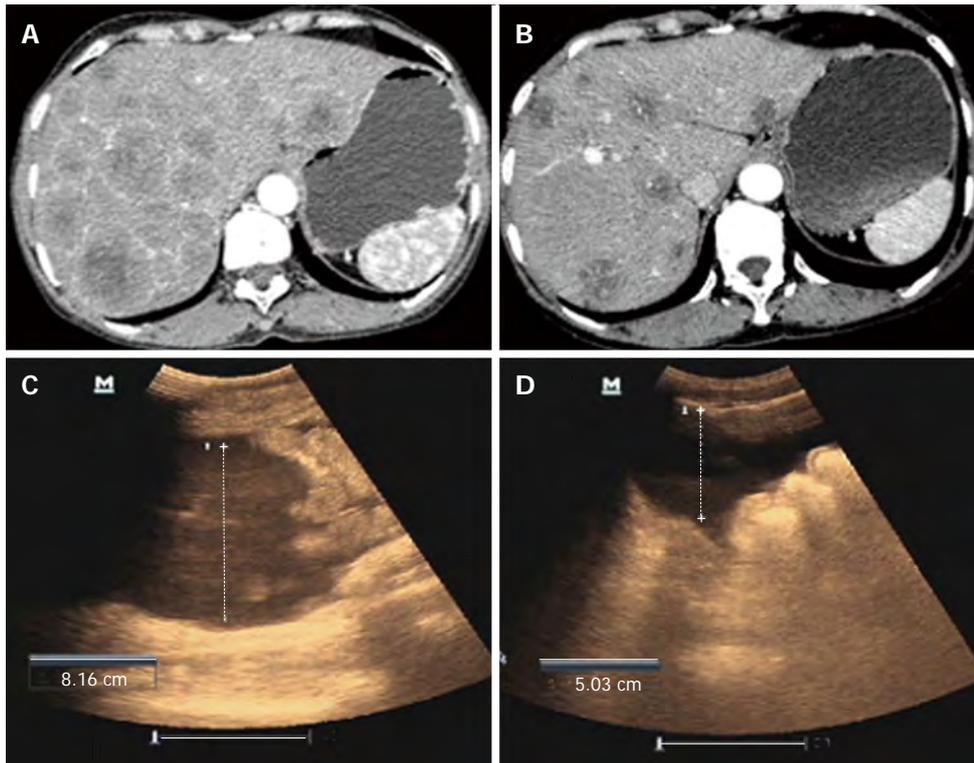
## DISCUSSION

Recently, targeted therapy and palliative chemotherapy have been the main treatment approaches for patients with advanced HCC. New targeted drugs such as sorafenib and sunitinib have improved clinical efficacy. However, the serious side effects and high costs of these drugs make it impossible for many patients to complete the entire treatment course<sup>[12,13]</sup>. Thus, it is necessary to investigate alternative therapies for patients with advanced HCC.

### Tumor killing ability of CIK cells

The special biological behavior of primary HCC, such as multiple lesions, existing hepatitis and cirrhosis, continuously suppresses cellular immunity and results in immune dysfunction. Autoimmune disorders in patients with HCC is manifested by lower levels of CD4<sup>+</sup> and CD3<sup>+</sup>CD56<sup>+</sup> cells and significantly higher levels of CD8<sup>+</sup> cells<sup>[14,15]</sup>. The ability to efficiently kill tumor cells is the ultimate requirement in immune effector candidates for adoptive immunotherapy. CIK cells have a MHC-independent tumor killing capacity in both solid and hematologic malignancies. The antitumor activity is mainly associated with a high percentage of the CD3<sup>+</sup>CD56<sup>+</sup> subset<sup>[16,17]</sup>. The exact mechanism involved in tumor recognition and killing is not completely known, but mainly involves the secretion of a cytokine to inhibit the growth of tumor cells<sup>[18-20]</sup>.

It has been demonstrated that CIK cells are effective against some solid malignancies including lung, gastro-



**Figure 2** Computed tomography scan. A: Typical case 1 with hepatocellular carcinoma before treatment; B: Typical case 1 with hepatocellular carcinoma after treatment; C: Typical case 2 with hepatocellular carcinoma before treatment; D: Typical case 2 with hepatocellular carcinoma after treatment.

intestinal and mesenchymal tumors both *in vitro* and *in vivo*<sup>[21,22]</sup>. Oliosio *et al*<sup>[23]</sup> reported 12 patients (6 advanced lymphomas, 5 metastatic kidney carcinomas and 1 HCC) who were intravenously transfused with  $28 \times 10^9$  (range,  $6 \times 10^9$  to  $61 \times 10^9$ ) CIK cells per patient during one cycle. The treatment schedule consisted of three cycles of CIK cell infusions at an interval of 3 wk. After treatment, the absolute median number of lymphocytes, CD3<sup>+</sup>, CD8<sup>+</sup> and CD3<sup>+</sup>CD56<sup>+</sup> cells significantly increased in peripheral blood. In our study, CD4<sup>+</sup>, CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup>CD56<sup>+</sup> cells increased after treatment from  $35.78\% \pm 3.51\%$ ,  $24.61\% \pm 4.19\%$  and  $5.94\% \pm 0.87\%$  to  $45.83\% \pm 2.48\%$  ( $P = 0.016$ ),  $39.67\% \pm 3.38\%$  ( $P = 0.008$ ) and  $10.72\% \pm 0.67\%$  ( $P = 0.001$ ), respectively. Our results were similar to those of Oliosio *et al*<sup>[23]</sup>.

#### **Intraperitoneal perfusion with local hyperthermia**

The most common causes of death in patients with advanced HCC are liver failure, ascites and obstruction. Control of the abdominal tumor is the principal goal of treatment, which can prolong survival and improve quality of life. Recently, intravenous infusion of CIK cells has been widely used in the treatment of HCC. However, the concentration of CIK cells is low in tumor tissues following intravenous infusion, and the anti-tumor effect is low. In this study, in order to achieve a high concentration of CIK cells in the abdomen, CIK cells were perfused intraperitoneally instead of intravenously. Intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia can improve efficacy in clinical practice. The primary reason for this may be that hyperthermia

accelerates the conjugation of CIK cells and tumor cells, which improves the reaction rate of CIK cells. Local RF hyperthermia after high-capacity perfusion of CIK cells improves the sensitivity of tumor cells, allowing CIK cells to permeate into tumor cells more effectively.

Local RF hyperthermia kills tumor cells by heating using physical energy and the temperature of tumor tissue rises to an effective treatment temperature. Studies have shown that high temperature can damage the membranes of mitochondria, lysosomes and endoplasmic reticulum and triggers the massive release of acid hydrolase from lysosomes, resulting in membranolysis, outflow of cytoplasm and death of cancer cells<sup>[24,25]</sup>. In addition, some studies have reported that hyperthermia improves immune function by stimulating the development of the anti-tumor immune effect and resolving the inhibition of blocking factors in the immune system<sup>[26]</sup>. The core points behind this treatment were to administer intraperitoneal perfusion of CIK cells to improve immune function to kill the tumor, and local hyperthermia to enhance the concentration of CIK cells which directly affect liver tumor tissues and small lesions on the abdominal wall, which can damage cancer cells and effectively reduce abdominal dropsy. Local hyperthermia plays an important role in enhancing the concentration of CIK cells which directly affect these parameters.

#### **Adverse events**

The most common adverse events in our study were low fever and slight abdominal erubescence, which resolved without treatment. No serious side effects were observed.

During the treatment process, 4 patients experienced low fever ranging from 37.5-38 °C, which resolved without treatment. This fever might have been caused by the application of IL-2. Some other symptoms, including erythema and lesser tubercle of abdominal subcutaneous fat, were observed, which were caused by high temperature and were relieved by simple symptomatic treatment. Intraperitoneal perfusion of autologous CIK cells can avoid immune rejection triggered by foreign cell infusion.

In conclusion, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia is safe and effective for advanced HCC. More clinical trials with a large sample size are warranted to provide evidence for further applications. The addition of palliative chemotherapy or targeted therapy is worthy of further investigation.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Recently, integrative therapy has become a useful treatment for patients with advanced HCC. Due to the close relationship between the pathogenesis of HCC and the autoimmune system, cellular immunotherapy has been used in the clinical treatment of advanced HCC. In addition, local hyperthermia increases the role of cytokine-induced killer (CIK) cells in killing tumor tissues.

### Research frontiers

Intraperitoneal perfusion of CIK cells in combination with local radio frequency (RF) hyperthermia can improve immune function in HCC patients, shrink the tumor, and relieve discomfort such as debilitation and abdominal distention.

### Innovations and breakthroughs

Recently, intravenous infusion of CIK cells has been widely used in the treatment of HCC. However, the concentration of CIK cells is low in tumor tissues following intravenous infusion, and the anti-tumor effect is low. In their research, intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia resulted in a high concentration of CIK cells in the abdominal cavity and effective killing of tumor tissues.

### Applications

The authors have been using this treatment strategy for more than 1 year. Forty-two patients accepted this treatment, of whom 31 were selected for this study. There were no serious adverse events following this treatment. A few patients showed low-grade fever or slight chest distress which resolved after symptomatic treatment. Following intraperitoneal perfusion of CIK cells in combination with local RF hyperthermia, abdominal distention resolved, tumor size diminished, and survival time was prolonged in some patients. This treatment strategy is worthy of further investigation.

### Terminology

CIK cells are alloplasmic cells with non-major histocompatibility complex (MHC) restriction anti-tumor activity which is acquired by multiple-cell factor-cultivation *in vitro* from human peripheral blood mononuclear cells. CD3<sup>+</sup>CD56<sup>+</sup> T is the main effector cell of the CIK cell group, and is also called nature killer cell-like T lympholeukocyte. These cells have powerful anti-tumor activity and MHC restriction of T lympholeukocytes. Local RF hyperthermia is used in the non-invasive treatment of malignant tissues. The difference between the complex dielectric constant (complex impedance) of malignant and healthy tissues makes it possible to select malignant tissues.

### Peer review

In this study, 31 patients with advanced HCC who could not be treated by surgical treatments or interventional therapies, were treated by CIK cell intraperitoneal perfusion in combination with RF local hyperthermia. The result showed that this treatment is safe and reliable, and worthy of further clinical studies of a larger sample. It is a new option of treatment for patients with advanced HCC.

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**P- Reviewers** Han SB, Introna M, Zhong H  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Xiong L



## Effect of Danzhi Jiangtang capsule on monocyte chemoattractant protein-1 mRNA expression in newly diagnosed diabetes subclinical vascular lesions

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Received: December 25, 2012 Revised: February 6, 2013

Accepted: February 28, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To investigate the effect of Danzhi Jiangtang capsule (DJC) on monocyte chemoattractant protein-1 (MCP-1) mRNA expression in newly diagnosed type 2 diabetes mellitus (T2DM) subclinical vascular lesions.

**METHODS:** Sixty-two patients with newly diagnosed T2DM subclinical vascular lesions were randomly divided into a control group and treatment group of 31 cases each. Oral antidiabetic therapy with routine western medicine was conducted in both groups, and the treatment group was additionally treated with DJCs. The treatment course for both groups was 12 wk. Before and after treatment, the total efficiency and traditional Chinese medicine (TCM) syndrome score were calculated. The fasting plasma glucose (FPG), 2-h plasma glucose (2hPG), fasting insulin (FINS), insulin resis-

tance index (IRI), hemoglobin (Hb)A1c, blood lipids, and hemorheology indices were determined. In addition, the levels of vascular endothelial growth factors including thrombomodulin (TM), von Willebrand factor (vWF), P-selectin and MCP-1 mRNA were determined.

**RESULTS:** After 12 wk of treatment, the TCM syndrome score was significantly decreased compared to before treatment in both groups. After treatment, FPG, 2hPG, HbA1c, FINS, IRI, total cholesterol, triglycerides, low-density lipoprotein, high-density lipoprotein, whole blood low shear specific viscosity, plasma specific viscosity, TM, vWF, P-selectin and MCP-1 mRNA were significantly improved compared to before treatment in both groups. After treatment, the total efficiency and TCM syndrome score in the treatment group were better than in the control group. FINS, IRI, whole blood high shear specific viscosity, plasma specific viscosity, TM, vWF, P-selectin and MCP-1 mRNA level in the treatment group were significantly reduced after treatment compared with control group.

**CONCLUSION:** DJCs are efficacious in supplementing qi, nourishing yin and invigorating blood circulation, and upregulate MCP-1 mRNA expression in patients with T2DM subclinical vascular lesions.

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**Key words:** Danzhi Jiangtang capsule; Type 2 diabetes mellitus; Subclinical vascular lesions; Monocyte chemoattractant protein-1

**Core tip:** The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key mediator of inflammation. MCP-1 also belongs to the chemokine superfamily. T2DM subclinical vascular lesions are a type of mild inflammation. En-

endothelial cell MCP-1 can be increased markedly. Danzhi Jiangtang capsule is a traditional Chinese medicine compound preparation. The technique can restrain mononuclear cell chemotactic activity by supplementing qi, nourishing yin and invigorating blood circulation, thereby preventing and treating subclinical vascular lesions.

Fang ZH, Liu Y, Bao TT, Ni YQ, Liu J, Shi GB, Wu JP, Yang JP, Zhang H. Effect of Danzhi Jiangtang capsule on monocyte chemoattractant protein-1 mRNA expression in newly diagnosed diabetes subclinical vascular lesions. *World J Gastroenterol* 2013; 19(19): 2963-2968 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i19/2963.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2963>

## INTRODUCTION

The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response, which then promotes the process of T2DM. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response. MCP-1 also belongs to the chemokine superfamily<sup>[1]</sup>. As shown in a large number of experimental studies, MCP-1 is a risk factor for the occurrence and development of insulin resistance (IR), T2DM and related vascular complications<sup>[2]</sup>.

Danzhi Jiangtang capsule (DJC) is a traditional Chinese medicine (TCM) compound preparation, and is efficacious in supplementing qi, nourishing yin and invigorating blood circulation. In this study, we investigated the effects of DJCs on general indexes, including fasting plasma glucose (FPG), 2-h postprandial plasma glucose (2hPG), fasting insulin (FINS), insulin resistance index (IRI), hemoglobin (Hb)A1c, blood lipids, and hemorheology indicators in newly diagnosed T2DM subclinical vascular lesions, and the effects on expression of vascular endothelial growth factors including thrombomodulin (TM), von Willebrand factor (vWF), P-selectin and MCP-1 mRNA. The objective was to investigate preliminarily the mechanism of TCM on delaying vascular lesions. This study has importance for achieving a comprehensive understanding of the efficacy of DJCs and illuminating the multichannel and multitarget regulating effects of TCM on newly diagnosed T2DM subclinical vascular lesions.

## MATERIALS AND METHODS

### General data

A total of 62 outpatients and inpatients with T2DM in the Department of Metabolism and Endocrinology, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine were enrolled in this study from 2009 to 2011. All patients conformed to the diagnostic criteria of diabetes according to World Health Organization (1999),

without clinical manifestations of heart, brain or kidney vascular lesions. Color Doppler ultrasound showed that the intima-media thickness of the common carotid, femoral and iliac arteries was > 0.6 cm. The exclusion criteria were as follows: patients with diabetes duration > 1 year; pregnant or lactating women; patients with metabolic disorders (*e.g.*, ketoacidosis) and complicated severe infection in the preceding month; patients complicated with a vascular lesion of the heart, brain, kidney or other region, or other severe primary diseases; patients taking anticoagulant, antiplatelet or antifibrinolytic drugs during the preceding month.

Patients were randomly divided into a control group (31 cases, 7 male and 14 female, mean age  $53.67 \pm 9.32$  years) and treatment group (31 cases, 16 male and 15 female, mean age  $51.90 \pm 10.13$  years). There was no significant differences in age, sex, FPG, 2hPG, HbA1c, FINS, body mass index and IRI between the two groups ( $P > 0.05$ ).

### Experimental methods

Basic treatments including diabetes education, alimentary control, and regular exercise were conducted in both groups. According to actual conditions, patients were treated with one or more antidiabetic drugs, with uniform administration in both groups. The antidiabetic drugs were as follows: metformin hydrochloride tablets (Glucophage; 0.5 g; Shanghai Squibb Pharmaceutical Co. Ltd., China), Acarbose (Glucoba; 50 mg; Bayer Pharmaceutical Co. Ltd., Leverkusen, Germany), pioglitazone hydrochloride (Kasiping; 15 mg; East China Pharmaceutical Group, Zhejiang, China), gliclazide sustained release tablets (Gliclazide tablets; 30 mg; Servier Pharmaceutical Company, Paris, France), and repaglinide tablets (NovoNorm; 1 mg; Novo Nordisk Pharmaceutical Industries, Bagsvaerd, Denmark). In the treatment group, in addition to the oral administration with the above western medicines, the patients were treated with DJCs (provided by the Pharmaceutical Formulations Centre, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine; 1.8 g of effective extract in each capsule) at a dose of five capsules, three times daily (a total daily dose of 15 capsules).

### Observational indexes

**Blood glucose and blood lipid:** Blood glucose was determined by the glucose hexokinase method. Total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) and high-density lipoprotein (HDL) with fasting for > 12 h were determined using a Hitachi-7600 fully automatic biochemical analyzer.

**Hemorheology indices:** High shear specific viscosity and low shear specific viscosity of whole blood, plasma specific viscosity, hematocrit and fibrinogen were determined using a rotary whole blood viscometer and plasma viscometer in the Experimental Centre, First Affiliated Hospital of Anhui College of Traditional Chinese Medicine.

**Vascular endothelial growth factors:** Fasting venous

**Table 1 Comparison of fasting plasma glucose, 2-h plasma glucose, haemoglobin A1c, fasting insulin, insulin resistance index, total cholesterol, triglycerides, low density lipoprotein, high density lipoprotein, haemorheology indices, thrombomodulin, von Willebrand factor, P-selectin levels and monocyte chemoattractant protein-1 mRNA levels**

| Index                                 | Control group             |                            | Treatment group           |                                |
|---------------------------------------|---------------------------|----------------------------|---------------------------|--------------------------------|
|                                       | Before treatment (n = 31) | After treatment (n = 29)   | Before treatment (n = 31) | After treatment (n = 30)       |
| FPG (mmol/L)                          | 8.74 ± 1.42               | 8.01 ± 1.09 <sup>b</sup>   | 9.02 ± 1.14               | 7.94 ± 0.91 <sup>b</sup>       |
| 2hPG (mmol/L)                         | 12.42 ± 1.76              | 10.03 ± 1.39 <sup>b</sup>  | 11.96 ± 1.53              | 9.81 ± 1.34 <sup>b</sup>       |
| HbA1c (%)                             | 7.98 ± 0.71               | 7.11 ± 0.69 <sup>b</sup>   | 8.03 ± 0.68               | 6.98 ± 0.65 <sup>b</sup>       |
| FINS (uIU mL <sup>-1</sup> )          | 12.05 ± 2.93              | 8.68 ± 1.83 <sup>b</sup>   | 11.93 ± 2.45              | 7.58 ± 1.82 <sup>b,c</sup>     |
| IRI                                   | 1.81 ± 0.24               | 1.60 ± 0.19 <sup>b</sup>   | 1.79 ± 0.23               | 1.48 ± 0.19 <sup>b,c</sup>     |
| TC (mmol/L)                           | 5.36 ± 0.76               | 4.55 ± 0.59 <sup>b</sup>   | 5.27 ± 0.69               | 4.32 ± 0.49 <sup>b</sup>       |
| TG (mmol/L)                           | 2.01 ± 0.38               | 1.78 ± 0.36 <sup>a</sup>   | 1.99 ± 0.42               | 1.76 ± 0.31 <sup>a</sup>       |
| LDL (mmol/L)                          | 3.15 ± 0.31               | 3.06 ± 0.29 <sup>a</sup>   | 3.24 ± 0.28               | 3.07 ± 0.28 <sup>a</sup>       |
| HDL (mmol/L)                          | 1.41 ± 0.19               | 1.66 ± 0.17 <sup>b</sup>   | 1.37 ± 0.21               | 1.77 ± 0.15 <sup>b,c</sup>     |
| High shear specific viscosity (mPa·s) | 5.37 ± 0.78               | 5.10 ± 0.71                | 5.43 ± 0.65               | 4.73 ± 0.54 <sup>b,c</sup>     |
| Low shear specific viscosity (mPa·s)  | 12.65 ± 0.89              | 11.91 ± 1.02               | 12.51 ± 0.92              | 11.84 ± 0.94 <sup>b</sup>      |
| Hematocrit (%)                        | 42.95 ± 3.69              | 42.89 ± 3.51               | 43.15 ± 4.31              | 41.98 ± 3.01                   |
| Plasma specific viscosity (mPa·s)     | 1.93 ± 0.35               | 1.72 ± 0.37                | 2.01 ± 0.31               | 1.54 ± 0.24 <sup>b,c</sup>     |
| Fibrinogen (mg/L)                     | 4.52 ± 0.48               | 4.36 ± 0.37                | 4.61 ± 0.38               | 4.39 ± 0.35                    |
| TM (μg/L)                             | 25.68 ± 6.54              | 23.27 ± 4.06               | 25.41 ± 6.28              | 19.32 ± 4.29 <sup>b,c</sup>    |
| vWF (U/L)                             | 1540.17 ± 74.70           | 1480.66 ± 72.32            | 1551.92 ± 82.23           | 1146.70 ± 94.45 <sup>b,d</sup> |
| P-selectin (ng/mL)                    | 25.03 ± 6.06              | 22.57 ± 5.56               | 25.39 ± 6.81              | 17.08 ± 5.82 <sup>a,c</sup>    |
| MCP-1 mRNA (%)                        | 0.537 ± 0.136             | 0.473 ± 0.134 <sup>a</sup> | 0.541 ± 0.148             | 0.421 ± 0.132 <sup>b,c</sup>   |

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs before treatment; <sup>c</sup> $P < 0.05$  vs control group. FPG: Fasting plasma glucose; 2hPG: 2-h plasma glucose; HbA1c: Haemoglobin A1c; FINS: Fasting insulin; IRI: Insulin resistance index; TC: Total cholesterol; TG: Triglycerides; LDL: Low density lipoprotein; HDL: High density lipoprotein; TM: Thrombomodulin; vWF: von Willebrand factor; MCP-1: Monocyte chemoattractant protein-1.

blood was obtained during the early morning. After anticoagulation with ethylenediaminetetraacetic acid and centrifugation (4 °C, 3000 g, 10 min), the plasma was separated and stored at -70 °C. TM was quantitatively determined using an enzyme-linked immunosorbent assay (ELISA) kit (ADL Company, CA, United States). vWF was quantitatively determined by the double antibody sandwich ELISA method (Shanghai Sun Biotechnology). P-selectin was quantitatively determined by the ABC-ELISA double antibody sandwich method (R and D Systems, Minneapolis, MN, United States).

**MCP-1 mRNA:** Expression of serum MCP-1 mRNA was determined using the reverse transcriptase polymerase chain reaction (Promega, Madison, WI, United States) according to the manufacturer's instructions.

### Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical software. Non-normally distributed data were logarithmically transformed to normally distributed data. Normally distributed measurement data are expressed as mean ± SD. Analysis of variance was performed for comparisons of multiple means. Student's *t* tests were used to analyze the differences between two samples.  $\chi^2$  tests were conducted to compare the enumerated data.  $P < 0.05$  or  $P < 0.01$  was considered to be statistically significant.

## RESULTS

### Comparison of blood glucose and blood lipid levels

Before treatment, there was no significant difference in FPG, 2hPG, HbA1c, FINS, IRI, TC, TG, LDL and HDL

level between the two groups ( $P > 0.05$  for all comparisons). After treatment with western medicines, the above indexes were significantly improved in both groups ( $P < 0.05$  or  $P < 0.01$ ). In addition, after treatment, FINS and IRI levels in the treatment group were significantly lower than in the control group ( $P < 0.05$ ), and HDL in the treatment group was significantly higher than in the control group ( $P < 0.05$ ) (Table 1).

### Comparison of hemorheology indices

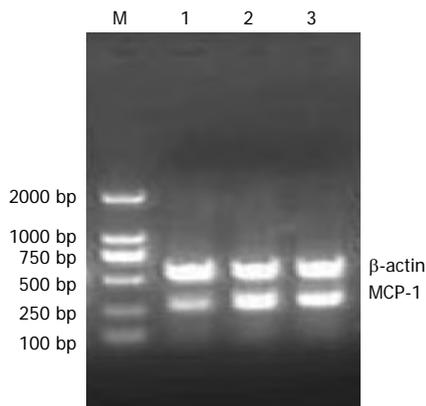
Before treatment, there was no significant difference in high shear specific viscosity and low shear specific viscosity of whole blood, plasma specific viscosity, hematocrit, and fibrinogen level between the two groups ( $P > 0.05$  for all comparisons). After treatment, the whole blood high shear specific viscosity, plasma specific viscosity and hematocrit in the treatment group were significantly lower than in the control group ( $P < 0.05$ ) (Table 1).

### Comparison of TM, vWF and P-selectin levels

In the control group, after treatment, TM, vWF and P-selectin levels decreased, but the differences compared with baseline values before treatment were not significant ( $P > 0.05$ ). In the treatment group, the above indexes were significantly reduced after treatment ( $P < 0.05$  or  $P < 0.01$ ) and compared with the control group ( $P < 0.05$  or  $P < 0.01$ ) (Table 1).

### Comparison of MCP-1 mRNA levels

Before treatment, there was no significant difference in MCP-1 mRNA levels between the two groups ( $P > 0.05$ ). After treatment, the MCP-1 mRNA level in the treatment group was significantly lower than before ( $P < 0.01$ ) and



**Figure 1** Electropherogram of monocyte chemoattractant protein-1 mRNA obtained by reverse transcription-polymerase chain reaction. MCP-1: Monocyte chemoattractant protein-1; M: Marker; 1: Normal group; 2: Control group; 3: Treatment group.

compared with control group ( $P < 0.05$ ) (Figure 1 and Table 1).

## DISCUSSION

The disease course of newly diagnosed T2DM is  $< 1$  year. There are different degrees of vascular lesions in 50% of patients with newly diagnosed T2DM. Subclinical vascular lesions exist in some patients with newly diagnosed T2DM, but the clinical symptoms are atypical<sup>[3-5]</sup>. They are the pathological basis of diabetic complications, with an extensive scope. Once the subclinical vascular lesions occur, they develop rapidly and both large and small vessels, arteries, capillaries and veins may be involved, with complicated lesions in many organs, such as angiopathy, brain, kidney, eyeground, nerve and skin. T2DM subclinical vascular lesions are caused by many factors including abnormal glucose metabolism, IR, lipid metabolism disorders, inflammatory factors, and oxidative stress<sup>[6,7]</sup>. Vascular endothelial injury is the initiating step of T2DM subclinical vascular lesions, and the anatomical endothelial layer is also the first involved site. During T2DM, continued hyperglycemia and high capillary pressure can cause vascular endothelial cell injury and dysfunction, changing angiostasis and hemodynamics, activate the coagulation system and platelets, and increase the vascular permeability, thus leading to a series of pathological changes<sup>[8]</sup>. Therefore, studying the change in endothelial cell function is beneficial for further preventing the occurrence and development of T2DM subclinical vascular lesions.

In recent years, TM has been confirmed as a molecular marker of endothelial cell injury<sup>[9,10]</sup>. In addition, it can be used as a marker for judging the severity of atherosclerosis of peripheral arteries and coronaries, and a sensitive detection index for the occurrence of vascular complications<sup>[11]</sup>. As found in prospective studies, vWF is involved in blood coagulation, and can promote platelet adhesion<sup>[12]</sup>. It is an important marker for predicting early subclinical endothelial cell injury in T2DM and develop-

ment of urinary albumin excretion rate<sup>[13]</sup>. In addition, it is an important risk factor for new microvascular lesions. P-selectin has been a research hotspot for vascular lesions in recent years. It is an important marker of endothelial cell injury and platelet activation, and is considered to be an important factor in the initial pathological process of atherosclerosis<sup>[14]</sup>.

MCP-1 is one member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response<sup>[15]</sup>. In T2DM, high-level blood glucose, non-enzymatic glycosylated protein, angiotensin, oxidative stress, interleukin-1, and tumor necrosis factor- $\alpha$  can all up-regulate MCP-1 expression<sup>[16-19]</sup>. MCP-1 can specifically induce blood monocytes to enter the endothelium of the lesion. This is an important mechanism in the occurrence and development of T2DM vascular lesions<sup>[20]</sup>. MCP-1 mainly attracts monocytes and T lymphocytes, and induces monocytes and endothelial cells to express adhesion molecules. It can promote various inflammatory cells, especially monocytes, to aggregate in the lesion sites, and respond to stimulation of the inflammatory cytokines<sup>[21]</sup>. In addition, MCP-1 can promote peripheral blood mononuclear cells to adhere, chemoattract and migrate to the intima, and then phagocytose lipids and transform into foam cells<sup>[22]</sup>. The activated leukocytes and vascular wall cells can release a variety of growth factors, which promote vascular smooth muscle cell proliferation, stimulate inflammation<sup>[23]</sup>, and participate in the occurrence and development of atherosclerosis. It has been found that MCP-1 plays an important role in atherosclerosis in diabetes, and can be used as a useful index for predicting T2DM subclinical atherosclerosis<sup>[24]</sup>. At the same time, MCP-1 is considered to be a potential biochemical marker for reflecting early atherosclerotic changes and prognosis<sup>[25]</sup>.

Therefore, MCP-1 is closely related to the occurrence and development of T2DM vascular lesions. Further study of MCP-1 will enrich the theoretical basis for pathogenesis of T2DM vascular lesions. Analysis of the chemokine dynamic change, regulatory mechanism and interactions, and treatment aimed at chemokines and receptors is a new method for therapy of T2DM vascular complications. The deepening awareness of the complexity of the chemokine network has provided new methods and means for prevention and treatment of T2DM vascular lesions.

TCM believes that the prethrombotic status of T2DM is similar to the blood stasis syndrome of emaciation-thirst disease. The pathogenesis of emaciation-thirst disease is that the dryness-heat, impairment of yin, and body fluid scorching will cause blood viscosity and blood stagnation. The long-term yin deficiency injures the healthy qi, leading to qi and yin deficiency and weakness, which aggravate the blood stasis. Deficiency of yin affects yang, and insufficiency of yang brings about cold syndrome. The cold coagulation causes blood stasis. The blood stasis is present throughout the disease course, and is a common pathological product and pathogenic factor of emaciation-thirst disease. In the treatment of this dis-

ease, supplementing qi, nourishing yin, invigorating blood circulation and dissolving stagnant blood should be carried out, and the treatment emphasis should be focused on regulating qi-blood circulation and improving blood circulation. DJCs are composed of Radix Pseudostellariae, Radix Rehmanniae, Cortex Moutan, Dodder, Alisma, leech and others. Radix Pseudostellariae can invigorate the qi of the spleen and kidney. Radix Rehmanniae can nourish the yin of the spleen and kidney. Dodder can tonify the kidney to arrest spontaneous emission. Cortex Moutan and leech can promote qi circulation, invigorate blood circulation, dissolve stagnant blood, and resolve kidney collateral obstruction. Alisma can clear heat and purge phlegm. The whole prescription can supplement qi and nourish yin, invigorate blood circulation and dissolve stagnant blood, thus achieving the efficacy of balancing yin and yang, dispelling blood stasis, and making the kidney pulse unobstructed. Modern pharmacological research shows that Radix Pseudostellariae, Radix Rehmanniae and Alisma have certain antidiabetic and antihypertensive effects. Moutan bark can inhibit a cyclooxygenase reaction, thus inhibiting platelet aggregation. The hirudin contained in leech can inhibit the effect of thrombin on fibrinogen, impede blood coagulation, and prevent thrombus formation. In addition, Leech also secretes a histamine-like substance, which can directly expand the blood vessels, activate plasmin, and inhibit collagen synthesis, thus reducing the blood viscosity and improving the hemorheology.

Results of this study have shown that, after treatment with DJCs, FINS and IRI levels are significantly lower than those obtained from basic treatment, with an increase in HDL level. In addition, after treatment with DJCs, the whole blood high shear specific viscosity, plasma specific viscosity and hematocrit, as well as TM, vWF and P-selectin expression levels are significantly reduced, with downregulated peripheral blood MCP-1 mRNA expression. This indicates that DJCs can alleviate lipid metabolism disorders, downregulate expression of TM, vWF, P-selectin and MCP-1 mRNA, lower whole blood viscosity and plasma specific viscosity, reduce hematocrit, antagonize platelet activation, inhibit platelet aggregation, and protect vascular endothelial cells, thus intervening in the newly diagnosed T2DM subclinical vascular lesions. It is believed that this therapy, by supplementing qi, nourishing yin and invigorating blood circulation, can enhance the anticoagulant and fibrinolytic activity, intervene in the hypercoagulable state, and improve the function of impaired endothelial cells. This has important significance in delaying vascular complications associated with T2DM.

## COMMENTS

### Background

The occurrence and development of type 2 diabetes mellitus (T2DM) is accompanied by an inflammatory response, which in turn promotes the process of T2DM. Monocyte chemoattractant protein (MCP)-1 is a member of the CC chemokine subfamily, and is a key cytokine mediator of the inflammatory response. MCP-1 also belongs to the chemokine superfamily. As shown in a large

number of experimental studies, MCP-1 is a risk factor for the occurrence and development of insulin resistance, T2DM and related vascular complications.

### Research frontiers

The disease course of newly diagnosed T2DM is < 1 year. There are different degrees of vascular lesions in 50% of patients with newly diagnosed T2DM. Subclinical vascular lesions exist in some patients with newly diagnosed T2DM, but the clinical symptoms are atypical. They are the pathological basis of diabetic complications, with an extensive scope.

### Innovations and breakthroughs

This study has important significance for comprehensive understanding of the efficacy of Danzhijiangtang capsules (DJCs) and illuminating the multichannel and multitarget regulating effects of traditional Chinese medicine on newly diagnosed T2DM subclinical vascular lesions.

### Applications

DJCs are efficacious in supplementing qi, nourishing yin and invigorating blood circulation, and can upregulate MCP-1 mRNA expression in patients with T2DM subclinical vascular lesions.

### Terminology

In recent years, thrombomodulin has been confirmed as a molecular marker of endothelial cell injury. In addition, it can be used as a marker for judging the atherosclerosis severity of peripheral arteries and coronaries, and a sensitive detection index for the occurrence of vascular complications.

### Peer review

This manuscript describes a traditional Chinese medicine DJCs for treatment of T2DM subclinical vascular lesions, and it has significant research interest.

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**P- Reviewers** Assy N, Ismail-Beigi F **S- Editor** Song XX  
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## Theory of mind deficits in patients with esophageal cancer combined with depression

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Received: November 10, 2012 Revised: March 5, 2013

Accepted: March 22, 2013

Published online: May 21, 2013

### Abstract

**AIM:** To characterize the two components of theory of mind (ToM) in patients with esophageal cancer combined with depression.

**METHODS:** Sixty-five patients with esophageal cancer combined with depression (depressed group) and 62 normal controls (control group) were assessed using reading the mind in the eyes test, faux pas task, verbal fluency test, digit span test and WAIS IQ test. The depressed group was divided into two subgroups including psychotic depressed (PD) group (32 cases) and nonpsychotic depressed (NPD) group (33 cases).

The clinical symptoms of patients were assessed using Beck depression inventory version II and brief psychiatric reacting scale (BPRS).

**RESULTS:** There was a significant difference between the depressed group and the control group on tasks involving ToM social perceptual components (mind reading:  $t = 7.39, P < 0.01$ ) and tests involving ToM social cognitive components (faux pas questions:  $t = 13.75, P < 0.01$ ), respectively. A significant difference was also found among the PD group, the NPD group and the control group on mind reading ( $F = 32.98, P < 0.01$ ) and faux pas questions ( $\chi^2 = 78.15, P < 0.01$ ), respectively. The PD group and NPD group performed worse than normal group controls both on mind reading and faux pas questions ( $P < 0.05$ ). The PD group performed significantly worse than the NPD group on tasks involving ToM (mind reading:  $F = 18.99, P < 0.01$ ; faux pas questions:  $F = 36.01, P < 0.01$ ). In the depressed group, there was a negative correlation between ToM performances and BPRS total score (mind reading:  $r = -0.35, P < 0.01$ ; faux pas questions:  $r = -0.51, P < 0.01$ ), and between ToM performances and hostile suspiciousness factor score (mind reading:  $r = -0.75, P < 0.01$ ; faux pas questions:  $r = -0.73, P < 0.01$ ), respectively.

**CONCLUSION:** The two components of ToM are both impaired in patients with esophageal cancer combined with depression. This indicates that there may be an association between ToM deficits and psychotic symptoms in clinical depression.

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**Key words:** Esophageal cancer combined with depression; Theory of mind; Social perceptual component; Social cognitive component

**Core tip:** In this study, the theory of mind deficits in patients with esophageal cancer combined with de-

pression was investigated, and the relation between ToM deficits and psychotic symptoms was discussed.

Cao Y, Zhao QD, Hu LJ, Sun ZQ, Sun SP, Yun WW, Yuan YG. Theory of mind deficits in patients with esophageal cancer combined with depression. *World J Gastroenterol* 2013; 19(19): 2969-2973 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2969.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2969>

## INTRODUCTION

Depression exists in about 24% of patients with esophageal cancer<sup>[1]</sup>. It is found that there are theory of mind (ToM) deficits in patients with depression<sup>[2-4]</sup>. In cognitive neuropsychiatry, the ToM is divided into a social perceptual component and a social cognitive component<sup>[5,6]</sup>, in terms of information processing theories. In recent years, the social cognitive impairment caused by depression and its potential cognitive neuropsychological mechanisms have become a research hotspot<sup>[2-4]</sup>.

It is reported that the occurrence of esophageal cancer combined with depression is closely related with cognition in disease<sup>[7-9]</sup>. Whether the cognitive biases include ToM deficits or not is not clear. In this study, the ToM deficits in patients with esophageal cancer combined with depression were investigated, and the relation between ToM deficits and psychotic symptoms was discussed.

## MATERIALS AND METHODS

### Research objects

Sixty-five patients with esophageal cancer combined with depression in Changzhou Second People's Hospital Affiliated to Nanjing Medical University (Changzhou, China) were studied from January to December in 2011. Their beck depression inventory version II (BDI-II)<sup>[10]</sup> scores were not less than 5. All patients had an education background of junior middle school and above. They were right-handed and had normal eyesight and hearing. There was no patient with a medical history of head trauma, central nervous system disease, metastatic brain tumor, mental illness or substance dependence. No patient had been treated with chemotherapy.

The patients were divided into a psychotic depressed (PD) group (32 cases) and a nonpsychotic depressed (NPD) group (33 cases), according to whether or not patients had psychotic symptoms [the brief psychiatric reacting scale (BPRS)<sup>[11]</sup> total score was > 35]. Psychotic symptoms were distinguished from schizophrenia. The ages of patients were 28-60 years, with an average age of  $48.50 \pm 4.53$  years. They had obtained 9-16 years of education, with an average education duration of  $12.57 \pm 1.64$  years. The average age of the NPD group and the PD group was  $45.00 \pm 5.02$  and  $46.28 \pm 4.19$  years, respectively. The average duration of education in the NPD

group and the PD group was  $12.18 \pm 1.06$  and  $11.37 \pm 1.25$  years, respectively.

The control group consisted of 62 cases of college students, physicians and people with normal physique. They had no medical history of neurological disease, mental illness, substance abuse, or family psychiatry. They had an education background of junior middle school and above. They were right-handed and had normal eyesight and hearing. Their ages were 27-62 years, with an average age of  $47.65 \pm 4.64$  years. The duration of education was 9-16 years, with an average education duration of  $11.59 \pm 2.01$  years.

All research participants had signed informed consent before enrolling in this study. The patients had obtained the agreement of guardians. There was no significant difference in gender, age, or duration of education between the depressed group and the control group ( $P > 0.05$ ) or between the PD group, the NPD group and the control group ( $P > 0.05$ ). The difference in disease course between the NPD group and the PD group was not statistically significant ( $t = 0.69$ ,  $P > 0.05$ ).

### Reading the mind in the eyes test

The reading the mind in the eyes test reflected the social perceptual component of ToM. It required the participants to conduct a perceptual processing of the mental activity status of the character in the eye area, and select one word from four alternatives which could most accurately reflect the character's mental activity status. The gender recognition task was used as a control task, which required the participant to recognize the gender of the character in the eye area. It reflected the perceptual processing of general social cues. Five min of learning of alternative vocabulary annotations was performed before test. One score was obtained for each correct answer. For a total of 34 questions (17 male and 17 female), there was a total score of 34 for reading the mind in the eyes test and the gender recognition task, respectively<sup>[12-14]</sup>.

### Faux pas task

The faux pas task (faux pas questions) belonged to the social cognitive component of ToM. It required the research object to plot the mental activity status of the character in the story and judge whether or not the character had said the words which should not be said or the embarrassing words, according to the storyline. The understanding of general text content in story was used as a control question. It reflected the understanding of the story content, and comprised 20 little stories (10 faux pas stories and 10 stories without faux pas). There were 2 faux pas questions and 2 control questions for each story. One score was obtained for each correct answer. The total score for faux pas questions and control questions was 20 and 40, respectively<sup>[13-15]</sup>.

### Clinical evaluation and neuropsychological test

The clinical symptoms of patients were assessed using BDI-II and BPRS ( $K = 0.83$ ). The WAIS-IQ test<sup>[16]</sup>, digit

**Table 1** General data of patients with esophageal cancer combined with depression

| Groups    | <i>n</i> | BDI-II score              | BPRS total score          | Anxiety depression factor score | Anergia factor score | Thought disturbance factor score | Activation factor score | Hostile suspiciousness factor score |
|-----------|----------|---------------------------|---------------------------|---------------------------------|----------------------|----------------------------------|-------------------------|-------------------------------------|
| PD group  | 23       | 33.00 ± 4.62 <sup>1</sup> | 43.72 ± 7.11 <sup>1</sup> | 14.28 ± 2.21 <sup>1</sup>       | 6.13 ± 3.14          | 6.34 ± 3.35                      | 4.99 ± 2.36             | 10.31 ± 2.95 <sup>1</sup>           |
| NPD group | 33       | 20.22 ± 3.28              | 27.13 ± 6.12              | 10.75 ± 2.69                    | 5.12 ± 3.46          | 5.91 ± 3.29                      | 4.97 ± 3.01             | 2.21 ± 1.95                         |

PD: Psychotic depressed; NPD: Nonpsychotic depressed. <sup>1</sup>*t* test,  $P < 0.01$  vs NPD group.

**Table 2** Comparisons of neuropsychological test and theory of mind test performance

| Groups          | <i>n</i> | IQ score      | VFT score                 | DST score    | Mind reading score          | Gender recognition score | Faux pas questions score    | Control questions score |
|-----------------|----------|---------------|---------------------------|--------------|-----------------------------|--------------------------|-----------------------------|-------------------------|
| Depressed group | 65       | 103.12 ± 5.18 | 32.11 ± 2.38 <sup>1</sup> | 13.02 ± 1.08 | 24.12 ± 2.19 <sup>1</sup>   | 30.11 ± 1.02             | 15.02 ± 1.63 <sup>1</sup>   | 37.25 ± 0.68            |
| PD group        | 32       | 103.42 ± 3.58 | 30.02 ± 2.16 <sup>2</sup> | 12.68 ± 1.02 | 22.89 ± 2.07 <sup>2,3</sup> | 30.12 ± 0.99             | 13.16 ± 1.71 <sup>2,3</sup> | 37.31 ± 0.71            |
| NPD group       | 33       | 103.77 ± 4.30 | 34.21 ± 2.08              | 13.79 ± 1.01 | 25.38 ± 2.32 <sup>2</sup>   | 30.22 ± 0.95             | 15.82 ± 1.13 <sup>2</sup>   | 37.52 ± 0.62            |
| Control group   | 62       | 104.11 ± 3.22 | 35.05 ± 2.01              | 13.88 ± 0.98 | 27.89 ± 2.05                | 30.57 ± 1.01             | 19.92 ± 1.01                | 37.51 ± 0.65            |

<sup>1</sup>*t* test,  $P < 0.01$  vs NPD group; <sup>2</sup>Bonferroni test or Mann-Whitney *U* test,  $P < 0.05$  vs control group; <sup>3</sup>*t* test,  $P < 0.01$  or  $0.05$  vs NPD group. VFT: Verbal fluency test; DST: Digit span test; PD: Psychotic depressed; NPD: Nonpsychotic depressed.

span test (DST)<sup>[17]</sup> and verbal fluency test (VFT)<sup>[18]</sup> were conducted on all research objects. The DST included the recitation and inverted recitation of digits. The total score was expressed as the sum of two recitation scores. The VFT required the participant to say as many names as possible of vegetables, fruits and animals. One score was obtained for each correct answer. There was no score for duplicated names.

### Statistical analysis

Statistical analysis was performed using SPSS 13.0 statistical software. The  $\chi^2$  test, independent samples *t* test, single factor analysis of variance (using Bonferroni correction for multiple comparisons) and Kruskal-Wallis test (using Mann-Whitney *U* test for multiple comparisons) were used to compare data according to the different types of variables. In the depressed group, the analysis of covariance was conducted on ToM performance using the BDI-II score as a covariant, and the partial correlation analysis was conducted between ToM performance and BPRS total score, and between ToM performance and other factor scores, respectively.

## RESULTS

### Comparisons of general data

There was no significant difference in IQ score between the depressed group and the control group ( $t = 0.52$ ,  $P > 0.05$ ). It was the same with IQ score among the NPD group, PD group and control group ( $F = 0.12$ ,  $P > 0.05$ ). The BDI-II score ( $t = 6.77$ ,  $P < 0.01$ ), BPRS score ( $t = 6.78$ ,  $P < 0.01$ ), anxiety-depression factor score ( $t = 3.56$ ,  $P < 0.01$ ) and hostile suspicion factor score ( $t = 10.95$ ,  $P < 0.01$ ) in the PD group were significantly higher than those in the NPD group. The differences in other factors scores were not significant between the two groups ( $P > 0.05$ ). Results were shown in Table 1.

### Comparisons of neuropsychological test performance

As shown in Table 2, the VFT score in the depressed group was significantly lower than that in the control group ( $t = 4.34$ ,  $P < 0.01$ ), but there was no significant difference in DST score between the two groups ( $t = 0.75$ ,  $P > 0.05$ ). The differences in VFT score among the NPD group, PD group and control group were statistically significant ( $F = 15.56$ ,  $P < 0.01$ ). The VFT score in the PD group was significantly lower than that in the control group and the NPD group ( $P < 0.05$ ), respectively, and there was no significant difference between the NPD group and the control group ( $P > 0.05$ ).

### Comparisons of ToM test performances

There was a significant difference between the depressed group and control group on tasks involving a ToM social perceptual component (mind reading:  $t = 7.39$ ,  $P < 0.01$ ) and tests involving a ToM social cognitive component (faux pas questions:  $t = 13.75$ ,  $P < 0.01$ ), respectively. But there was no significant difference for the gender recognition score and control questions score ( $t = 0.47$ ,  $P > 0.05$ ;  $t = 0.52$ ,  $P > 0.05$ ), respectively. A significant difference was also found among PD group, NPD group and control group on mind reading ( $F = 32.98$ ,  $P < 0.01$ ) and faux pas questions ( $\chi^2 = 78.15$ ,  $P < 0.01$ ). Results of multiple comparisons showed that the PD group was worse than the NPD group and the NPD group was worse than the control group on mind reading ( $P < 0.05$  for both). In addition, on faux pas questions, the PD group performed worse than the control group and the NPD group, respectively, (Mann-Whitney *U* = 153.08,  $Z = -7.38$ ,  $P < 0.05$ ; Mann-Whitney *U* = 127.95,  $Z = -4.26$ ,  $P < 0.05$ ), and the NPD group performed worse than the control group (Mann-Whitney *U* = 153.13,  $Z = -6.81$ ,  $P < 0.05$ ). In addition, the PD group performed significantly worse than the NPD group on tasks involving ToM (mind reading:  $F = 18.99$ ,  $P < 0.01$ ; faux pas questions:  $F = 36.01$ ,  $P < 0.01$ ).

### Partial correlation analysis between ToM performance and BPRS score

In the depressed group, the faux pas questions score was positively correlated with the mind reading score ( $r = 0.56$ ,  $P < 0.01$ ). There was a negative correlation between ToM performances and BPRS total score (mind reading:  $r = -0.35$ ,  $P < 0.01$ ; faux pas questions:  $r = -0.51$ ,  $P < 0.01$ ), and between ToM performances and hostile suspiciousness factor scores (mind reading:  $r = -0.75$ ,  $P < 0.01$ ; faux pas questions:  $r = -0.73$ ,  $P < 0.01$ ), respectively. There was no correlation between ToM performances and other factors score related to BPRS ( $P > 0.05$ ).

## DISCUSSION

This study aims to investigate the components of ToM in patients with esophageal cancer combined with depression. Results show that the depressed group performed worse than the control group on tasks involving ToM social perceptual components and test involving ToM social cognitive components, respectively. This suggests that the ToM deficits exist in patients with esophageal cancer combined with depression. These results are in accordance with the studies of Inoue *et al.*<sup>3,41</sup> and Lee *et al.*<sup>191</sup>, in which ToM deficits are found in patients with depression in ToM tests.

In this study, there are impairments in the social perceptual component and social cognitive component of ToM. The social perceptual component of ToM occurs mainly in the right cerebral hemisphere, and the left cerebral hemisphere is mainly responsible for the social cognitive component<sup>16,121</sup>. This indicates that there are bilateral brain impairments in patients with esophageal cancer combined with depression. These results are similar to those of the study by Rotenberg<sup>201</sup> which suggests that depression is related not only to right hemisphere dysfunction, but also to left hemisphere prefrontal hypo-function. This indicates that there is a cognitive neuropsychology mechanism of bilateral brain impairments for depression.

In this study, the ToM performances in the PD group are significantly worse than those in the NPD group. Results of partial correlation analysis show that there was a negative correlation between ToM performances and BPRS total score, and between ToM performances and hostile suspiciousness factor score, respectively. This indicates that there is a positive correlation between ToM deficits and psychiatric symptoms in clinical depression. Therefore, early detection and intervention of ToM deficits and psychiatric symptoms in patients with esophageal cancer combined with depression is helpful for reducing the perniciousness of disease.

There is no metastatic brain tumor or chemotherapy in these patients, which has purified the samples to a certain extent. But the remote effects of tumors on neuropsychiatric function can not be excluded. Therefore, the follow up is very important for enriching and improving the final results.

## COMMENTS

### Background

Twenty-four percent of esophageal cancer patients also have depression, which may aggravate the disease condition and affect the treatment and rehabilitation. Previous studies suggest that there are a variety of cognitive disorders including theory of mind (ToM) deficits in patients with depression. The occurrence of esophageal cancer complicated with depression is closely related to cognition on disease. Whether cognitive biases include ToM deficits is not clear.

### Research frontiers

ToM deficits are social cognitive disorders. In this study, ToM deficits in esophageal cancer patients complicated with depression are observed using "Reading the Mind in the Eyes" test and Faux pas Task.

### Innovations and breakthroughs

Previous studies on cognitive impairment in esophageal cancer patients complicated with depression mainly focus on episodic memory impairment, executive function decline, psychomotor slowing and attention deficits, except ToM deficits. In this study, ToM deficits in esophageal cancer patients complicated with depression have been investigated. This contributes to prevention and treatment of esophageal cancer with depression, and improvement of patient's social adaptability.

### Applications

Results of this study can be applied to preventing and treating esophageal cancer with depression, and improving the rehabilitation level and social adaptability of patients.

### Terminology

ToM refers to the knowledge system on speculation of psychological state, namely the cognitive system on judgment of psychological state such as belief, intention, wish, need, motive and emotion. It is one of the most basic abilities of an individual for adapting into society. In cognitive neuropsychiatry, ToM is divided into a social perceptual component and social cognitive component, in terms of information processing theory.

### Peer review

This is good research. It finds ToM deficits in esophagus cancer patients complicated with depression, and has analyzed the damage characteristics of two subcomponents. This study is helpful for clinical assessment of cognitive disorders in esophageal cancer patients complicated with depression, and prevention and treatment of disease. In addition, it can provide new approaches for improving the social adaptabilities of patients.

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**P- Reviewers** Inoue Y, Kataria K **S- Editor** Wen LL  
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## Successful liver resection in a giant hemangioma with intestinal obstruction after embolization

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Author contributions: Zhou JX, Wu H and Zeng Y designed this study; Zhou JX and Huang JW collected, analyzed and interpreted data; Zhou JX drafted the article; all authors critically reviewed and approved the manuscript; Zhou JX and Zeng Y are principal investigators for the project.

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Received: October 9, 2012 Revised: January 17, 2013

Accepted: March 28, 2013

Published online: May 21, 2013

### Abstract

Hepatic hemangiomas are the most common benign tumor of the liver. Most hepatic hemangiomas remain asymptomatic and require no treatment. Giant hepatic hemangiomas with established complications, diagnostic uncertainty and incapacitating symptoms, however, are generally considered an absolute indication for surgical resection. We present a case of a giant hemangioma with intestinal obstruction following transcatheter arterial embolization, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. A 21-year-old Asian man visited our hospital for left upper quadrant pain. Examinations at the first visit revealed a left liver hemangioma occupying the abdominal cavity, with a maximum diameter of 31.5 cm. Embolization of the left hepatic artery was performed and confirmed a decrease in its size. However, the patient was readmitted to our hospital one month after embolization for intestinal obstruction. A left hepatectomy was completed through a herringbone incision, and safely removed a giant hemangioma of 26.5 cm × 19.5 cm × 12.0 cm in

size and 3690 g in weight. Pre-operative arterial embolization is effective for reducing tumor size, but a close follow-up to decide the time for hepatectomy is important.

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**Key words:** Hepatic hemangioma; Transcatheter arterial embolization; Intestinal obstruction; Complications; Hepatectomy

**Core tip:** Hepatic hemangiomas are the most common benign tumor of the liver. Most hepatic hemangiomas remain asymptomatic and require no treatment; giant hepatic hemangiomas with established complications, diagnostic uncertainty and incapacitating symptom, however, are generally considered an absolute indication for surgical resection. We present a case of a giant hemangioma with intestinal obstruction following transcatheter arterial embolization, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. Our experience indicates the effectiveness of pre-operative arterial embolization to reduce tumor size, and the importance of a close follow-up to decide when to perform the surgery.

Zhou JX, Huang JW, Wu H, Zeng Y. Successful liver resection in a giant hemangioma with intestinal obstruction after embolization. *World J Gastroenterol* 2013; 19(19): 2974-2978 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i19/2974.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i19.2974>

### INTRODUCTION

Liver hemangiomas are the most common benign tumors of the liver, with an estimated prevalence of 3%-20%<sup>[1-3]</sup>. Most of them are small in size (< 4 cm in diameter) and asymptomatic and are discovered incidentally during

screening tests by modern diagnostic procedures. Giant hemangiomas are defined as tumors with a diameter > 4 cm, and symptoms rarely appear unless the tumor size exceeds 4 cm<sup>[4-6]</sup>. Although the majority of hepatic hemangiomas remain asymptomatic, symptomatic hepatic hemangiomas can present with abdominal pain, hemorrhage, biliary compression, or a consumptive coagulopathy.

A range of treatment options exists for liver hemangiomas, from observation to various radiological and surgical procedures. When treatment is needed, surgical excision of the hemangioma is most effective, and associated with low morbidity and mortality<sup>[7,8]</sup>. Other treatments, including transcatheter arterial embolization (TAE)<sup>[9]</sup>, arterial ligation<sup>[10]</sup>, radiotherapy<sup>[11]</sup>, radiofrequency ablation<sup>[12]</sup>, corticosteroid therapy<sup>[13]</sup>, and liver transplantation<sup>[14,15]</sup>, have been employed for large unresectable lesions. Apart from liver transplantation, however, the long-term effect of these methods usually cannot be anticipated.

Here, we report a case of a giant hemangioma with intestinal obstruction following TAE treatment, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy.

## CASE REPORT

A 21-year-old Asian man visited a local hospital for left upper quadrant pain as the chief complaints. Ultrasonography, performed during the visit, detected huge hypoechoic lesions, and under the diagnosis of a giant hemangioma, he was recommended for surgery and transferred to our hospital. The patient's past or family medical history was unremarkable. Physical examination revealed a grossly distended abdomen without a fluid wave, and tenderness and bounce pain in the left upper quadrant.

On admission, the patient's laboratory values were notable for an international normalized ratio of 1.16, a decreased fibrinogen level of 1.99 g/L (normal range, 2.0-4.0) and D-dimer levels of 8.12 mg/L fibrinogen equivalent unit (normal range, < 0.55 mg/L). The results of blood routine and liver function tests were normal, including a total bilirubin level of 0.99 mg/dL, an albumin level of 40.2 g/L, and an indocyanine green retention rate at 15 min of 5.6%. Serum tumor markers were all within normal range.

Multi-detector computed tomography (MDCT) on admission revealed a huge hemangioma, 31.5 cm × 24.8 cm × 11.1 cm, located on the left liver, and replacing the parenchyma of the left liver. His abdomen was distended by the huge hemangioma extending to the pelvis. The non-contrast phase showed a homogenous hypodense lesion contrasted with the surrounding liver parenchyma. On arterial phase images, the lesion remained hypodense relative to normal liver, but early central enhancement was detected. On delayed phase images, the lesion showed progressive fill-in. These findings indicated a giant hemangioma. The left hepatic artery and its branches were extremely stretched, and the left portal vein was compressed and occluded by the tumor. The left hepatic

duct was slightly dilated as a result of compression by the hemangioma. The left hepatic vein was completely occluded, while the hepatic vena cava and the middle and right hepatic veins remained patent (Figure 1A-C). The gastrointestinal tract was compressed with no sign of intestinal obstruction. Volumetric analysis revealed a tumor volume of 6503 mL and a right hemiliver volume of 1140 mL. The findings of MDCT were further confirmed by magnetic resonance imaging (Figure 2).

TAE of the left hepatic artery was performed with lipiodol. Thereafter, we planned to calculate and investigate the tumor volume, anatomical positions, and recanalizations by dynamic MDCT once a month, not to misjudge the timing of operation. However, the patient was readmitted to our emergency department one month after TAE, with abdominal pain for two days and clinical characteristics of intestinal obstruction. MDCT on admission revealed the thickening and swelling of the small bowel, dilatation and multiple liquid gas level of the lower part of esophagus, stomach and small bowel, and the colon collapsed. Volumetric analysis revealed that the tumor volume had decreased to 3988 mL (Figure 1D-F). The patient was initially managed conservatively by gastrointestinal decompression and intravenous therapy. Close observation and timely treatment were conducted. The patient's condition improved in 7 d.

The remarkable volume reduction of the tumor allowed for a safe approach to the portal vein and hepatic artery. Through a herringbone incision, a left hepatectomy was safely conducted after the ligation of left hepatic artery, middle hepatic artery and left branch of portal vein. There were extensive adhesions formed between the hemangioma and the jejunum, ileum, sigmoid colon, peritoneum and omentum. No dilated intestinal loops were found. The duration of operation was 280 min and intraoperative blood loss was 400 mL. The resected tumor was 26.5 cm × 19.5 cm × 12.0 cm in size and 3690 g in weight (Figure 3). Histologically, it was diagnosed as a cavernous hemangioma with local subcapsular necrosis, calcification and fibrous tissue proliferation.

The patient's post-operative course was uneventful and he was discharged from the hospital 16 d after surgery. At 6 mo following surgery, he enjoys an improved quality of life with normal liver function.

## DISCUSSION

Cavernous hemangiomas of the liver are benign, and usually small (< 4 cm) in size, but when they are larger than 4 cm in diameter, they are classified as giant cavernous hemangiomas<sup>[16]</sup>. They arise from the mesoderm and are composed of blood-filled cavernous spaces of varying size lined with a single layer of flat endothelial cells, which may be separated by fibrous septa of variable thickness<sup>[16,17]</sup>. 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 infarc-

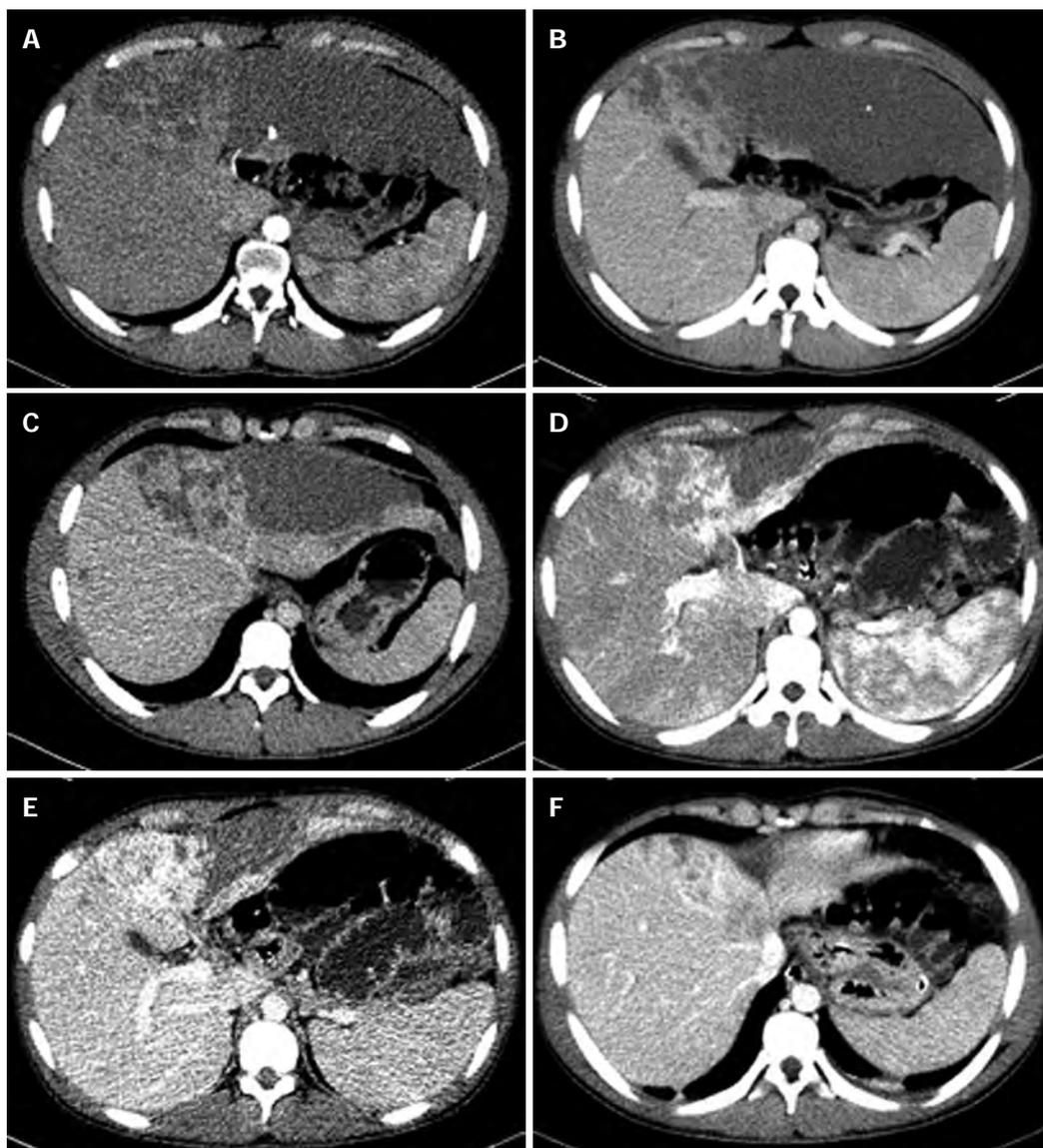


Figure 1 Axial images of multi-detector computed tomography. Multi-detector computed tomography (MDCT) images at the first visit (A-C), and corresponding MDCT slices just before the operation (*i.e.*, one month after transcatheter arterial embolization) (E-G).

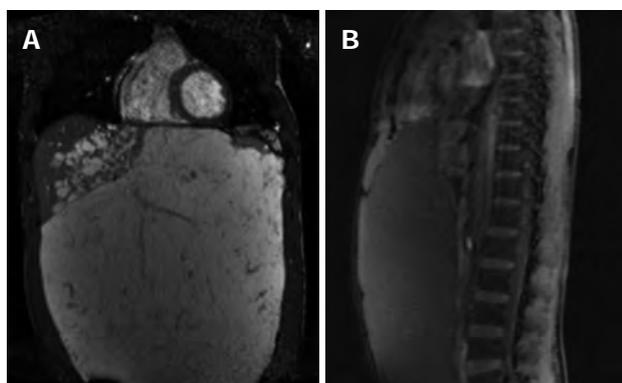


Figure 2 Coronal (A) and sagittal (B) views of the hemangioma from magnetic resonance imaging.



Figure 3 Intra-operative photograph of the tumor.

tion (and consequent stomachache), Kasabach-Merritt syndrome, and spontaneous rupture. Out of the mass effect, it might produce symptoms such as obstructive

jaundice, biliary colic, and gastric outlet obstruction<sup>[18]</sup>.

A recent major argument in the treatment of liver hemangiomas is the indications of operation. Considering the benign and non-progressive nature of the dis-

ease, it is currently accepted that a giant hemangioma is not necessarily an indication for surgery just because of its size, and continued observation in asymptomatic patients or patients with minimal abdominal symptoms seems to be justified<sup>[19,20]</sup>. Surgery remains the only consistently effective curative treatment for giant hemangiomas and should be considered for patients with established complications, diagnostic uncertainty and incapacitating symptoms, where operative risk is acceptable, or where the diagnosis remains uncertain despite appropriate specialist investigation<sup>[21]</sup>. Various other treatment methods have been reported but their long-term results have been poor<sup>[20]</sup>.

In our case, the patient complained of abdominal pain. For the treatment of a giant hemangioma accompanied with symptoms, surgical resection is primarily recommended. Based on the liver function tests and remnant liver volume, urgent primary tumor resection seemed possible. But we considered that an urgent resection at the first admission would be dangerous because it seemed difficult to approach the bifurcation of hepatic artery and portal vein behind the tumor. Liver transplantation was also considered an option, but the patient strongly refused because of donor shortage and high expenses for transplantation. Although some authors reported that symptomatic giant liver hemangiomas can be managed successfully and non-invasively by TAE with a satisfactory decrease in symptoms and tumor volume<sup>[22]</sup>, the effect of TAE generally seems to be variable and sometimes even results in a volume increase<sup>[14,15]</sup>. However, there were reports that TAE for giant hemangiomas, performed prior to surgical resection, facilitated the mobilization of the liver by shrinking the hemangioma and, consequently, decreased intraoperative hemorrhage<sup>[9,23]</sup>. We also performed TAE before surgery to ensure the safety of the future radical resection of the tumor, which resulted in a decrease in the size of hemangioma, as shown in the previous report.

Considering the various complications and vascular recanalization after TAE which might postpone the operation and result in the loss of an opportunity for the radical resection, some authors recommend urgent operation after TAE<sup>[9,24]</sup>. In the present case, the patient was readmitted to our hospital one month after TAE with intestinal obstruction. To our knowledge, intestinal obstruction after embolization of a giant hepatic hemangioma has not previously been reported in the English literature, we speculated that it might be related to the tumor shrinkage, and inflammatory adhesions formed between the tumor and small bowel. Based on this speculation and the remarkable volume reduction of the tumor, we decided to perform a left hepatectomy. Fortunately, the operation was safely conducted without any complication, and our speculation was confirmed by the laparotomy and histological examination. However, when to operate should be decided on a case-by-case basis, with close follow-up and meticulous assessment by skillful surgeons and radiologists, not to misjudge the appropriate timing for the radical surgery.

We report a case of a single huge hemangioma with intestinal obstruction following TAE treatment, by which the volume of the hemangioma was significantly reduced, and it was completely resected by a left hepatectomy. The outcome in our case indicates the importance of pre-operative management to reduce tumor size before surgery, and surgeons should pay attention to the complications of TAE.

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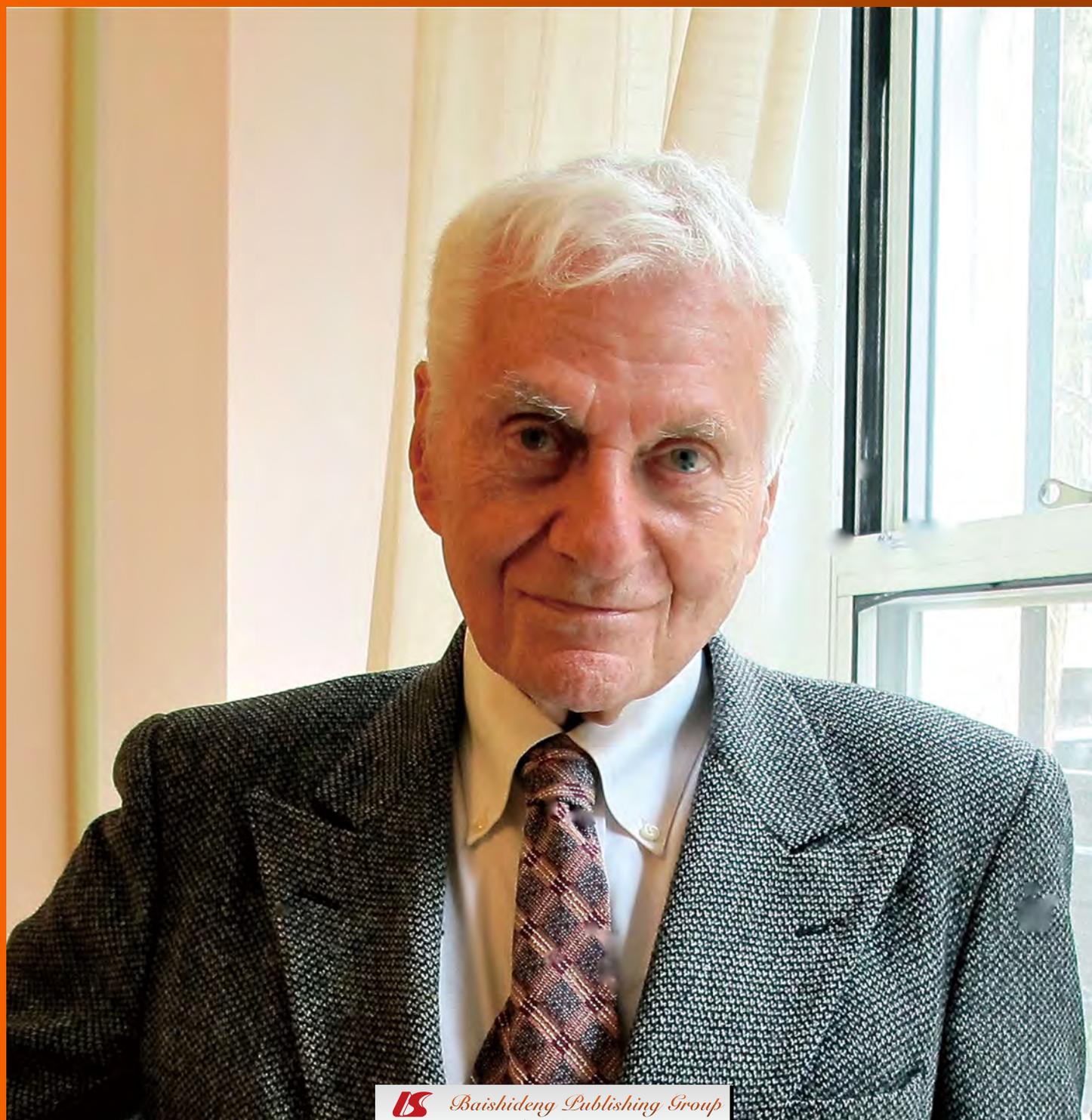
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- FRONTIER**                      **2979**    Expert opinion: Experience with 6-mercaptopurine in the treatment of inflammatory bowel disease  
*Korelitz BI*
- REVIEW**
- 2985**    Role of microRNAs in the immune system, inflammation and cancer  
*Raisch J, Darfeuille-Michaud A, Nguyen HTT*
- 2997**    Colon cancer stem cells: Controversies and perspectives  
*Puglisi MA, Tesori V, Lattanzi W, Gasbarrini GB, Gasbarrini A*
- ORIGINAL ARTICLE**
- 3007**    A silybin-phospholipids complex counteracts rat fatty liver degeneration and mitochondrial oxidative changes  
*Grattagliano I, Diogo CV, Mastrodonato M, de Bari O, Persichella M, Wang DQH, Liquori A, Ferri D, Carratù MR, Oliveira PJ, Portincasa P*
- 3018**    Incidence and mortality of acute and chronic pancreatitis in the Netherlands: A nationwide record-linked cohort study for the years 1995-2005  
*Spanier BWM, Bruno MJ, Dijkgraaf MGW*
- 3027**    Matrix metalloproteinase-9 in the initial injury after hepatectomy in mice  
*Ohashi N, Hori T, Chen F, Jermanus S, Nakao A, Uemoto S, Nguyen JH*
- BRIEF ARTICLE**
- 3043**    *MGMT* and *MLH1* methylation in *Helicobacter pylori*-infected children and adults  
*Alvarez MC, Santos JC, Maniezzo N, Ladeira MS, da Silva ALC, Scaletsky ICA, Pedrazzoli Jr J, Ribeiro ML*
- 3052**    Neoadjuvant-intensified treatment for rectal cancer: Time to change?  
*Musio D, De Felice F, Bulzonetti N, Guarnaccia R, Caiazzo R, Bangrazi C, Raffetto N, Tombolini V*
- 3062**    Glucomannan for abdominal pain-related functional gastrointestinal disorders in children: A randomized trial  
*Horvath A, Dziechciarz P, Szajewska H*
- 3069**    Efficacy and safety of 0.4 percent sodium hyaluronate for endoscopic submucosal dissection of gastric neoplasms  
*Kim YD, Lee J, Cho JY, Kim SW, Kim SH, Cho YK, Jang JS, Han JS, Cho JY*

- 3077** Robotic cholecystectomy with new port sites  
*Kim JH, Baek NH, Li G, Choi SH, Jeong IH, Hwang JC, Kim JH, Yoo BM, Kim WH*
- 3083** High-intensity focused ultrasound ablation: An effective bridging therapy for hepatocellular carcinoma patients  
*Cheung TT, Fan ST, Chan SC, Chok KSH, Chu FSK, Jenkins CR, Lo RCL, Fung JYY, Chan ACY, Sharr WW, Tsang SHY, Dai WC, Poon RTP, Lo CM*
- 3090** Development and application of a real-time polymerase chain reaction method for *Campylobacter jejuni* detection  
*Zhang MJ, Qiao B, Xu XB, Zhang JZ*
- 3096** Prediction of risk factors for lymph node metastasis in early gastric cancer  
*Ren G, Cai R, Zhang WJ, Ou JM, Jin YN, Li WH*
- 3108** Rectal gastrointestinal stromal tumors: Imaging features with clinical and pathological correlation  
*Jiang ZX, Zhang SJ, Peng WJ, Yu BH*
- 3117** Synchronous adenocarcinoma and gastrointestinal stromal tumors in the stomach  
*Cai R, Ren G, Wang DB*
- 3124** Risk factors for proton pump inhibitor refractoriness in Chinese patients with non-erosive reflux disease  
*Niu XP, Yu BP, Wang YD, Han Z, Liu SF, He CY, Zhang GZ, Wu WC*
- 3130** Expression of huCdc7 in colorectal cancer  
*Chen HJ, Zhu Z, Wang XL, Feng QL, Wu Q, Xu ZP, Wu J, Yu XF, Qian HL, Lu Q*
- 3134** Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes  
*Lv WS, Sun RX, Gao YY, Wen JP, Pan RF, Li L, Wang J, Xian YX, Cao CX, Zheng M*
- 3143** Value of circulating cell-free DNA in diagnosis of hepatocellular carcinoma  
*Chen K, Zhang H, Zhang LN, Ju SQ, Qi J, Huang DF, Li F, Wei Q, Zhang J*
- 3150** Protective effects of two *Lactobacillus plantarum* strains in hyperlipidemic mice  
*Wang LX, Liu K, Gao DW, Hao JK*

## CASE REPORT

- 3157** Small undifferentiated intramucosal gastric cancer with lymph-node metastasis: Case report  
*Odagaki T, Suzuki H, Oda I, Yoshinaga S, Nonaka S, Katai H, Taniguchi H, Kushima R, Saito Y*
- 3161** Intraductal papillary neoplasm of the bile duct accompanying biliary mixed adenoneuroendocrine carcinoma  
*Onishi I, Kitagawa H, Harada K, Maruzen S, Sakai S, Makino I, Hayashi H, Nakagawara H, Tajima H, Takamura H, Tani T, Kayahara M, Ikeda H, Ohta T, Nakanuma Y*
- 3165** Behçet's disease complicated by multiple aseptic abscesses of the liver and spleen  
*Maeshima K, Ishii K, Inoue M, Himeno K, Seike M*
- 3169** Esophageal reconstruction with remnant stomach: A case report and review of literature  
*Xie SP, Fan GH, Kang GJ, Geng Q, Huang J, Cheng BC*

**APPENDIX I-VI Instructions to authors**

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| <p><b>NAME OF JOURNAL</b><br/><i>World Journal of Gastroenterology</i></p> <p><b>ISSN</b><br/>ISSN 1007-9327 (print)<br/>ISSN 2219-2840 (online)</p> <p><b>LAUNCH DATE</b><br/>October 1, 1995</p> <p><b>FREQUENCY</b><br/>Weekly</p> <p><b>EDITORS-IN-CHIEF</b><br/><b>Ferruccio Bonino, MD, PhD, Professor</b> of Gastroenterology, Director of Liver and Digestive Disease Division, Department of Internal Medicine, University of Pisa, Director of General Medicine 2 Unit University Hospital of Pisa, Via Roma 67, 56124 Pisa, Italy</p> <p><b>Myung-Hwan Kim, MD, PhD, Professor, Head</b>, Department of Gastroenterology, Director, Center for Biliary Diseases, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpa-gu, Seoul 138-736, South Korea</p> <p><b>Kjell Öberg, MD, PhD, Professor</b>, Department of Endocrine Oncology, Uppsala University Hospital, SE-751 85 Uppsala, Sweden</p> | <p><b>Matt D Rutter, MBBS, MD, FRCP</b>, Consultant Gastroenterologist, Senior Lecturer, Director, Tees Bowel Cancer Screening Centre, University Hospital of North Tees, Durham University, Stockton-on-Tees, Cleveland TS19 8PE, United Kingdom</p> <p><b>Andrzej S Tarnawski, MD, PhD, DSc (Med), Professor of Medicine, Chief Gastroenterology</b>, VA Long Beach Health Care System, University of California, Irvine, CA, 5901 E. Seventh Str., Long Beach, CA 90822, United States</p> <p><b>EDITORIAL OFFICE</b><br/>Jin-Lei Wang, Director<br/>Xiu-Xia Song, Vice Director<br/><i>World Journal of Gastroenterology</i><br/>Room 903, Building D, Ocean International Center, No. 62 Dongsihuan Zhonglu, Chaoyang District, Beijing 100025, China<br/>Telephone: +86-10-59080039<br/>Fax: +86-10-85381893<br/>E-mail: wjg@wjgnet.com<br/>http://www.wjgnet.com</p> <p><b>PUBLISHER</b><br/>Baishideng Publishing Group Co., Limited<br/>Flat C, 23/F, Lucky Plaza,<br/>315-321 Lockhart Road, Wan Chai, Hong Kong, China</p> | <p>Fax: +852-65557188<br/>Telephone: +852-31779906<br/>E-mail: bpgoffice@wjgnet.com<br/>http://www.wjgnet.com</p> <p><b>PUBLICATION DATE</b><br/>May 28, 2013</p> <p><b>COPYRIGHT</b><br/>© 2013 Baishideng. Articles published by this Open-Access journal are distributed under the terms of the Creative Commons Attribution Non-commercial License, which permits use, distribution, and reproduction in any medium, provided the original work is properly cited, the use is non commercial and is otherwise in compliance with the license.</p> <p><b>SPECIAL STATEMENT</b><br/>All articles published in this journal represent the viewpoints of the authors except where indicated otherwise.</p> <p><b>INSTRUCTIONS TO AUTHORS</b><br/>Full instructions are available online at <a href="http://www.wjgnet.com/1007-9327/g_info_201100315215714.htm">http://www.wjgnet.com/1007-9327/g_info_201100315215714.htm</a></p> <p><b>ONLINE SUBMISSION</b><br/><a href="http://www.wjgnet.com/esp/">http://www.wjgnet.com/esp/</a></p> |
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## Expert opinion: Experience with 6-mercaptopurine in the treatment of inflammatory bowel disease

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Author contributions: Korelitz BI solely contributed to this paper.

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Received: December 26, 2012 Revised: April 9, 2013

Accepted: April 17, 2013

Published online: May 28, 2013

### Abstract

Arbitrarily, modern day treatment of inflammatory bowel disease begins with the introduction of immunosuppressives for ulcerative colitis. Clinical improvement with sulfasalazine had been meaningful but modest. Treatment with adrenocorticotrophic hormone and corticosteroids led to clinical responses never before realized but it took much too long to recognize that they were not capable of maintaining remission, that adverse reactions were subtle but potentially devastating and that some other agent would be necessary to capitalize on their transient advantage. This of course was true in the treatment of Crohn's disease as well. Not much was ever made of the role of sulfasalazine for Crohn's disease, but with the severing of the diazobond and the elimination of the sulphur component, the 5-aminosalicylic acid (5-ASA) products clearly led to clinical improvement, especially in cases of Crohn's colitis and those with ileitis where the 5-ASA product was released in the terminal ileum and more proximal in the small bowel as well as in ulcerative colitis. The induction of remission was first demonstrated by 6-mercaptopurine (6-MP) with case reports and uncontrolled trials in patients with ulcerative colitis, but its placebo controlled trial for Crohn's disease firmly established its role in inducing remission. No subsequent trial has confirmed

its similar role for ulcerative colitis, but nevertheless clinicians know well that 6-MP works at least as well and probably more effectively for ulcerative colitis than for Crohn's disease. What changes have taken place utilizing 6-MP in the management of inflammatory bowel disease since its introduction in the 1960's and 1970's and its trial for Crohn's disease published in the *New England Journal of Medicine* in 1980?

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**Key words:** 6-Mercaptopurine; Crohn's disease; Ulcerative colitis

**Core tip:** Calculation of dose, utilization of serological tests, maintenance therapy, desensitization to 6-mercaptopurine (6-MP), toxicity to 6-MP, post-operative prevention, extraintestinal manifestations, perirectal fistulas and other fistulas, pregnancy, role of biologicals in management, brand name *vs* generic 6-MP and azathioprine.

Korelitz BI. Expert opinion: Experience with 6-mercaptopurine in the treatment of inflammatory bowel disease. *World J Gastroenterol* 2013; 19(20): 2979-2984 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/2979.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.2979>

### CALCULATION OF DOSE

In the original controlled study, the dose of 6-mercaptopurine (6-MP) chosen by Present and myself was based on weight at 1 ½ mg per kilogram. This precedent has subsequently been used in almost all other studies around the world when the immunosuppressive of choice was 6-MP rather than Azathioprine. Despite this and due to my own experience based on the wide range of leukopenia as influenced by dose, I changed my preference from dose by weight and started all patients at 50 mg/d. Since

in both cases my policy was to have blood drawn for a complete blood count (CBC), at one, two and three weeks, a rapid fall in the white blood cell count (WBC) would be recognized early and the drug could be reduced or stopped accordingly. On the contrary should there be no fall in the WBC and there also be no clinical improvement in the inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), I would have the opportunity to increase the dose within the 3 wk period<sup>[1-5]</sup>.

If the patient were still on steroids when the 6-MP was started and there was clinical improvement, I have reduced the steroid dose early to minimize its duration, also keeping in mind that the WBC was elevated because of the steroids and their reduction might accelerate the leukopenia. Reintroduction of 6-MP after stabilization on recovery from the leukopenia would be by 25-50 mg less than before. Some patients tolerate only as little as 1/2 tablet or 25 mg/wk while others require an increase up to 300 mg/d, the low and the high doses being equally effective for the individual case.

This exercise has been modified by the availability of biologicals since the severity of disease and lack of response to early doses of 6-MP will serve to accelerate the decision to add the biological to the therapy<sup>[6-8]</sup>.

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## UTILIZATION OF SEROLOGICAL TESTS TO INFLUENCE THE DOSE OF 6-MP

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Since these tests were not available in the 1960's and 1970's, we were permitted a prolonged period of experience without them and then later learned that I never had to utilize them. By the time I would decide to start 6-MP the first CBC was drawn and interpreted before there was even time to receive a report on thiopurine methyltransferase, TGN or TPPT. Furthermore, a favorable clinical response was documented as early as possible since the blood counts were entered on a monitor sheet followed by directions by me when the patient called as told to do so. If the patient did not call on time, it was the practice of my office to call them should a change in dose be warranted. With increased experience, I would also get liver function tests including a GGTP early on and then periodically to recognize mild abnormalities consistent with an acceptable "transaminitis" versus a progressive abnormality of true liver damage. One monitor sheet included a column for WBC, Hb, Hct, and platelets and another for bilirubin, serum glutamic oxaloacetic transaminase, serum glutamic pyruvate transaminase, alkaline phosphatase, GGTP and Amylase<sup>[9-12]</sup>.

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## MAINTENANCE THERAPY WITH 6-MP FOLLOWING SUCCESSFUL INDUCTION

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As an outcome of the original controlled trial, we learned that the mean time to induction of remission was three months. Even then, however, it was clear that some patients responded faster, even as little as three weeks, and

others might take up to a year and still be successful. The differences were modified by recognizing the success of a small dose of 6-MP leading to that dose becoming the maintenance dose, and the failure of the initial dose leading to a relatively rapid increase until the maintenance dose was found without requiring a whole year or even six months. Again, should the patient be on steroids when the 6-MP was started, tapering and elimination of the steroids was part of the goal, always mindful that any reduction of steroids increased the risk for leukopenia.

The role of immunosuppressives in top down vs bottom up straddles these two approaches to therapy. This is because 6-MP has maintenance as well as induction value as opposed to steroids which work quickly but have no maintenance value and infliximab which also works quickly and does have maintenance value. The 6-MP, however, is slower in its action than both of the above in most cases. Therefore 6-MP will be more appropriate as a Step Up drug unless used from the outset as a Top Down in conjunction with infliximab as done in the Sonic Trial.

At the other end of the spectrum, after prolonged remission on 6-MP [ $\pm$  a 5-aminosalicylic acid (5-ASA) product but no biological as yet], some patients wanted to stop the drug, others refused to stop it remembering the severity of their disease before its introduction, and others accepted my recommendation based on clinical judgment. The time between CBC's and LFT's was already extended due to persistence of normality, so that some patients reached an interval of three months. I have chosen not to extend the interval beyond this period. If I then chose to reduce the dose on clinical grounds (usually because of patients' persistent fear of toxicity), I would deduct 25-50 mg. from the daily dose and keep that level for a trial of one year before considering a further reduction. This means that some patients would be maintained on one tablet/day (reduced from 2/d or 1 1/2/d) and others on 1/2 every other day reduced from 1/2/d or 1/2 on two out of three days.

At this time I will add that in a few cases I eventually stopped the 6-MP entirely. I have not yet analyzed the results but have witnessed many examples of recurrence on both stopping and reducing the dose of the 6-MP<sup>[13-16]</sup>.

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## DESENSITIZATION TO 6-MP IN THE COURSE OF TREATMENT OF IBD

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Next to leukopenia, allergic reactions to 6-MP are the next most common adverse effects. Whether nausea and malaise are also on an allergic basis has not been clear. In my own experience, the most common allergic reactions are fever, skin rash, joint pains and back pain. Unfortunately, the 6-MP has often been terminated on this basis and both CD and UC have frequently progressed in severity thereafter, leading to surgical resections which might not have been necessary. Just as we have found earlier that desensitization to sulfasalazine not only is frequently successful, and treatment with that drug resulted in remissions of IBD, particularly UC, desensitization has been fruitful for 6-MP. This has been done by starting

with as little as 1/8 tablet and increasing the dose every few days or switching to Azathioprine at either full dose or the same fractions. In my experience the only allergic reaction to which desensitization has rarely been successful has been pancreatitis. Once desensitization to 6-MP has been accomplished, remission has been maintained on this drug for many years<sup>[17,18]</sup>.

## TOXICITY TO 6-MP IN THE COURSE OF TREATMENT OF IBD

Other than leukopenia and allergic reactions to 6-MP, concern about neoplasm has prevailed. After many years of experience, I am convinced that malignant tumors such as breast, lung, liver, pancreas, kidney, prostate and brain are not more common in those treated with 6-MP than in the entire IBD population or the general population. The problem of lymphoma has warranted intense observation and it is statistically significantly increased but still rare. I accept the conclusion that a lymphoma occurs in somewhere between 1:2000 and 1:4000 cases. If it occurs its prognosis is no different than lymphomas in IBD patients not treated with 6-MP or Azathioprine or no IBD. The exception to this rule is the hepato-splenic lymphoma which carries the worst prognosis of the lymphomas and it occurs in children, particularly male children who have the most virulent IBD, so that even in this group of patients it is challenging not to use 6-MP as well as to using it. Most patients with this type of lymphoma have been on combination therapy (6-MP or Azathioprine plus IV infliximab) but the onus is on the immunosuppressive since the lymphoma rarely occurs with infliximab alone. The one neoplasm which theoretically might be increased with immunosuppressive therapy is colon cancer superimposed on ulcerative or Crohn's colitis. My own studies and those of others have shown that this is not the case. If anything, treatment with immunosuppressives has reduced the risk of colon cancer, probably as a result of eliminating inflammation due to successful therapy.

Unfortunately, skin cancers are common in IBD patients treated with immunosuppressives. While basal cells can be successfully resected and don't often lead to terminating 6-MP, this is not so true of squamous cell carcinomas which I see fairly commonly in patients who have received the drug for many years. I have rarely seen a melanoma in patients on 6-MP.

Opportunistic infections are rare. When they occur they correlate best with situations when the disease is not controlled by the immunosuppressive drug and usually when the patient is still being treated with steroids.

Pancreatitis usually occurs within three weeks of onset of treatment with 6-MP. In our original studies, we encountered pancreatitis in 3% of patients. I rarely see it anymore.

Thrombocytopenia is fairly common in patients on 6-MP and if it occurs it is usually in conjunction with leukopenia. Anemia due to 6-MP is rare and if it occurs is most likely accompanied by a pancytopenia and requires

stopping the immunosuppressive therapy<sup>[19-26]</sup>.

## POST OPERATIVE PREVENTION WITH 6-MP

Trials of available drugs for prevention of recurrent ileitis after ileo-colic resection have been disappointing. My own study of 6-MP for this indication showed statistically significant but not impressive results. Nevertheless, I have had many patients who started 6-MP following surgery who have remained without clinical recurrence for many years. It is my impression that endoscopic recurrence may occur despite taking the 6-MP, but its progress to a point of clinical recurrence is extremely retarded. I have also reassessed the results of my own study and learned that when the 6-MP is started in the immediate peri-operative period, protection against the recurrence is far more effective. This is an area where infliximab is proving to be more effective than immunosuppressives, again best when started immediately postoperatively. More studies in the area of which drug, both, and when are needed to resolve this question<sup>[27-29]</sup>.

## THE ROLE OF IMMUNOSUPPRESSIVES FOR EXTRAINTestinal MANIFESTATIONS

Before the advent of biologicals, treatment of pyoderma gangrenosum, erythema nodosum, arthritic manifestations and uveitis were often successful with the introduction of 6-MP. If however, the extraintestinal manifestation occurs while the patient is already taking an immunosuppressive, the need to introduce a biological is clear.

## PERIRECTAL FISTULAS AND OTHER FISTULAS

Many of my own studies have demonstrated the favorable effect on all fistulas with 6-MP treatment. Perirectal fistulas and abscesses often require incision and drainage in conjunction with 6-MP but are more likely to be recurrent without immunosuppressive therapy at the same time. Infliximab also has been very effective in closing fistulas. Nevertheless, it is fairly common to see persistent drainage from fistulas despite treatment with either drug alone and even with the combination. Fortunately, the severity of the residual fistula is not great and if the primary CD has been brought into remission, the patient tolerates the drainage well<sup>[30,31]</sup>.

## PREGNANCY AND IMMUNOSUPPRESSIVES

The issue of 6-MP and AZA before and during pregnancy prevails since the most common years of onset

of Crohn's disease and ulcerative colitis are during the ages of greatest fertility and Crohn's disease occurs more often in females than males. Furthermore, consideration of continuing immunosuppressives during pregnancy is markedly diluted as an issue since pregnancy usually takes emotional priority over treatment of the disease in female IBD patients who want to stop all medications and so often the obstetrician is encouraging them to do so.

The evidence favoring continuing 6-MP/AZA during the pregnancy is based on the following: (1) The largest reported study on pregnancy and adverse outcomes possibly attributed to 6-MP from Mount Sinai has concluded that these drugs are safe; (2) Most adverse reactions to 6-MP/AZA occur early, soon after the drug is started. Therefore, the coincidence of any other toxicity to 6-MP in pregnancy most likely must be attributed to active disease; and (3) If the most virulent factor with toxic complications during pregnancy is active Crohn's disease and if the patient is in a remission just achieved by the drug, it should not be stopped. On the other hand, in a study from Lenox Hill Hospital there was a 23% incidence of spontaneous abortions (*vs* 13% in IBD controls), a 3% incidence of ectopic pregnancies (compared with none in IBD controls) and finally an abnormal amniocentesis in 2 patients (and none in the IBD controls).

Statistically speaking, no one is yet certain of the risk or the safety of immunosuppressives taken before or during pregnancy and therefore no conclusion should yet be drawn. Logically there must be a compromise solution: (1) Given that the most important issue is active Crohn's disease at conception, if the patient has already been started on the immunosuppressive drug it should be continued and the dose even increased if the clinical severity of the disease warrants it; (2) If the IBD is in remission and has been for months or for years, I find no contraindication to stopping the drug at or before the diagnosis of pregnancy since our experience has shown that any exacerbation is not likely to occur immediately or for that matter even for months, by which time the pregnancy may be ended or at least the fetus is protected through the first trimester when theoretically it would be most susceptible to any danger. Should an exacerbation occur earlier in the pregnancy, the choice may be made to reintroduce the drug; (3) The risk of toxicity to the pregnancy when the father is the one who has the IBD and is taking 6-MP/AZA raises a special consideration. If the male has been in remission, it might be prudent to stop the drug for 1 to 3 mo before conception. Since the timing of the pregnancy is so infrequently controlled, this opportunity does not occur often; and (4) Decisions whether to continue 6-MP/AZA in pregnant women and their husbands who are taking the drug for IBD require rigorous clinical judgement. For example, if the woman has been in remission for a long time, it seems reasonable to stop the drug until delivery since recurrence is very unlikely. If recurrence does develop, then the drug can be restarted at that time. If either the pregnant female patient or the husband with IBD have active Crohn's

disease or have been in remission only briefly following a severe attack, I recommend continuing the drug. This is an area where rules should not be rigid<sup>[32-36]</sup>.

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## THE ROLE OF 6-MP SINCE THE AVAILABILITY OF BIOLOGICALS; WHEN TO ADD ONE TO THE OTHER, WHEN TO TERMINATE ONE OR THE OTHER AND WHICH ONE

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Some of the most challenging therapeutic decisions have been raised since the publication of articles suggesting that once a patient with Crohn's disease is in clinical remission while being treated with both infliximab and 6-MP/AZA, there is no advantage to continuing the immunosuppressive drug. These studies do not adequately allow for the duration of treatment with the 6-MP, when it was started in reference to infliximab, or the duration and dose of infliximab required to bring the patient into remission. Furthermore, it does not allow for the conclusions of the Study of Immunomodulator Naïve Patients in Crohn's Disease which demonstrated that the therapeutic efficacy of the combination of infliximab and 6-MP/AZA is greater than either drug alone.

The following are the my suggested options for changing therapy for Crohn's disease and ulcerative colitis in regard to either 6-MP or AZA alone, infliximab or other biological alone, and 6-MP/AZA and a biological together.

### *No response or beginning failure with 6-MP/AZA alone*

Increase the dose if WBC or platelets permit; add a biological; add a 5-ASA product (this is a particularly good opportunity to add a once daily dose product for compliance reasons.); surgery, usually the last resort, but influenced by location and specific complication of Crohn's disease.

### *No response or failure with a biological*

Increase the dose, decrease the interval between infusions or injections; add 6-MP or AZA; add a 5-ASA product; change the biological; Measure serum infliximab and antibody levels for guidance; brief rescue therapy with intravenous corticosteroids; Surgery, usually the last resort, but influenced too by location and specific complication of the Crohn's disease.

### *Failure with combined therapy of immunosuppressive and biological*

Increase the dose of the immunosuppressive if WBC or platelet counts permit; decrease the interval between infusions or injections; add a 5-ASA product; brief rescue therapy with intravenous corticosteroids; measure serum infliximab and antibody levels for guidance; stop biological if degree of immunogenicity is high and accompanied by allergic symptoms such as joint pains; stop 6-MP or

AZA if complications suspected of being attributed to these drugs are evident, such as nausea, malaise, fever, and worsening liver or pancreatic function tests; surgery.

### **Eliminating 6-MP/AZA after remission with combined therapy of immunosuppressives and biologicals**

Complications of drug or disease; reduce the dose - especially for persistent leukopenia; patients' fear of late complications; in some cases of pregnancy or anticipated pregnancy; continuation influenced by earlier severity of the disease.

### **Eliminating biologicals (when used alone) after remission**

Fear of complications of the drug; lack of compliance; now substitute 6-MP/AZA; first extend interval for infusion or injection; first reduce the dose; add a 5-ASA product if not already done.

### **Eliminating the biological or immunosuppressive after remission with both**

To be considered preferably only after 1 full year of maintenance therapy and full dose of both after remission achieved; first reduce dose of the immunosuppressives; eventually eliminate the 6-MP/AZA or the biological; the author's preference is to eliminate the 6-MP/AZA and continue the biological; later reduce the dose of the biological as well.

Once remission of the IBD has been maintained for at least a year, there are many considerations. While some patients do not wish to change the therapeutic program because of its success, others are fearful of complications of either or both drugs and are anxious to eliminate or reduce. In some cases the specific indication for starting the program remains tolerable but not eliminated, in which case I encourage the patient to persist. In other cases where the indication for starting one or both drugs is gone and indeed mucosal healing has been accomplished as well (mucosal healing to me requires histological healing), I undertake a dose reduction.

Despite the efforts made in rationalizing the reduction of 6-MP vs infliximab, the subsequent management is currently influenced by the subsequent course of the primary disease rather than patient hardships or drug complications. I have witnessed exacerbations of Crohn's disease and ulcerative colitis following elimination or reduction of both drugs. The course of management is then clear by reinstating the appropriate drug, preferably to the dose of 6-MP at which the longest remission was maintained or the infliximab at the dose and frequency of infusions in which remission was achieved.

One other option for failure or intolerance to 6-MP or lack of response to Remicade is the substitution of a different biological. My experience is biased by the long period of time with the availability of only the Remicade. With the subsequent introduction of Adalimumab and Certolizumab pegol, I had already learned how to use the infliximab well and had no need to change, or if the infliximab had failed so then did the other biological that I

then tried. Furthermore, I don't consider self administration of the biological an advantage since the patient has been known to alter the dose or the frequency for whatever the rationale whenever the guidance of the managing gastroenterologist is reduced or otherwise modified. Rapport between patient and doctor remains an influential factor in successful therapy even though the scientific evidence for this might be lacking<sup>[37-39]</sup>.

## **BRAND NAME VS GENERIC 6-MP AND AZATHIOPRINE**

While there are no controlled trials to resolve this issue, I have seen more recurrences of IBD after switching to the generic than when continuing with the brand (Purinethol or Imuran). Therefore, I continue using the brand name when feasible.

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**P- Reviewers** Capasso R, Fitzpatrick LR, Kaymakoglu S  
**S- Editor** Wen LL **L- Editor** A **E- Editor** Zhang DN



## Role of microRNAs in the immune system, inflammation and cancer

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Author contributions: Raisch J and Nguyen HTT wrote the paper; Darfeuille-Michaud A supervised the manuscript.

Supported by The Ministère de la Recherche et de la Technologie (JE2526, France), Inserm and University of Auvergne (UMR1071), INRA (USC-2018); Grants from the Association François Aupetit (AFA to Arlette Darfeuille-Michaud), and the European Union FP7 People Marie Curie International Incoming Fellowship (IIF to Hang Nguyen)

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Received: January 24, 2013 Revised: March 29, 2013

Accepted: April 10, 2013

Published online: May 28, 2013

### Abstract

MicroRNAs, a key class of gene expression regulators, have emerged as crucial players in various biological processes such as cellular proliferation and differentiation, development and apoptosis. In addition, microRNAs are coming to light as crucial regulators of innate and adaptive immune responses, and their abnormal expression and/or function in the immune system have been linked to multiple human diseases including inflammatory disorders, such as inflammatory bowel disease, and cancers. In this review, we discuss our current understanding of microRNAs with a focus on their role and mode of action in regulating the immune system during inflammation and carcinogenesis.

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**Key words:** MicroRNAs; Immune response; Inflammation; Inflammatory bowel disease; Colorectal cancer

**Core tip:** MicroRNAs (miRNAs), a key class of gene expression regulators, have emerged as crucial players in various biological processes such as cellular proliferation and differentiation, development and apoptosis. A better understanding of the function of miRNAs is providing new insights into the molecular basis of human pathologies, and new biomarkers for disease diagnosis and therapy.

Raisch J, Darfeuille-Michaud A, Nguyen HTT. Role of microRNAs in the immune system, inflammation and cancer. *World J Gastroenterol* 2013; 19(20): 2985-2996 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/2985.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.2985>

### INTRODUCTION

MicroRNAs (miRNAs, miR) are small (approximately 20-22 nucleotides), non-coding RNAs that post-transcriptionally regulate gene expression by binding to the 3'-untranslated region of target mRNAs, leading to mRNA degradation or translational inhibition<sup>[1]</sup>.

Since the identification of the first miRNA, *lin-4*, in *Caenorhabditis elegans* in 1993<sup>[2,3]</sup>, thousands of miRNA genes have been identified in animal and plant genomes<sup>[4]</sup>. As a class, miRNAs account for about 1%-2% of genes in worms, flies, and mammals<sup>[5]</sup>. Each miRNA can target hundreds of mRNAs within a given cell type, and a single mRNA is often the target of multiple miRNAs, and thus over half of the human transcriptome is predicted to be under miRNA regulation, embedding this post-transcriptional control pathway within nearly every biological process<sup>[5]</sup>.

Given its fundamental biological roles, it is not sur-

prising that miRNA expression is tightly controlled and that its deregulation can lead to various diseases. In this review, we summarize our current knowledge about the physiological role of miRNAs in mammalian biology and the manner in which miRNA activities contribute to diseases including inflammatory disorders and cancer.

## MIRNA BIOSYNTHESIS AND REGULATION

### Biosynthesis

Our knowledge of miRNA biogenesis and regulation has been greatly expanded in recent years<sup>[1]</sup>. The canonical miRNA biogenesis takes place in a multi-step process and involves two RNase III endonucleases, Dicer and Drosha. MiRNAs are encoded by genomic DNA and are most commonly transcribed by RNA polymerase II, which generates a primary miRNA (pri-miRNA) transcript. Within the primary transcripts, miRNAs form stem-loop structures, which contain the mature miRNA as part of an imperfectly paired double-stranded stem connected by a short terminal loop. Pri-miRNAs are then processed by a microprocessor complex, a multiprotein complex with the two core components, Drosha and DiGeorge Syndrome critical region 8 (DGCR8)<sup>[6-8]</sup>. This results in the formation of a hairpin-shaped RNA molecule of 70-100 bp called miRNA precursor or pre-miRNA, which is then exported into the cytoplasm in a process involving the nucleocytoplasmic shuttle Exportin-5 and in a Ran-GTP-dependent manner<sup>[9-11]</sup>. In cytoplasm, the pre-miRNA hairpin is cleaved by the endonuclease DICER into an imperfect miRNA:miRNA\* duplex of 21-23 nucleotides in length<sup>[12]</sup>. After separation of the two strands of the duplex, one of the strands (the mature miRNA) is transferred into an Argonaute (Ago) protein located in the RNA-induced silencing complex (RISC or miRISC), which is involved in the repression of gene expression by leading miRNAs to specific target mRNAs, whereas the other strand (the star-strand) is degraded. It has been shown that strand selection and RISC assembly in mammals are accomplished by a complex that contains Dicer, Ago and the double-stranded RNA binding protein TRBP<sup>[13-15]</sup>. MiRNAs target mRNAs by interacting with sites of imperfect complementarity. Short “seed” sequences at the 5'-ends of miRNAs (nucleotides 2-8) are critical, and in some cases fully sufficient, for target selection<sup>[16,17]</sup>.

### Regulation

Although there have been recent advances in our knowledge of the biogenesis of the miRNA pathway, relatively little is known about the mechanisms regulating the activity of the pathway's components. Several recent studies indicate that the regulation of miRNA expression and function occurs at three levels: transcription, processing and subcellular localization<sup>[17,18]</sup>.

The first, and one of the most important, mechanisms controlling miRNA abundance is the regulation of pri-

miRNA transcription, which could be positively or negatively regulated by different factors such as transcription factors, enhancers, silencers and epigenetic modification in miRNA promoters<sup>[16]</sup>. For example, the oncogene c-myc can bind to the promoter of the miR-17-5p cluster, thereby up-regulating expression of the miRNAs encoded by the cluster<sup>[19,20]</sup>. Similarly, the tumour suppressor p53 has been shown to upregulate the transcription of miR-34 family members, inhibiting important factors of cell proliferation and survival, such as Bcl2 and Cdk4 and 6<sup>[21-24]</sup>. A region of miRNA genes is located within CpG islands involving the epigenetic control of miRNA transcription. It is estimated from recent works that 5%-10% of mammalian miRNAs are epigenetically regulated<sup>[19,25-27]</sup>.

Several post-transcriptional regulatory mechanisms that affect miRNA processing at different stages, from the pri-miRNA transcripts to the delivery of mature miRNAs to their target mRNAs, have recently been investigated<sup>[18]</sup>. For example, p53 can form a complex with Drosha, which increases the processing of pri-miRNAs to pre-miRNAs<sup>[28]</sup>. Histone deacetylase I can enhance pri-miRNA processing by deacetylating the protein DGCR8 of the microprocessor complex<sup>[29]</sup>. Cytokines such as interferons have been shown to inhibit Dicer expression, decreasing the processing of pre-miRNAs<sup>[30]</sup>.

## MICRORNAS AND IMMUNE SYSTEM

The immune system has evolved to maintain self-tolerance and to recognize efficiently specific pathogens. The innate immune system acts as a first protector providing an immediate response to pathogens, and propagation of the innate response activates the adaptive immune system. Both innate and adaptive immune responses are highly regulated, and recent studies have shed light on the role of miRNAs in this intricate system<sup>[31,32]</sup>. The role of miRNAs in immune responses will be discussed in this section.

### MicroRNAs and innate immune response

The innate immune system is activated *via* recognition of pathogen-associated molecular patterns by toll-like receptors (TLRs)<sup>[33]</sup>, which will recruit adaptor proteins to the receptor, followed by activation of downstream signalling pathways such as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway<sup>[34]</sup>. This signal transduction ultimately leads to induction of immune gene expression.

The first study examining the effect of lipopolysaccharide (LPS)-mediated activation of TLR signalling on miRNA production identified miR-155, miR-146a and miR-132, which are induced in human macrophages by LPS<sup>[35]</sup>. Further analysis showed that miR-155 is induced by LPS, cytokine IFN- $\beta$  and various TLR ligands in murine macrophages<sup>[36,37]</sup>. MiR-155, once induced, is involved in the activation of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-6 (IL-6), enzyme linked immunosorbent assay pathway *via* targeting the Fas-associated

death domain protein, I B kinase  $\epsilon$ , and receptor (TNF receptor superfamily)-interacting serine-threonine kinase 1<sup>[37]</sup>. MiR-155 plays a role in the innate immune response by regulating suppressor of cytokine signalling (SOCS)-1, a negative regulator of dendritic cell antigen-presenting capacity<sup>[37-39]</sup>. Likewise, miR-155-deficient dendritic cells exhibit impaired antigen presentation and therefore are unable to activate T cells to promote inflammation<sup>[39]</sup>. One study demonstrated that in human myeloid-derived DCs, knockdown of miR-155 expression significantly increased protein expression of the pro-inflammatory cytokine IL-1<sup>[40]</sup>. The same study also showed that miR-155 directly inhibited expression of the pro-inflammatory signalling protein TAK1-binding protein 2 (TAB2, also known as MAP3K7IP2), which could be a mechanism underlying its anti-inflammatory property<sup>[40]</sup>. In contrast, other studies have shown that miR-155 can enhance inflammatory responses. Overexpression of miR-155 in mouse bone marrow leads to a myeloproliferative phenotype that is similar to that observed transiently after LPS stimulation<sup>[41]</sup>. MiR-155 can negatively regulate SHIP1, an important negative regulator of phosphoinositide 3-kinase (PI3K) and the downstream AKT pathway<sup>[42,43]</sup>. SHIP1, which is similar to SOCS1, is a negative regulator of TLR4 signaling<sup>[44]</sup>, and hence repression of SHIP1 by miR-155 may counter this negative regulation and increase downstream AKT signalling.

Like miR-155, miR-146a is induced by LPS, TNF- $\alpha$  and IL-1 $\beta$  in a NF- $\kappa$ B-dependent manner. MiR-146a in turn inhibits expression of two components of the TLR4 signaling pathway, IL-1 receptor associated kinase and TNF receptor-associated factor-6<sup>[35]</sup>. Thus, miR-146a functions as a negative feedback regulator of the TLR/NF- $\kappa$ B pathway. MiR-155 and miR-146 expression is increased in macrophages in response to LPS stimulation, while miR-125b expression is decreased. MiR-125b can target TNF- $\alpha$  mRNA, and a decrease in its expression leads to elevated TNF- $\alpha$  production and consequently increased inflammatory response<sup>[37]</sup>.

Macrophage inflammatory response to infection involves the upregulation of several miRNAs, such as miR-21, miR-9 and miR-147<sup>[45-47]</sup>. These miRNAs can also be induced by TLR signaling, and can negatively regulate activation of inflammatory pathways in myeloid cells. MiR-9 represses NF- $\kappa$ B subunit 1 (NFKB1/p50 unit) and helps to maintain a constant level of NF- $\kappa$ B1 protein expression during TLR4-mediated activation of monocytes and neutrophils<sup>[46]</sup>. MiR-147 has been shown to attenuate TLR2, TLR3 and TLR4-mediated production of inflammatory proteins such as TNF- $\alpha$  and IL-6<sup>[47]</sup>. Induction of miR-21 inhibits PDCD4, an IL-10 inhibitor, thereby derepressing IL-10. IL-10 in turn inhibits miR-155, allowing SHIP1 to be derepressed and inhibit TLR signaling<sup>[45,48]</sup>. Hence, immune responses are highly regulated by TLRs-mediated upregulation of different miRNAs.

In addition to miRNA induction by TLR signaling, recent studies have also reported inflammatory repres-

sion, such as miR-155 repression, in response to anti-inflammatory cytokine IL-10<sup>[49]</sup>.

### MicroRNAs and adaptive immune response

In addition to their role in regulating the innate immune system, miRNAs have been implicated in adaptive immunity by controlling the development and activation of T and B cells.

#### T cells

Specific miRNA expression profiles have been reported in different T cell subsets and stages of development<sup>[50-52]</sup>, suggesting that miRNA-mediated regulation of signalling networks in T cells, and probably other immune cells, is dynamic and highly regulated. Interestingly, miRNA profiling in naive, effector and memory CD8<sup>+</sup> T cells has revealed that a few highly expressed miRNAs are dynamically regulated during antigen-specific T-cell differentiation<sup>[52]</sup>. Mice exhibiting T-cell specific deletion of Dicer had lower numbers of mature T cells with abnormally developed T-cell subsets than wild-type mice, indicating that miRNAs are required for T cell development<sup>[53,54]</sup>. Two specific miRNAs have been implicated in T cell development, and probably account for some of the phenotype of Dicer-deficient T cells. The miR-17-92 cluster suppresses expression of pro-apoptotic proteins, including BCL-2-interacting mediators of cell death (BIM or BCL2L11) and phosphatase and tensin homologue. This miRNA cluster is thought to increase T cell survival during development and is expressed during the double negative 2 stage of thymopoiesis<sup>[55]</sup>.

The role of miRNAs in the differentiation of T cells into distinct effector T helper cell subsets has been recently reported. It was demonstrated that miR-326 regulates differentiation of Th17 cells both *in vitro* and *in vivo*<sup>[56]</sup>. MiR-155 is implicated in regulatory T (T<sub>reg</sub>) cell formation and function, since forkhead box P3 (FOXP3), a transcription factor that is required for the development and function of T<sub>reg</sub> cells, may directly regulate the expression of this miRNA<sup>[57]</sup>. Furthermore, miRNA-155-deficient mice are immunodeficient, indicating the implication of miR-155 in homeostasis and the immune system<sup>[39]</sup>. Similarly, using genetic deletion and transgenic approaches, Thai and colleagues showed the important role of miR-155 in the mammalian immune system, specifically in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T cell-dependent antibody response<sup>[58]</sup>. Certain miRNAs, such as the miR-17-92 cluster, might be involved in the development and function of T follicular helper cells (specialized T cells that provide selective signals to supporting germinal center B cells), which are essential for long-lived antibody responses<sup>[59,60]</sup>. In addition, miR-181a, which is increased during early T cell development and down-regulated in mature CD4 T cells such as Th1 and Th2 effector cells, can enhance TCR signaling strength by inhibiting multiple phosphatases that negatively regulate the TCR signaling cascade<sup>[61]</sup>. Finally, conditional deletion

of *Dicer* or *Drosha* in T<sub>reg</sub> cells led to lethal autoimmune inflammatory disease, accompanied by impaired development or function of T<sub>reg</sub> cells, indicating the role of miRNAs in T<sub>reg</sub> cell biology<sup>[62-64]</sup>.

### B cells

Distinct miRNA profiles in naive, germinal central and post-germinal central B cells have been reported<sup>[65-67]</sup>, suggesting the implication of miRNAs in B cell development and maturation. A pioneer study showed that miR-181 is highly expressed in B cells of mouse bone marrow, and its ectopic expression in hematopoietic stem and progenitor cells resulted in an increase in the percentage of B-lineage cells but not in T cells or myeloid cells<sup>[68]</sup>, indicating the role of lineage-specific miRNAs in regulating lymphocyte development. Conditional deletion of *Dicer* in B cells completely arrested B cell development in mice, which is thought to be due to dysregulated expression of the pro-apoptotic protein BIM, probably during the selection of effective antigen receptors<sup>[69]</sup>. Notably, B cells lacking miR-17-92 family and *Dicer*-deficient B cells exhibited similar gene expression profiles<sup>[70]</sup>, suggesting that this miRNA cluster could play a determining role in the regulation of B cell development.

Recent studies have explored the role of miR-150, a miRNA specifically expressed by mature lymphocytes, in B cell differentiation<sup>[51,71,72]</sup>. MiR-150 expression increases during B-cell maturation in bone marrow, and its constitutive expression blocked B cell development at the transition from the pro-B-cell to pre-B-cell developmental stage, leading to severe defects in the production of mature B cells<sup>[71]</sup>. MiR-150-deficient mice exhibited a 2-fold increase in splenic B-1 cell numbers, with a relative decrease in those of B-2 cells, but had no apparent defect in the development of other lymphoid-derived T- and B-cell types<sup>[72]</sup>. Mice expressing a miR-150 transgene early in life also had dramatically impaired B cell development with normal T cell levels. These defects in miR-150 gain- and loss-of-function were further shown to be due to dysregulation of c-Myb, a target of miR-150 and a transcription factor that controls multiple steps of lymphocyte development<sup>[72]</sup>. MiR-155-deficient B cells showed defects in antibody class switching and differentiation into plasma cells, resulting in an impaired humoral response to T cell-dependent antigenic stimulation<sup>[39,58,73]</sup>. The constitutive expression of miR-34a blocked B cell development at the pro-B to pre-B cell transition, leading to a reduction in mature B cells<sup>[74]</sup>. This block appeared to be mediated by miR-34a-inhibited expression of the transcription factor Foxp1<sup>[74]</sup>, which is an essential regulator of B cell development<sup>[75]</sup>. Together, these studies show the important role of miRNAs in normal B cell development.

## MICRORNAS AND INFLAMMATORY BOWEL DISEASE

As miRNAs play a critical role in the regulation of the immune system, failure of miRNA regulation is associ-

**Table 1 MicroRNAs dysregulated in ulcerative colitis and/or Crohn's disease**

|               | Up-regulated  | Down-regulated  | Source, reference  |
|---------------|---|---|--|
| UC vs healthy | miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195, let-7f, miR-21, miR-155   | miR-192, miR-375, miR-422b  | Sigmoid colon <sup>[77]</sup>                                      |
|               | miR-7, miR-31, miR-135b, miR-223, miR-29a, miR-29b, miR-126*, miR-127-3p, miR324-3p   | miR-188-5p, miR-25, miR-320a, miR-346   | Colon <sup>[78]</sup><br>Colonic mucosa <sup>[79]</sup>            |
| CD vs healthy | miR-28-5p, miR-151-5p, miR-199a-5p, miR-340*, miRplus-E1271, miR-103-2*, miR-362-3p, miR-532-3p   | miR-505*  | Peripheral blood <sup>[80]</sup>                                   |
|               | miR-9, miR-126, miR-130a, miR-181c, miR-375, miR-26a, miR-29b, miR-30b, miR-34c-5p, miR-126*, miR127-3p, miR-133b, miR-155, miR-196a, miR324-3p, miR-21, miR-22, miR-29c, miR-31, miR-106a, miR-146a, miR146b-3p, miR-150 |   | Colonic mucosa <sup>[79]</sup>                                     |
| UC vs CD      | miR-199p-5a, miR-362-3p, miR-340*, miR-532-3p, miRplus-E1271  | miR-149*, miRplus-F1065   | Peripheral blood <sup>[80]</sup>                                   |
|               | miR-28-5p, miR-103-2*, miR-149*, miR-151-5p, miR-340*, miR-532-3p, miRplus-E1153  | (miR-150, miR-196b, miR-199a-3p, miR-199b-5p, miR-223, miR-320a) <sup>2</sup> | Colonic mucosa <sup>[79]</sup><br>Peripheral blood <sup>[80]</sup> |

<sup>1</sup>miRNAs upregulated specifically in non-inflamed colonic mucosa; <sup>2</sup>miRNAs downregulated specifically in non-inflamed tissue colonic mucosa. UC: Ulcerative colitis; CD: Crohn's disease.

ated with several human disorders such as inflammatory bowel disease (IBD) (Table 1), which is a chronic inflammatory gastrointestinal disorder. Although the etiology of IBD remains largely unknown, extensive studies in the last decades have suggested that it involves environmental and genetic factors that lead to dysfunction of the epithelial barrier with consequent deregulation of the mucosal immune system and responses to gut microbiota<sup>[76]</sup>.

Distinguished miRNA expression profiles have been recently described in tissues of patients with active and inactive UC, CD, irritable bowel syndrome (IBS), infectious colitis (IC), and microscopic colitis (MC)<sup>[77]</sup>. Wu and colleagues demonstrated that active UC was associated

with the differential expression of 11 miRNAs (3 significantly decreased and 8 significantly increased in UC tissues). MiR-192, the expression of which is decreased in active UC, was predominantly localized to colonic epithelial cells, and targeted macrophage inflammatory peptide (MIP)-2 $\alpha$ , a chemokine expressed by epithelial cells<sup>[77]</sup>. In colonic epithelial cells, TNF- $\alpha$ -induced MIP-2 $\alpha$  expression was inhibited by a miR-192 mimic. In contrast, miR-21 is significantly increased in patients with active UC compared to healthy subjects. In inactive UC patients, miR-375 and miR-422 expression was increased, while that of miR-192 was unaltered compared to healthy subjects<sup>[77]</sup>. Inactive UC showed similar expression levels of miR-375, miR-422b, and miR-23a to IBS and IC tissues. The miRNAs differently expressed in active UC were not dysregulated in MC and CD. This study highlights the specific miRNA expression patterns in active and inactive IBD tissues, and suggests that miRNAs could regulate expression of proteins implicated in the pathogenesis.

Another study showed the upregulated expression of several miRNAs in active UC compared to healthy colonic biopsies, suggesting that upregulation of miRNAs may be responsible for the development of intestinal inflammation in UC<sup>[78]</sup>. MiR-21 was found among the upregulated miRNAs, which is consistent with the findings of Takagi *et al.*<sup>[78]</sup>.

Of interest, Fasseu and colleagues identified restricted subsets of miRNAs abnormally expressed in inactive colonic mucosa of IBD patients<sup>[79]</sup>. This elegant study identified 14 (in UC) and 23 (in CD) miRNAs with significantly altered expression (> 5-fold increase or < 0.05-fold decrease) in quiescent colonic mucosa compared to healthy control tissues. Eight of the miRNAs (miR-26a, -29a, -29b, -30c, -126\*, -127-3p, -196a, -324-3p) were commonly dysregulated in non-inflamed UC and CD. Six miRNAs (miR-196b, -199a-3p, -199b-5p, -320a, -150, -223) displayed significantly distinct dysregulation of expression between non-inflamed UC and CD colonic biopsies. Interestingly, several miRNA genes with dysregulated expression mapped within acknowledged IBD-susceptibility loci. In addition, significant dysregulated expression of four and five miRNAs specific to inflamed UC or CD tissues, respectively, compared to healthy controls was observed<sup>[79]</sup>. This study sheds light on the role of miRNAs as contributors to IBD susceptibility, in particular their implication in the onset and/or relapse of inflammation from quiescent mucosa of IBD patients.

There have been recent reports of differential miRNA expression profiles in the peripheral blood of IBD patients<sup>[80]</sup>. Four miRNAs (miR-199a5p, -362-3p, -532-3p and miRplus-E1271) were upregulated and one miRNA (miRplus-F1065) was downregulated in the peripheral blood of patients with active CD, but not inactive CD, compared to healthy controls<sup>[80]</sup>. Both active and inactive CD patients had increased expression of miR-340 and decreased expression of miR-149 in the blood. Expression of three miRNAs (miR-103-2, 262-3p, 532-3p) was increased in the blood of both active and inactive UC

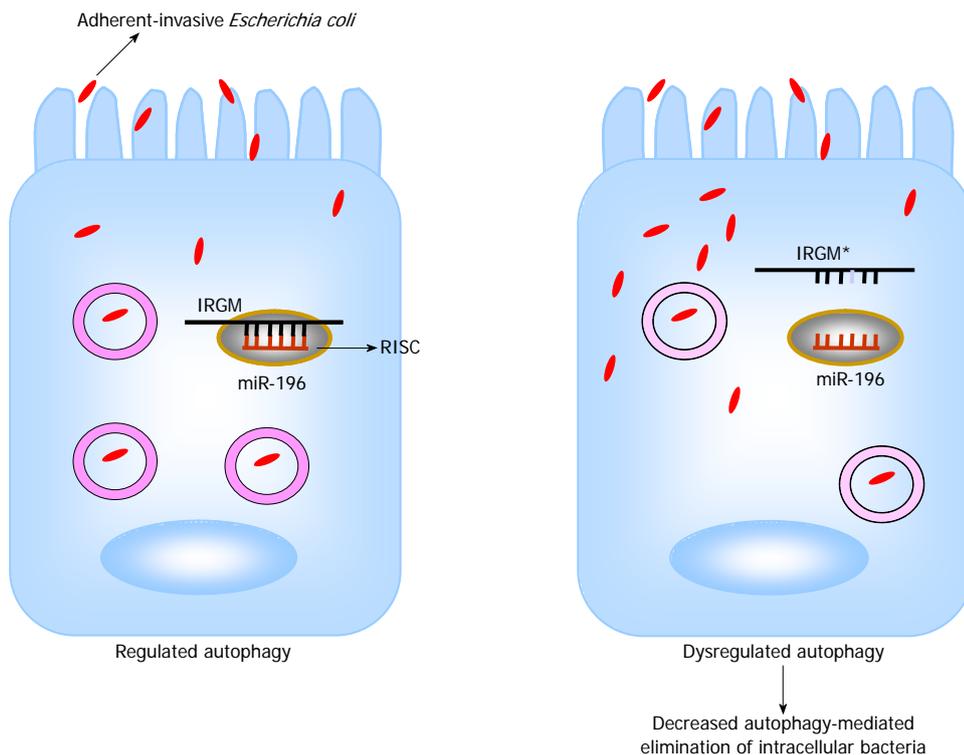
patients. In addition, a subset of 11 miRNAs can distinguish active CD from active UC<sup>[80]</sup>. This study importantly supports the evidence that distinct peripheral blood miRNA profiles in different circulating immune cell types are associated with IBD.

Efforts have been made to understand the mechanisms underlying the implication of miRNAs in the pathogenesis of IBD. The potential association between single nucleotide polymorphisms (SNPs) in pre-miRNA coding regions and IBD susceptibility has been analyzed. A study in a Japanese cohort of 170 UC patients and 403 healthy controls revealed the association of three SNPs (rs11614913, rs2910164, and rs3746444) in coding regions of pre-miR-196a2, pre-miR-146a and pre-miR-499<sup>[81]</sup>. Of particular interest, the CD-associated SNP C313T in immunity-related GTPase family, M (*IRGM*) gene caused a loss in binding of miR-196<sup>[82]</sup> (Figure 1). *IRGM* plays an important role in the immune system by its involvement in the autophagy process. In addition, miR-196 is overexpressed in the inflamed epithelium of CD patients and downregulates the *IRGM* protective variant (c.313C) but not the risk-associated allele (c.313T)<sup>[82]</sup>. Loss of tight regulation of *IRGM* expression by miR-196 resulted in defects in autophagy-mediated control of intracellular replication of adherent-invasive *Escherichia coli* (AIEC), leading to abnormal persistence of AIEC in host cells (Figure 1). This suggests that the association of *IRGM* with CD could arise from abnormal miRNA-mediated *IRGM* regulation, which affects the efficacy of autophagy, thereby contributing a synonymous polymorphism as a likely causal variant.

Intestinal microbiota is increasingly recognized as a risk for, and a causal factor of, IBD. Our recent studies showed that miRNAs are involved in the regulation of host gene expression by gut microbiota<sup>[83]</sup>. In another study, we showed that miRNAs play a role in determining the unique physiological characteristics of intestinal epithelial cells, such as their differentiation during migration along the crypt/villus axis<sup>[84]</sup>. In particular, expression of CD98, a transmembrane glycoprotein that regulates integrin signalling, cellular homeostasis and innate immune response in the gut<sup>[85]</sup>, and its function are directly under the control of miRNAs during the differentiation of intestinal epithelial cells<sup>[86]</sup>. MiRNAs could also be involved in the upregulation of CD98 during intestinal inflammation and IBD<sup>[86]</sup>. The biological importance of miRNAs in the pathogenesis of IBD is becoming clearer, and targeting miRNAs in the gastrointestinal tract may be a promising approach for future therapeutic opportunities.

## MICRORNAS AND COLORECTAL CANCERS

The transformation of a normal epithelium into a cancerous state involves modifications in several genes that are involved in different stages of carcinogenesis such as apoptosis, proliferation, limitless replicative potential of tumor cells, angiogenesis, migration and invasion<sup>[87]</sup>.



**Figure 1** Hypothetical model for the involvement of miR-196 in the pathogenesis of Crohn's disease. MicroRNAs (miRNAs, miR) 196 normally targets immunity-related GTPase family, M (IRGM) mRNA within RNA-induced silencing complex (RISC) for a negative regulation (left panel). The IRGM risk allele (IRGM\*) mRNA lacks the binding site for miR-196 and therefore is not regulated by this miRNA (right panel). During Crohn's disease, loss of tight regulation of IRGM\* expression by miR-196 may lead to defects in autophagy with most intracellular bacteria replication occurring in dysfunctional vacuoles<sup>[82]</sup> (dotted cycle). This consequently results in abnormal persistence of pathogens in host cells, which could further worsen disease status<sup>[82]</sup>.

Colorectal cancer (CRC) is one of the most common cancers worldwide. Its incidence is greater in industrial countries than in developing countries<sup>[88]</sup>. MiRNAs have been shown to play an important role in oncogenesis by regulating the expression of genes involved in cancer initiation, promotion and development<sup>[89]</sup>. Hundreds of miRNAs mapped to the human genome regions that are known to be altered in cancer, and a similar number of miRNAs are aberrantly expressed in cancerous tissues<sup>[90,91]</sup>. By analyzing miRNA expression profile (miRNome) of prostate, stomach, pancreas, lung, breast and colon tumors, Volinia and colleagues identified a solid cancer miRNA signature including those with well-characterized cancer association, such as miR-17-5p, miR-20a, miR-21, miR-92, miR-106a, and miR-155<sup>[92]</sup>. In particular, 21 miRNAs are up-regulated and 1 is down-regulated in colon tumors compared to normal tissue<sup>[92]</sup>. MiRNA profiles can identify different tissue and tumor types better than mRNA expression patterns, making them attractive targets for development as cancer biomarkers<sup>[93]</sup>. Distinguished miRNA profiles can even be found in the serum of patients with cancers. The functions of such circulating miRNAs have not been identified, but profiling of serum miRNAs might be a powerful approach for early cancer diagnosis. The cancer-associated miRNAs may function as oncogenes or tumor suppressors depending on their role in carcinogenesis. Some of the best examples of such miRNAs will be discussed in this section

(Figure 2).

### Oncogenic miRNAs

MiR-21 is one of the most up-regulated miRNAs in various cancers, including CRC<sup>[92,94]</sup>, and was identified as an independent predictor of overall survival in the validation set containing tumor samples from 113 patients with CRC<sup>[95]</sup>. It has been shown that miR-21 is involved in invasion, intravasation and metastasis processes by targeting the tumor suppressor PDCD4<sup>[96]</sup>, and in CRC tissues expression of miR-21 is inversely correlated with that of PDCD4 compared to normal tissue<sup>[97]</sup>. Shibuya and colleagues suggested that miR-21 expression may predict poor prognosis in CRC<sup>[98]</sup>. Likewise, these authors also examined the prognostic value of miR-155 in CRC since its expression is up-regulated in tumor tissues compared to normal adjacent tissues from CRC patients<sup>[98]</sup>. MiR-155 was previously shown to target the tumor protein 53-induced nuclear protein 1 (TP53INP1), a pro-apoptotic stress-induced p53 target, and significant reduction or loss of TP53INP1 expression was detected during adenocarcinoma progression<sup>[99]</sup>.

MiR-17-92 and miR-106b-25 clusters are known to be up-regulated in CRC stromal tissues compared with normal stroma<sup>[100]</sup>. They include, respectively, multiple mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b1, miR-92-1<sup>[101]</sup>, and miR-106b, miR-93 and miR-25<sup>[102]</sup>. These miRNA clusters play an important role

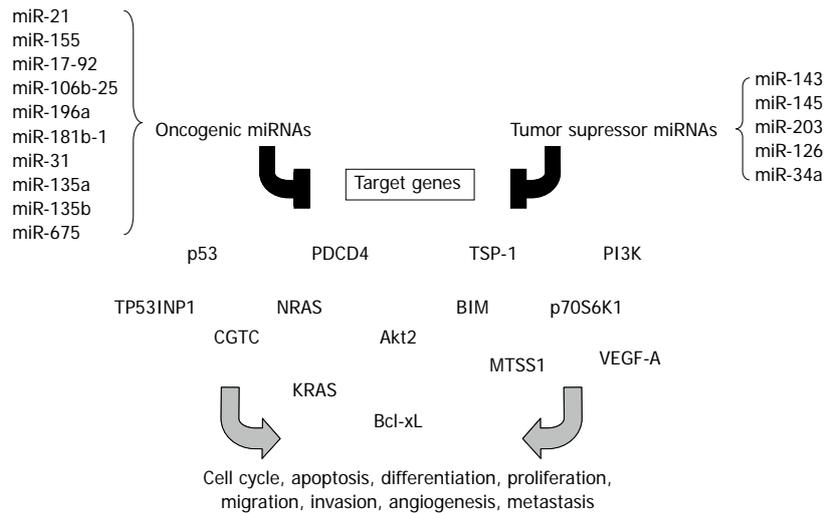


Figure 2 Overview of “oncogenic” and “tumor suppressor” microRNAs related to colorectal cancer described in this review, their targets and different carcinogenesis pathways in which they have been implicated.

during carcinogenesis<sup>[92,100,103,104]</sup>. An anti-apoptotic effect of miR-17-92 appears to be one of the mechanisms underlying its procarcinogenic role in CRC development and progression<sup>[105]</sup>. Abrogation of miR-92a leads to cell apoptosis, and there is a correlation between the miR-17-92 overexpression in tumors of CRC patients and the downregulated expression of BIM, a member of the Bcl-2 family that promotes apoptosis<sup>[105]</sup>. Some works have reported that there is an interconnection between the expression of miR-17-92 cluster and angiogenesis, which occurs later in tumor development and is one of the most important stages in carcinogenesis. Dews and colleagues demonstrated that the anti-angiogenic factors thrombospondin-1 (tsp-1) and connective tissue growth factor (CTGF) are down-regulated by this cluster in intestinal epithelial cells expressing constitutively the oncogene c-myc<sup>[106]</sup>, which was shown to be involved in regulation of miR-17-92 expression<sup>[20]</sup>. MiR-18 targets tsp-1 and miR-19 modulates the expression of CTGF<sup>[107]</sup>.

Other miRNAs have also been identified as causal factors in colon carcinogenesis. For example, miR-196a had higher expression level in CRC tissues than in normal epithelial tissues<sup>[108]</sup>. MiR-196a exerts a pro-oncogenic influence in CRC as a high level of its expression promotes the oncogenic phenotype of colorectal cancer cells such as increased cell detachment, migration and invasion<sup>[109]</sup>. MiR-31 is often up-regulated in CRC and its high expression associated with advanced tumor stage but the clinical significance is unclear<sup>[110]</sup>. MiR-181b-1, miR-135a, miR-135b, miR-675 are also known to be up-regulated in CRC tumors<sup>[111]</sup>. MiR-135a is able to promote the growth and invasion of CRC cells by targeting the metastasis suppressor 1<sup>[112]</sup>.

### Tumor suppressor miRNAs

Mir-143 and miR-145 are among the best examples of tumor suppressor miRNAs. The expression of these miRNAs is down-regulated in CRC tumors, and in other

cancers such as breast, prostate, cervical and lymphoid cancer<sup>[113-115]</sup>. Many studies have reported that down-regulation of miR-143 and miR-145 correlates with poor prognosis<sup>[110,115,116]</sup>. The expression and post-transcriptional maturation of these miRNAs were recently shown to be enhanced by the tumor suppressor p53 in response to DNA damages in CRC cell lines<sup>[28,117]</sup>. In particular, miR-143 is involved in inhibition of oncogene KRAS expression<sup>[118]</sup>. MiR-145 is reported to inhibit tumor growth and angiogenesis by directly targeting p70S6K1<sup>[117]</sup>, which is activated by mTOR, and its overexpression in cancer cells induces tumor angiogenesis<sup>[119-121]</sup>. Another study reported that this miRNA is able to inhibit tumor growth and angiogenesis in breast cancer by targeting N-RAS and VEGF-A, which are key players in carcinogenesis<sup>[122]</sup>.

It was recently demonstrated that miR-34a is down-regulated in colon tumors and also in circulating blood<sup>[123]</sup>. Furthermore, ectopic expression of miR-34a in CRC cell line reduces cell proliferation, demonstrating that this miRNA has a tumor suppressive function in colon carcinogenesis<sup>[124]</sup>. Several studies conducted in 2007 revealed that miR-34a can target p53, leading to apoptosis and cell cycle arrest<sup>[21-24,125]</sup>. MiR-203 is identified as another tumor suppressor miRNA. Its low expression was found *in vitro* in CRC cell lines and was correlated with tumor size in CRC. MiR-203 can inhibit proliferation of cancer cell lines<sup>[126]</sup>. Li *et al.*<sup>[127]</sup> showed that miR-203 overexpression significantly decreased cell proliferation and survival and induced cell apoptosis in the p53-mutated CRC cells. The tumor suppressive role of miR-203 was mediated by negatively regulating Akt2 expression *via* mRNA degradation. In addition, overexpression of miR-203 decreased expression of the anti-apoptotic gene Bcl-xL, leading to a resistance to apoptosis<sup>[127]</sup>. MiR-126 is specifically expressed in endothelial cells and is known to be down-regulated in CRC compared to normal tissue *via* an unknown mechanism<sup>[128]</sup>. *In vitro* studies suggested that a loss in negative regulation of p85 subunit of PI3K

by miR-126 could lead to a selective growth advantage during colon carcinogenesis<sup>[129]</sup>.

## CONCLUSION

MiRNAs are a class of gene regulators that have recently emerged as key players in the innate and adaptive immune system. Changes in miRNA expression are observed in many human diseases such as inflammatory bowel disease and cancers. Dysregulated miRNA expression profiles in IBD have been reported and could be used as diagnostic biomarkers but further studies are needed to examine the mechanism of their action in the etiopathogenesis of this disease and their clinical utility. Emerging evidence suggests that miRNAs play important roles in the pathogenesis of a limited range of human cancers. Some miRNAs may be directly involved in cancer development by controlling cell differentiation and apoptosis, while others may be involved in cancers by targeting cancer oncogenes and/or tumor suppressors. Given the critical role of miRNAs, current studies are focusing on their association with CRC incidence and prognosis and on the possibility of using circulating miRNAs or fecal miRNA expression as noninvasive early detection biomarkers. These data suggest that miRNAs may be potential molecular classifiers, early detection biomarkers, and therapeutic targets for CRC. Finally, miRNA-based cancer therapy has been limited to targeting a single miRNA<sup>[130,131]</sup>. However, it has been recently shown that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the miRNA-processing machinery by binding to TRBP<sup>[132]</sup>. Thus, if most cancers are characterized by a dysregulation of global mature miRNA expression, restoration of the global miRNome may be an attractive approach in cancer therapy. In conclusion, a better understanding of the function of miRNAs is providing new insights into the molecular basis of human pathologies, and new biomarkers for disease diagnosis and therapy.

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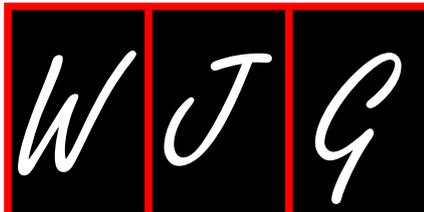
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**P- Reviewers** Chi SG, Monticelli S **S- Editor** Gou SX  
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## Colon cancer stem cells: Controversies and perspectives

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Received: January 12, 2013 Revised: March 25, 2013

Accepted: April 3, 2013

Published online: May 28, 2013

### Abstract

Tumors have long been viewed as a population in which all cells have the equal propensity to form new tumors, the so called conventional stochastic model. The cutting-edge theory on tumor origin and progression, tends to consider cancer as a stem cell disease. Stem cells are actively involved in the onset and maintenance of colon cancer. This review is intended to examine the state of the art on colon cancer stem cells (CSCs), with regard to the recent achievements of basic research and to the corresponding translational consequences. Specific prominence is given to the hypothesized origin of CSCs and to the methods for their identification. The growing understanding of CSC biology is driving the optimization of novel anti-cancer targeted drugs.

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**Key words:** Colon cancer stem cells; Colorectal cancer; CD133; Therapy; Chemoresistance

**Core tip:** According to the “cancer stem cell” (CSC) theory, tumor growth and spread are driven by a minority of cancer cells which exhibit characteristics similar to normal stem cells. Although CSCs have been implicated in colon carcinogenesis, due to the complexity of their biology and unsolved technical issues, an unequivocally approved identification and isolation strategy is still a matter of debate. Several markers have been used to identify colon CSCs but the function of these proteins in CSC biology has not yet been clarified. Moreover, the possibility that CSCs might contribute to the failure of existing chemotherapies to eradicate malignant tumors, indicate that targeting of CSCs may represent a promising strategy to eradicate chemoresistant cancers. Aim of this review was to acquire more information on the biology of human colon CSCs and shed light on the role of this cells in the onset and the maintenance of colon cancer.

Puglisi MA, Tesori V, Lattanzi W, Gasbarrini GB, Gasbarrini A. Colon cancer stem cells: Controversies and perspectives. *World J Gastroenterol* 2013; 19(20): 2997-3006 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/2997.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.2997>

### INTRODUCTION

The intestine epithelium is subjected to a rapid and continuous regeneration, supported by crypt intestinal stem cells (ISCs). This feature severely increases the risk for malignant conversion<sup>[1]</sup>. Indeed, colorectal cancer (CRC) is one of the most common type of neoplasm worldwide, representing the second leading cause of morbidity and mortality from cancer in both Europe and the United States. This means that CRC can also be considered one of the main national emergencies, both in terms of morbidity and in terms of social and economic costs<sup>[2]</sup>.

Studies on CRC pathogenesis have been originally focusing on the clonal selection process, a model of carcinogenesis postulated in 1975<sup>[3]</sup>. The characterization

of the genetic mechanisms underlying this process has been performed, for the first time, in the early 90s by Bert Vogelstein, who developed the molecular model of CRC progression known as “Vogelstein model”. According to it, the CRC develops from epithelial cells lining the gastrointestinal tract, which undergo sequential mutations in specific DNA sequences that disrupt normal mechanisms of proliferation and self-renewal<sup>[3,4]</sup>. This pathogenic model still represents a paradigm of tumor growth and provides a standard rationale to dissect the molecular mechanisms responsible for CRC. Though, current anticancer treatments are often unable, even those based on molecular targeted strategies, to eradicate the disease. Otherwise, the cellular origin of human cancers is still controversial and the mechanisms responsible for the complexity and heterogeneity of tumors remain to be defined<sup>[3,4]</sup>. In recent years, converging evidence has suggested that human cancer can be considered as a stem cell disease. Therefore, the “stochastic” theory for the cellular origin of cancer, based upon the assumption that all cancer cells are equally malignant and able to give rise to tumors, has been abandoned in favor of the “hierarchical” theory. The latter assumes that: (1) tumors are hierarchically organized; and (2) only a rare subpopulation of undifferentiated cells at the apex of this hierarchy have the unique biological properties necessary for tumor initiation, maintenance and spreading. Given the similarities between tumor-initiating cells and normal stem cells (SC), the tumor-initiating cells have been termed “cancer stem cells” (CSCs)<sup>[5]</sup>. In its simplicity, this hypothesis suggests that tumorigenesis is an “aberrant organogenesis”, supported by a minority of cancer cells, that by consecutive genetic changes, can differentiate to give rise to different phenotypes in the neoplastic population<sup>[6]</sup>.

The hierarchical model implies that within tumors there are cells with different tumorigenic potential: cells that have lost the ability to propagate the tumor and cells that retain their clonogenic ability. Indeed, biologically distinct populations of CSCs have been identified within hematological malignancies<sup>[7]</sup> and in most solid tumors, including colon cancer<sup>[8,9]</sup>. The origin of CSCs is still unclear but the discovery of stem cells in the majority of normal tissues, including colon crypts, support the hypothesis that normal SC might represent a possible target for tumorigenic mutations, due to both their long life and their capacity of self-renewal<sup>[10]</sup>.

Cancer stem cells theory has profound translational implications. Current treatments are hardly able to completely eradicate cancer cells, being often complicated by the occurrence of tumor recurrence and/or metastasis and are burdened by toxicity issues. The failure of chemotherapy may at least in part lie in its capacity of targeting the bulk of cancer without affecting stem cells. These can on turn replicate after treatment and, eventually, develop selective features responsible for the occurrence of drug-resistance, which usually characterize and complicate the course of the disease<sup>[11-13]</sup>.

Several studies have suggested that the CSC fraction

may be identified within a variety of human cancers, including CRC, by the expression of the CD133 surface marker<sup>[8,9,14,15]</sup>. CD133 (also known as prominin-1 in rodents or AC133 in humans), a 120 kDa transmembrane and cell surface protein, has been shown to characterize normal and cancer stem cells in several human tissues, including the colonic mucosa. Hence, CD133 has been used to identify and isolate tumor initiating cells from human colon cancers. CD133+ cells are able to maintain themselves as well as to differentiate and re-establish tumor heterogeneity upon serial transplantation *in vivo*<sup>[8,9]</sup>. However, despite constant research efforts, the molecular mechanisms and signaling pathways that regulate the behavior of CD133-expressing cells remain unknown. Aim of this review was to acquire more information on the biology of human colon CSCs and shed light on the role of CD133+ tumor-initiating cells in the onset and the maintenance of colon cancer.

## CANCER STEM CELLS: PROPERTIES

It is widely accepted the concept that tumor is an heterogeneous entity derived from a small subpopulation of undifferentiated cancer cells, the CSCs. CSCs are defined as cells having three unique properties: the capacity of self-renew indefinitely, the ability to recreate the full repertoire of cancer cells of the parent tumor and the expression of a distinctive set of surface biomarkers<sup>[16]</sup>. It must be emphasized that self-renewal is not synonymous with proliferation. Self-renewal involves the ability of SC populations to precisely maintain their numbers through a combination of symmetric and asymmetric SC division, giving rise to one or both daughter cells identical to the mother and retaining SC properties<sup>[17]</sup>. In the case of CSCs, mechanisms involved in self-renewal are deregulated and seem to lead to CSC overpopulation. The underlying mechanisms for generating excess of CSC numbers are believed to relate to increases in symmetric CSC division (which produces two CSC daughters) relative to asymmetric CSC division (which produces one CSC daughter and one non-CSC daughter). Many authors have documented this concept quantitatively, using mathematical modeling<sup>[17]</sup> or fluorescent dye assay<sup>[18]</sup>. These authors have showed that only increased symmetric division of CSCs could account both for the biologic observation that there is an exponential increase in CSC populations in tumoral tissues and for the known long lag phase, which is typical in the development of many cancers, including colorectal cancer<sup>[17]</sup>. Although CSCs have the capacity for self-renewal, they are relatively quiescent; that is, they have proliferative capacity but are often not cycling. Indeed, they have been shown to have significantly longer cell cycle times than proliferating non-SCs. This is presumably due to the arrest of SCs at a G0-like cell cycle phase or checkpoint<sup>[19]</sup>.

Another property of CSCs is their potential for multilineage differentiation. This is consistent with the concept that CSCs, like normal SCs, give rise to a hierarchical

organization of cell populations that underlie organogenesis<sup>[20]</sup>.

CSCs also display alterations of DNA repair, due to the presence of cytoprotective properties (including telomerase activation and high expression of anti-apoptotic factors), and a relatively low proliferative potential. In addition, they express high levels of proteins belonging to the ABC membrane transporters family, involved in chemotherapeutic resistance (*i.e.*, to paclitaxel, cisplatin, 5-fluorouracil, mitoxantrone, methotrexate, anthracyclines, etoposide, vinca alkaloids, camptothecins, topotecan, imatinib)<sup>[20]</sup>. These unique properties of CSCs would explain the failure of many antitumor therapies, which affect rapidly dividing cells, determining only a reduction of tumor cells number, while CSCs divide slowly and are not sensitive to the cytotoxic drugs. New insight in the molecular mechanisms that underlie these processes were obtained by studying the intracellular regulator of gene expression<sup>[21]</sup>.

On this regard, Bitarte and colleagues showed that the micro RNA miR-451 was downregulated in colonospheres, obtained from different colon carcinoma cells, versus parental cells. The expression of miR-451 caused a decrease in self-renewal, tumorigenicity, and chemoresistance to irinotecan of colonospheres. Authors demonstrated that miR-451 downregulation allows the expression of the target gene macrophage migration inhibitory factor, which induces cyclooxygenase-2 (COX-2) expression. In turn, COX-2 allows Wnt activation, which is essential for CSC growth. Furthermore, miR-451 restoration decreased expression of the ATP-binding cassette drug transporter ABCB1 and resulted in irinotecan sensitization. These findings correlated well with the lower expression of miR-451 observed in patients who did not respond to irinotecan-based first-line therapy compared with patients who did<sup>[22]</sup>. Moreover, various signaling pathways have already been identified and described in CSCs. It is known that standard pathways for self-renewal of normal stem cells, such as Wnt, Notch and Hedgehog signaling, are also present in CSCs and have an important role in their function. Targeting critical steps in those pathways, however, will be complicated by intense crosstalks as well as main safety issues related to the pleiotropic effects of these signaling molecules<sup>[23]</sup>. Nevertheless, there are already several reports indicating that CSCs can be selectively targeted without damaging normal stem cells<sup>[24]</sup>. These and other findings could reasonably pave the way to the development of novel, more efficient and less toxic anti-cancer medications.

## CANCER STEM CELLS: DEFINITION

In operational terms, CSCs can only be defined experimentally on the basis of their ability to regenerate continuously the tumor. The implementation of this approach, explains the use of alternative terms in the literature. For example, the term “tumor-initiating cells” is frequently used to describe putative CSCs. However, both

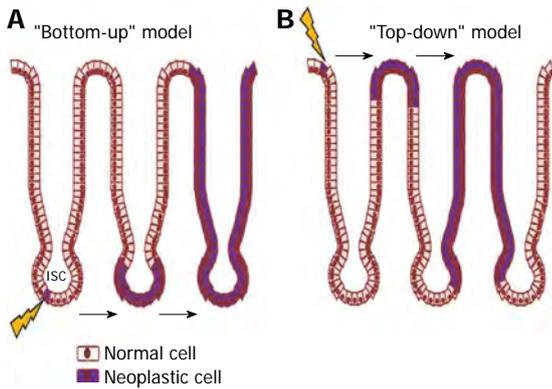
these terms (cancer stem cell and tumor-initiating cells) can cause confusion about the cell type to which they relate<sup>[16]</sup>. In fact, the term CSCs might suggest that these cells arise from normal stem cells, which have acquired a number of genetic mutations sufficient to induce malignant transformation. This assumption is probably true in many cancers, but may not be the case of all tumors. It is plausible, indeed, that in some tumors, a number of differentiated cells can acquire the capacity for self-renewal, through multiple mutagenic events, and thus “reacquire” stem-like properties<sup>[25]</sup>.

On the other hand, the term “tumor-initiating cells”, according to experimental evidence, refers to the ability of these cells to initiate tumors when transplanted in a xenograft model. In this case, it could be incorrectly inferred that the cell that gives rise to the xenograft tumor is the same cell in which the first oncogenic mutation occurred. This is unlikely, since it is clear that the cells that drive aberrant growth at one precise moment may differ from those acting during different stages in tumor evolution or during metastasis. Furthermore, both genetic and epigenetic instability can induce cellular heterogeneity within the stem and non-stem cell populations of the tumor<sup>[26]</sup>. It has been argued that species differences alone might account for the selective growth of subpopulations of cells in xenotransplantations. Indeed, the great majority of cells in a mouse lymphoma were shown recently to possess tumor initiating capacity when allografted into syngenic mice<sup>[27]</sup>.

## WHICH IS THE ORIGIN OF COLON CANCER STEM CELLS?

Although, as mentioned earlier, the sequence of events in CRC has been intensively studied, the cell of origin for cancer formation is still poorly known. Two possible hypotheses have been suggested: the so called “bottom-up” and the “top-down” theories. The first proposes that an ISC, either a progenitor or a differentiated cell, is the first transformed cell, as a consequence of anomalous differentiation, giving rise directly to cancer cells or reprogramming itself, acquiring SC behavior before inducing cancer<sup>[28-30]</sup> (Figure 1).

ISCs represents the ideal target for neoplastic transformation, due to their extended life span that alter their behavior as a result of genetic and epigenetic changes. Also, similar signalling pathways may regulate self-renewal in stem cells and cancer cell, so that the transformation of an SC would require fewer mutations compared to a progenitor cell<sup>[31,32]</sup>. Conversely, histological evidence suggested that colon cancer might arise from late progenitors or even an early differentiated cell<sup>[33]</sup>, sustaining the “top-down model” of CRC development. By contrast, genetic observation recently support strongly the bottom-up theory. Indeed, the identification of specific genes expressed in the stem cells of the intestine has recently allowed to show that the cell of origin of adenomas, induced by a constitutively active Wnt signaling, is the stem



**Figure 1** Schematic presentation of two possible ways of colon adenocarcinoma formation. A: “Bottom-up” theory: intestinal stem cells (ISCs, arrow) at the base of the crypt, within the stem cell zone, are the cell of origin of neoplasia as a consequence of anomalous differentiation; B: “Top-down” theory: a progenitor or differentiated cell is the first transformed cell that can acquire stem cells (arrow) behavior before inducing cancer.

cell of the small intestine<sup>[30,34,35]</sup>. These studies have demonstrated that ISC-specific deletion of both functional adenomatous polyposis coli (APC) alleles using *Bmi1*-, *CD133*- and (leucine-rich-repeat containing G-protein-coupled receptor 5) *Lgr5*- CRE recombinase mice leads to efficient tumor formation. Interestingly, Barker and colleagues also showed that tumor formation does not occur when APC is deleted in progenitors or differentiated cells<sup>[30]</sup>. These results show that the cell giving rise to adenomas in the small intestine is the stem cell. It is still pending, though, whether *BMI*-, *LGR5*-expressing cells or *CD133*-expressing cells of the tumor, are able alone to induce tumor progression and therefore are markers of the intestinal CSC.

Recently, Schwitalla *et al.*<sup>[36]</sup> have suggested that these models do not exclude each other and that tumor-initiating mutations can occur in both *Lgr5*+ crypt stem cells or in more differentiated *Lgr5*-cells, as long as these initially negative cells dedifferentiate and re-express *Lgr5*. Indeed, the authors have demonstrated, in a genetic model of intestinal tumor initiation, that epithelial non-stem cells can re-express stem cell markers and be converted into tumor-initiating cells. This phenomenon is strictly dependent on the degree of Wnt activation and can only be observed when Wnt signaling is markedly elevated<sup>[37]</sup>.

It has also been discussed that even cells from outside the tumor, for example, bone marrow-derived cells, might also serve as CSC’s ancestors. This phenomenon has been firstly demonstrated in a murine model of gastric cancer induced by *Helicobacter pylori*, in which SC derived from bone marrow were able to generate the tumor<sup>[37,38]</sup>.

Emerging evidence suggests that bone-marrow-derived mesenchymal stem cells (BM-MSCs) contribute to tissue regeneration in the colon partly by promoting neovascularization or arteriogenesis<sup>[39,40]</sup>. Although tumor stromal fibroblasts are mainly recruited from local tissue fibroblasts, it has been proposed that BM-MSCs are recruited into the stroma of developing tumors<sup>[41-43]</sup>. Several studies have demonstrated that BM-MSCs can se-

**Table 1** Summary of characteristics and controversies about colon cancer stem cells

|                        |   |
|------------------------|---|
| Origin                 | “Bottom-up” theory <sup>[30-32,34,35]</sup><br>“Top-down” theory <sup>[33]</sup>  |
| Identification assays  | CSCs can derive from epithelial non-stem cells that re-express stem cell markers <sup>[36]</sup><br>CSCs can derive from bone marrow cells <sup>[37-43]</sup>   |
|                        | Serial transplantation in immune-compromised mice <sup>[8,9,27]</sup><br>Expression of cell surface marker <i>CD133</i> <sup>[5,8,9,14,31,44-46,48,49]</sup><br>Side Population <sup>[52-61]</sup>  |
| Therapeutic strategies | Induction of CSC differentiation by salinomycin or <i>BMP4</i> <sup>[72,73]</sup>   |
|                        | Monoclonal antibodies directed against cell surface molecules, such as <i>CD133</i> , <i>CD44</i> , <i>EGFR</i> (cetuximab) and <i>VEGF</i> (bevacizumab) <sup>[74,76-79]</sup>   |
|                        | Blockage of self-renewal pathways, including Wnt, Notch, <i>PTEN</i> , <i>Hedgehog</i> , <i>EMT</i> and <i>IL-4</i> pathway by microRNA or selected small-molecule antagonists <sup>[65-70,80-86]</sup><br>Target the Warburg effect <sup>[87,88]</sup> |

CSC: Colon cancer stem cell; EGFR: Epidermal growth factor receptor; VEGF: Vascular endothelial growth factor; EMT: Epithelial-to-mesenchymal transition.

lectively migrate to sites of mucosal damage and wound healing including colorectal cancers, where a number of tumor-related inflammatory reactions and abnormal tissue regeneration phenomena take place actively. It also has been shown that cancer cells release specific factors that induce BM-MSC mobilization and recruitment to the tumor stroma where they eventually contribute to the formation of a tumor-supportive microenvironment<sup>[44]</sup> (Table 1).

## IDENTIFICATION OF COLON CANCER STEM CELLS: LIMITS AND CONTROVERSIES

The correct identification and isolation of the cells responsible for tumor formation is always challenging in cancer research. Although CSCs have been implicated in colon carcinogenesis, due to the complexity of their biology and unsolved technical issues, an unequivocally approved identification and isolation strategy is still a matter of debate<sup>[32]</sup>. The gold standard for identifying a CSC is the capacity to propagate tumors as xenografts in immunocompromised mice. However, it has been argued that species differences alone might account for the selective growth of subpopulations of cells in these assays. Indeed, the great majority of cells in a mouse lymphoma were shown recently to possess tumor initiating capacity when allografted into syngenic mice<sup>[27]</sup>. Moreover, serial transplantation experiments with animal models are laborious and time-consuming, hence the need to develop reliable surrogate assays.

Several *in vitro* assays have been used to identify CSCs can derive, including sphere assays, surface cell markers and the Hoechst dye efflux properties, which identify the

so-called Side-Population (SP). Studies have also been performed to define putative CSC genetic signatures. However, each of these methods has potential pitfalls that complicate the interpretation of results<sup>[25]</sup>.

It is clearly not sufficient to define a stem cell based only on surface markers. Moreover, none of the markers used to isolate stem cells in various normal and cancerous tissues is expressed exclusively by the stem cell fraction. Indeed most markers used for colon CSC isolation are chosen either because they are expressed in normal stem cells or as they were found to identify CSCs in other malignancies, either hematological or solid. The disadvantage of choosing markers in this fashion is that the functional effect of expression of the marker in CSCs is usually unknown.

For instance, focusing on CRC, several studies have suggested that the CSC fraction within colon cancer might be identified by the expression of the cell surface marker CD133<sup>[8,9]</sup>. CD133 is a trans-membrane glycoprotein, expressed by normal progenitors belonging to neuronal, hematopoietic, epithelial and endothelial lineages. In the last years, CD133 has become the “molecule of the moment”, being recognized as a putative CSC marker for many human solid tumors, including liver, pancreas and colon neoplasms<sup>[14,45]</sup>. However, despite constant research efforts, the molecular mechanisms and signaling pathways that regulate the behavior of CD133-expressing CSC, remain unknown.

In particular, we demonstrated the existence of a population of self renewing cells expressing CD133 within primary and metastatic human CRC<sup>[5]</sup>. This antigen was expressed in significantly higher percentage in CRC samples, compared to the respective normal tissues. CD133-positive cells were also found in liver metastases (up to 10%), while they were hardly detectable in the healthy liver tissue<sup>[5]</sup>. In addition, CD133+ cells, isolated from different human colonic adenocarcinoma lines (CaCo-2, HT29, LoVo), were highly clonogenic *in vitro* and gave rise to tumors following transplantation in mice. Conversely, the CD133-negative fraction of all cell lines had a lower clonogenic potential in soft agar assays and did not generate tumors in secondary recipients<sup>[45]</sup>, confirming the tumor initiating properties of CD133+ CSC. Interestingly, we also provided the original demonstration that modulation of CD133 expression in the CaCo-2 colon cancer cell line was associated with corresponding variations in the expression levels of both Endothelin-1 and nuclear receptor subfamily 4, group A, member 2<sup>[46]</sup>, both known to play an important role in the proliferation and metastasis processes. This modulation was associated with a significant inhibition of the cells' clonogenic and migration ability, thus further confirming a role of the CD133 molecule in the definition of the CSC phenotype<sup>[46]</sup>.

There are though still some controversies on the role of CD133 as a CSC marker in CRC; the opposing theories emerge from the evidence that most CD133 antibodies target glycosylation-dependent epitopes<sup>[35]</sup>, whose

presence is related to the differentiation stage of the cell. Experimental data from colon and glioblastoma cells suggested that the differential glycosylation of specific epitopes may mask the presence of CD133 on cells previously characterized as negative<sup>[47,48]</sup>. Moreover, CD133 has been found to be expressed by the full spectrum of undifferentiated and differentiated colonic epithelial cells, both in humans and in mice<sup>[49]</sup>. Shmelkov *et al.*<sup>[49]</sup> have demonstrated that primary and metastatic colon cancers contain CD133+ and CD133- parenchymal tumor cells, and both types of cells are capable of tumor initiation, as observed in a xenotransplantation model. A similar lack of specificity has been also observed for other potential CSC markers of CRC, such as CD44, CD166, CD29, CD24, Lgr5, and nuclear beta-catenin<sup>[50]</sup>. In fact, the vast majority of cells that express these markers are not stem cells<sup>[51]</sup>.

Another approach to identify CSCs is their presence within the so-called “Side Population”. SP cells have been first described within the hematopoietic system. In particular, bone marrow stem cells contain a subpopulation that extrude the DNA-binding dye, Hoechst 33342, out of the cell membrane. Comparing the fluorescence intensity on the wavelength of blue against the red, the SP appears as a tail of cells with low fluorescence. This phenotype is attributed to the activity of the ABC membrane transporter proteins that can confer drug resistance to stem cells. These proteins can be blocked by inhibitors of efflux pumps, such as verapamil<sup>[52-54]</sup>. The SP fractions have been identified in various human tissues<sup>[55,56]</sup>, cancer specimens<sup>[57,58]</sup> and tumor cell lines<sup>[59,60]</sup> and it has been suggested that they may represent the true stem cell population. However, as with cell surface markers, the SP phenotype is not synonymous with stemness. Indeed, a recent article claimed that both SP and non-SP cells, isolated from gastrointestinal cancer cell lines, displayed similar clonogenic and tumorigenic potential *in vitro* and *in vivo*, and showed identical expression of putative stem cell markers. Therefore, the Authors concluded that the SP does not enrich for stem cells in gastrointestinal cell lines<sup>[61]</sup>. Also, possible toxicity of the dye, in cells not capable of extrusion, should also be considered as a caveat to interpreting functional assays of SP cells.

Without a better understanding of normal tissue stem cells and their susceptibility to neoplastic transformation, it will be difficult to conduct conclusive studies of the existence and origins of CSCs (Table 1).

## WHICH STRATEGY TO TARGET COLON CSCS?

According to the CSC model, the few self-renewing CSCs that mediate tumor growth are difficult to kill and their persistence might explain tumor recurrence after therapy<sup>[62]</sup>. Indeed, chemotherapeutics interfere with the ability of rapidly growing cells to divide, so CSCs may be spared, leading to tumor recurrence and metastasis<sup>[11-13]</sup>. Therefore, to assess the efficacy of therapeutics, it is

necessary to accurately distinguish tumorigenic from non-tumorigenic cancer cells and to understand which progression model occurs in the tumor. Unfortunately, the complex network of mechanisms that regulate SC renewal and carcinogenesis is not clear.

Chemotherapeutic resistance is exerted either through a shift from active state to quiescence or through a wide spectrum of protective mechanisms that characterize CSC biology; these include altered DNA damage repair, altered cell-cycle checkpoint control, malfunction of apoptosis, expression of drug transporters and detoxifying enzymes, and a high expression of proteins belonging to the ABC membrane transporters family<sup>[63,64]</sup>.

Moreover, the plasticity of CSCs and the epithelial-to-mesenchymal transition (EMT) complicate therapeutic approaches because participate in the acquisition of both *de novo* and acquired drug resistance<sup>[65]</sup>. Indeed, EMT can trigger reversion to a CSC-like phenotype, providing an association between EMT, CSCs and drug resistance<sup>[66]</sup>. Several key signaling pathways contribute to this process, such as transforming growth factor  $\beta$  and Wnt, that are well known to induce EMT and promote stem cell maintenance<sup>[67,68]</sup>. Recent studies have implicated microRNA functionality in these processes; indeed the dysregulation of microRNA expression is likely to be a major contributing factor in the etiology of some cancers<sup>[69,70]</sup>.

It is therefore essential to renovate the therapeutic repertoire by designing new treatments that specifically target CSCs and, at the same time, also eliminate the non-CSC population, intervening in the process of EMT<sup>[65,71]</sup>. Novel therapeutic strategy based on targeting EMT pathways and CSC maintenance might be a promising tool for CRC defeat.

Moreover, CSCs can be functionally antagonized by inducing their differentiation. Differentiation therapy forces cells to shift into a mature phenotype, lose their self-renewal abilities, and therefore become vulnerable to conventional treatment. For instance, salinomycin, a highly selective potassium ionophore, was recently described as the first compound that can selectively eradicate the tumor through induction of terminal epithelial differentiation of CSCs<sup>[72]</sup>. Gupta *et al.*<sup>[72]</sup> revealed that salinomycin decreases the proportion of CD44<sup>high</sup>/CD24<sup>low</sup> breast cancer cells, whereas paclitaxel has opposing effects. Importantly, cells exposed to salinomycin were less capable of inducing tumors following injection into mice; salinomycin also slowed the growth of the animals' tumors through unknown mechanisms<sup>[71]</sup>. Salinomycin is thought to inhibit potassium-positive channel-regulated migration and interfere with EMT and metastasis<sup>[72]</sup>. Also induction of differentiation in colon CSCs by exposing these cells to Bone Morphogenetic Protein 4 (BMP4), which can initiate a differentiation program as well as mediate apoptosis, sensitizes CRC cells to the effects of 5-Fluorouracil (5-FU) or oxaliplatin *in vivo*, resulting in complete and long term regression of colon xenografts<sup>[73]</sup>.

The potential toxic effect, that might occur from the impact on normal SCs, can be minimized by target-

ing molecules or pathways that are preferentially active in CSCs<sup>[73]</sup>. Monoclonal antibodies could be directed against cell surface molecules, such as CD133, CD44, or even drug transporters, resulting in reduction of tumor size, metastatic potential, and resistance to chemoradiotherapy<sup>[74,75]</sup>.

Advances in high-throughput technologies and bioinformatics will allow developing additional compounds targeting CSC signaling pathways. Currently there are two established targets for such therapies: epidermal growth factor receptor (EGFR), which belongs to the ErbB family of tyrosine kinase receptors and is abnormally activated in many tumors<sup>[76]</sup>, and vascular endothelial growth factor (VEGF), which is known to promote formation of new vessels by inducing growth and differentiation of endothelial cells<sup>[77,78]</sup>. Several clinical trials have demonstrated that introduction of targeted therapies with monoclonal antibodies against EGFR (cetuximab) and VEGF (bevacizumab) in addition to 5-FU, resulted in a significant survival increase in patients with advanced disease<sup>[79]</sup>.

Another rational target includes blockage of various self-renewal pathways, including Wnt, Notch, PTEN, and Hedgehog<sup>[80]</sup>. Small-molecules that inhibit the Wnt pathway and  $\gamma$ -secretases that inhibit the Notch pathway have been recently identified as novel approaches to CRC<sup>[73]</sup>. The Wnt/ $\beta$ -catenin pathway has been implicated in the maintenance of the intestinal crypt stem cell phenotype and Wnt signaling dysregulation through either loss of APC function or oncogenic  $\beta$ -catenin mutations has been shown to cause the majority of sporadic cancer cases<sup>[81]</sup>. Disruption of Tcf/ $\beta$ -catenin complexes by selected small-molecule antagonists has been shown to antagonize cellular effects of  $\beta$ -catenin and to result in inhibition of cellular proliferation in colon cancer cells<sup>[82]</sup>. Similarly, the Notch signaling pathway has been reported to be overexpressed in colon CSCs, where it was found to play a role in colon CSC viability, tumorigenicity, and self-renewal<sup>[83-87]</sup>. Van Es *et al.*<sup>[87]</sup> have demonstrated that blocking the Notch cascade with a gamma-secretase inhibitor induced goblet cell differentiation in adenomas in mice carrying a mutation of the APC tumor suppressor gene and subsequent tumor growth arrest. Moreover, Hoey *et al.*<sup>[88]</sup> have demonstrated that by inhibiting delta-like 4 ligand (DLL4), an important component of the Notch pathway, with human monoclonal antibody 21M18 in colon carcinoma xenografts, the tumor growth as well as the CSC frequency, was decreased compared to control. Interestingly, even though treatment of the xenografts with irinotecan, a chemotherapeutic often used in colon cancer, slowed down tumor growth, and the clonogenicity was increased. Combination treatment of irinotecan with anti-hDLL4 reduced again the tumor growth and stem cell frequency, at even higher levels than the anti-DLL4 treatment alone. This indicates that inhibiting Notch signaling reduces CSC frequencies and sensitizes tumor cells for irinotecan treatment.

It has recently been observed that the inhibition of

the interleukin (IL)-4 pathway with an anti-IL-4 antibody or an IL-4 receptor antagonist in CD133+ colorectal CSCs augmented the antitumor effects of conventional chemotherapeutics<sup>[89-90]</sup>. Indeed, colon carcinomas produce IL-4 that functions in an autocrine manner, promoting antiapoptotic pathways in these tumors. Inhibiting IL-4 by blocking antibodies sensitizes the cells for killing by 5-FU and oxaliplatin IL-4<sup>[89-90]</sup>.

Reversing chemoresistance and radioresistance represents a promising proposal. This can be achieved through interference with a plethora of cellular components, including inactivation of drug transporters and DNA checkpoint kinases, depletion of reactive oxygen species scavengers, and inhibition of signal transduction pathways.

Interestingly, little is as of yet known with regard to the metabolism of CSC population, leaving an exciting avenue unstudied in the dawn of the emerging field of metabolomics. The Warburg effect, the premise of which is that cancer cells restrict use of fatty-acid oxidation in favor of glycolysis as an ATP energy source, can also be harnessed to create novel broad-spectrum anticancer agents<sup>[91-94]</sup>. A recent study by Akao *et al.*<sup>[95]</sup> provided initial evidence of metabolic changes in therapy-resistant cell populations by demonstrating significant overexpression of a metabolic “master-regulator” Sirt1 in DLD-1 5-FU-resistant cells.

Altogether, these data illustrate the therapeutic utility of the cancer stem cell concept, which, by enabling specific examination of more aggressive cancer-initiating and cancer propagating subpopulations, provides the tools for discovery of novel mechanisms of cancer therapeutic resistance (Table 1).

## CONCLUSION

Understanding the details of CSCs' biology is a primary goal in basic oncology research but would also pave the way for a better clarification of CRC progression. The translation implication of such information is clearly deducible. In particular, the combination of previously known and new markers defining CSC specificity, could lead to the development of a better oriented anticancer therapy, possibly targeting CSCs.

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**P- Reviewers** Bommireddy R, Wu W, Zhu YL  
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## A silybin-phospholipids complex counteracts rat fatty liver degeneration and mitochondrial oxidative changes

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Supported by Grants from MIUR (Ministero Università e Ricerca Scientifica COFIN2006) and "Fondi Ateneo Ricerca Scientifica 2005/2006" from the University of Bari, Italy

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Received: September 14, 2012 Revised: November 6, 2012

Accepted: November 11, 2012

Published online: May 28, 2013

### Abstract

**AIM:** To investigate the effectiveness of antioxidant compounds in modulating mitochondrial oxidative alterations and lipids accumulation in fatty hepatocytes.

**METHODS:** Silybin-phospholipid complex containing

vitamin E (Realsil®) was daily administered by gavage (one pouch diluted in 3 mL of water and containing 15 mg vitamin E and 47 mg silybin complexed with phospholipids) to rats fed a choline-deprived (CD) or a high fat diet [20% fat, containing 71% total calories as fat, 11% as carbohydrate, and 18% as protein, high fat diet (HFD)] for 30 d and 60 d, respectively. The control group was fed a normal semi-purified diet containing adequate levels of choline (35% total calories as fat, 47% as carbohydrate, and 18% as protein). Circulating and hepatic redox active and nitrogen regulating molecules (thioredoxin, glutathione, glutathione peroxidase), NO metabolites (nitrosothiols, nitrotyrosine), lipid peroxides [malondialdehyde-thiobarbituric (MDA-TBA)], and pro-inflammatory keratins (K-18) were measured on days 0, 7, 14, 30, and 60. Mitochondrial respiratory chain proteins and the extent of hepatic fatty infiltration were evaluated.

**RESULTS:** Both diet regimens produced liver steatosis (50% and 25% of liver slices with CD and HFD, respectively) with no signs of necro-inflammation: fat infiltration ranged from large droplets at day 14 to disseminated and confluent vacuoles resulting in microvesicular steatosis at day 30 (CD) and day 60 (HFD). In plasma, thioredoxin and nitrosothiols were not significantly changed, while MDA-TBA, nitrotyrosine (from  $6 \pm 1$  nmol/L to  $14 \pm 3$  nmol/L day 30 CD,  $P < 0.001$ , and  $12 \pm 2$  nmol/L day 60 HFD,  $P < 0.001$ ), and K-18 (from  $198 \pm 20$  to  $289 \pm 21$  U/L day 30 CD,  $P < 0.001$ , and  $242 \pm 23$  U/L day 60 HFD,  $P < 0.001$ ) levels increased significantly with ongoing steatosis. In the liver, glutathione was decreased (from  $34.0 \pm 1.3$  to  $25.3 \pm 1.2$  nmol/mg prot day 30 CD,  $P < 0.001$ , and  $22.4 \pm 2.4$  nmol/mg prot day 60 HFD,  $P < 0.001$ ), while thioredoxin and glutathione peroxidase were initially increased and then decreased. Nitrosothiols were constantly increased. MDA-TBA levels were five-fold increased from  $9.1 \pm 1.2$  nmol/g to  $75.6 \pm 5.4$  nmol/g on day 30,  $P < 0.001$  (CD) and doubled with HFD on day 60. Realsil

administration significantly lowered the extent of fat infiltration, maintained liver glutathione levels during the first half period, and halved its decrease during the second half. Also, Realsil modulated thioredoxin changes and the production of NO derivatives and significantly lowered MDA-TBA levels both in liver (from  $73.6 \pm 5.4$  to  $57.2 \pm 6.3$  nmol/g day 30 CD,  $P < 0.01$  and from  $27.3 \pm 2.1$  nmol/g to  $20.5 \pm 2.2$  nmol/g day 60 HFD,  $P < 0.01$ ) and in plasma. Changes in mitochondrial respiratory complexes were also attenuated by Realsil in HFD rats with a major protective effect on Complex II subunit CII-30.

**CONCLUSION:** Realsil administration effectively contrasts hepatocyte fat deposition, NO derivatives formation, and mitochondrial alterations, allowing the liver to maintain a better glutathione and thioredoxin antioxidant activity.

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**Key words:** Fatty liver; Glutathione; Lipid peroxidation; Nitrosothiols; Nitrotyrosine; Thioredoxin

Grattagliano I, Diogo CV, Mastrodonato M, de Bari O, Persichella M, Wang DQH, Liquori A, Ferri D, Carratù MR, Oliveira PJ, Portincasa P. A silybin-phospholipids complex counteracts rat fatty liver degeneration and mitochondrial oxidative changes. *World J Gastroenterol* 2013; 19(20): 3007-3017 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3007.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3007>

## INTRODUCTION

Simple steatosis of the liver without inflammation is considered a rather benign condition, although it represents a favoring substrate for the potentially damaging effects of a second hit (*i.e.*, ischemia-reperfusion, starvation)<sup>[1]</sup>. However, severe fatty degeneration represents a leading factor of hepatocyte dysfunction (mitochondrial respiration, microsomal metabolism, biliary secretion)<sup>[2]</sup> and is associated with excess delivery of nitrosative and oxidative stress molecules<sup>[3]</sup>, thus potentially rendering the liver a major source of systemic alterations in patients with metabolic syndrome<sup>[4]</sup>. Also, while several adaptive metabolic mechanisms have been described during the early phase of fatty infiltration<sup>[1,5]</sup> including expression of intracellular sensors and signaling molecules for lipid metabolism and oxidative stress pathways<sup>[6,7]</sup>, the threshold above which fat infiltration becomes dangerous is not clear, so far. Indeed, it has been observed that transient hepatocellular triglycerides accumulation is essential for normal liver regeneration<sup>[8]</sup> and represents a mechanism of liver protection from lipotoxicity. Buffering free fatty acids might, therefore, prevent the formation of liver steatosis<sup>[9]</sup>. By contrast, several observations suggest that ongoing fatty degeneration indeed exposes hepatocytes to higher risk of oxidative damages<sup>[10]</sup>.

Experimental rat models of liver steatosis are characterized by accumulation of triglycerides, decreased mitochondrial function, and increased activity of microsomal enzymes<sup>[2]</sup>. The altered functions of these subcellular organelles favor the enhanced production of reactive oxygen species (ROS) and NO derivatives with consequent morphological and functional modifications of crucial structures, thus rendering fatty hepatocytes particularly susceptible to additional injuring factors<sup>[3]</sup>.

A number of natural or chemical compounds are able to counteract the damages induced by oxidative and nitrosative stress and therefore are claimed to have antioxidant properties. Vitamin E efforts a remarkable protection against lipid oxidation<sup>[11]</sup> and, if vehiculated within a phospholipid complex together with silybin, a *silybum marianum* extract, protects against pro-fibrotic oxidative injury<sup>[12]</sup>. Less is known about the effectiveness of such a compound to block or modulate ROS/NO production and their pro-oxidant effects. Also, it would be of interest to know if the administration of such an antioxidant complex may contribute to break off the intracellular mechanisms leading to a progressive accumulation of neutral lipids in fatty hepatocytes and in particular, mitochondrial dysfunction.

Therefore, this study aimed to evaluate the effect of a silybin-phospholipid complex containing vitamin E (Realsil<sup>®</sup>) on hepatocyte fatty degeneration and nitrosative/oxidative stress in two different rat dietary models [choline deficiency (CD) and high fat diet (HFD)] of fatty liver. Both diets induce fatty degeneration without major inflammation and fibrosis, representing therefore ideal models for assessing changes associated with simple steatosis without the metabolic consequences depending on inflammation. The results of this study contribute to clarify both pathophysiologic mechanisms of damage in fatty hepatocytes and of pharmacological protection.

## MATERIALS AND METHODS

Male Wistar rats (b.w. 250-270 g, Harlan, S. Pietro al Natissone, Italy) were kept under controlled conditions of temperature and humidity and on a 12 h dark/light cycle. Animals were divided into five groups: rats fed a CD diet (Dyets, Bethlem, PA); rats fed a CD diet plus daily administration of Realsil by gavage (one pouch diluted in 3 mL of water and containing 15 mg vitamin E and 47 mg silybin complexed with phospholipids); rats fed a HFD (20% fat content), containing 71% total calories as fat, 11% as carbohydrate, and 18% as protein (Altromin Rieper, Vandoies, Italy); rats fed a HFD supplemented by daily administration of Realsil by gavage; control group fed a normal semi-purified diet containing adequate levels of choline, 35% total calories as fat, 47% as carbohydrate, and 18% as protein. The control diet, CD, and HFD were nutritionally adequate, calorically equivalent (1 kcal/mL), and contained equal amounts of fat as olive and safflower oil with excess corn oil added to the HFD.

Following an overnight fast, five rats per group were

**Table 1** Characteristics and fat infiltration of rats fed a choline-deficient diet or a high fat diet with or without Realsil or a normal choline-supplemented diet (controls)

| Characteristics       | Controls day 30 | CD day 30               | CD + Realsil day 30      | HFD day 30              | HFD + Realsil day 30   | Controls day 60 | HFD day 60              | HFD + Realsil day 30      |
|-----------------------|-----------------|-------------------------|--------------------------|-------------------------|------------------------|-----------------|-------------------------|---------------------------|
| Body weight (g)       | 305 ± 8         | 311 ± 12                | 304 ± 11                 | 302 ± 10                | 308 ± 12               | 355 ± 11        | 364 ± 13                | 351 ± 12                  |
| Liver weight (g)      | 12.7 ± 0.5      | 19.7 ± 1.3 <sup>a</sup> | 16.7 ± 0.7 <sup>a</sup>  | 15.7 ± 1.7 <sup>a</sup> | 13.1 ± 1.3             | 12.7 ± 0.5      | 18.8 ± 1.1 <sup>a</sup> | 15.1 ± 0.6 <sup>a,c</sup> |
| ALT (IU/L)            | 30 ± 8          | 92 ± 14 <sup>a</sup>    | 68 ± 12 <sup>a,c</sup>   | 42 ± 10 <sup>a</sup>    | 31 ± 7 <sup>c</sup>    | 30 ± 8          | 69 ± 11 <sup>a</sup>    | 52 ± 8 <sup>a,c</sup>     |
| % area                | 3 ± 2           | 311 ± 12                | 304 ± 11                 | 16 ± 4                  | 8 ± 3                  | 4 ± 1           | 20 ± 4                  | 15 ± 3 <sup>a,c</sup>     |
| No. of droplets       | 11 ± 5          | 19.7 ± 1.3 <sup>a</sup> | 16.7 ± 0.7 <sup>a</sup>  | 570 ± 47 <sup>a</sup>   | 356 ± 23               | 12 ± 6          | 389 ± 31 <sup>a</sup>   | 519 ± 26 <sup>a,c</sup>   |
| Mean droplet diameter | 1.1 ± 0.3       | 9.2 ± 1.4 <sup>a</sup>  | 6.8 ± 1.2 <sup>a,c</sup> | 4.2 ± 1.0 <sup>a</sup>  | 3.2 ± 0.7 <sup>c</sup> | 0.9 ± 0.5       | 4.4 ± 1.1 <sup>a</sup>  | 3.4 ± 0.9 <sup>a,c</sup>  |

Data represent percentage of fat in the whole area, number of fat droplets and mean diameter of the droplets. Results are mean ± SD of *n* = 5 different slides per rat per group at each time point. <sup>a</sup>*P* < 0.05 vs control rats; <sup>c</sup>*P* < 0.05 vs choline-deficient diet (CD) or high fat diet (HFD) only. ALT: Alanine transaminase.

anesthetized with xylazine/ketamine (5.45 mg/36.4 mg/kg *im*) and then sacrificed by decapitation on days 0, 7, 14, and 30 for the CD and 0, 14, 30 and 60 for the HFD groups. Blood was collected into heparinized tubes and centrifuged at 4000 × *g* for 5 min to obtain serum. Livers were removed immediately and homogenized in an ice-cold potassium-phosphate buffer containing 5 mmol/L EDTA (pH 7.4). General and liver parameters are reported in Table 1.

The protocol was conducted according to the Guide Principles for the care and use of laboratory animals and was approved by the local committee for animal experimentation.

### Biochemical determinations

Total glutathione (GSH) determination was performed by precipitating tissue homogenates with 15% sulfosalicylic acid and processing the supernatant by the oxidized glutathione recycling procedure<sup>[13]</sup>. Protein thiols were measured with an Elmann's procedure modification<sup>[14]</sup>. The hepatic and serum levels of thiobarbituric acid malondialdehyde complex (MDA-TBA) were first separated by high-performance liquid chromatography using an analytical column Spherisorb ODS 5 μmol/L (250 mm × 4.6 mm) eluted with 60% (v/v) potassium phosphate buffer 50 mmol/L, pH 6.8 and 40% (v/v) methanol at a flow rate of 1 mL/min. Next, spectrophotometric detection of the MDA-TBA adducts occurred at 532 nm<sup>[15]</sup>. Glutathione Peroxidase (GPx) activity was assessed by use of the method described by Flohè *et al.*<sup>[16]</sup>. Calculations were made with 1 unit enzyme considered as the amount consuming 1.15 μmol of nicotinamide adenine dinucleotide phosphate reduced (NADPH) in 1 min at 37 °C (pH 7.0). Thioredoxin levels were quantitated by a standardized ELISA method in serum and liver homogenates. Procedure followed the manufacturer's instructions (Histo-line Laboratories S.r.l., Milan, Italy); samples were located in micro-wells previously coated with a polyclonal antibody (LF-PA0002) and successively with a primary (LF-MA0077) and a secondary HRP-conjugated anti-mouse antibody (81-6720). After the addition of a chromogenic system, the reaction was stopped and absorbance read at 492 nm. Nitrosothiols were measured according to the method of Cook *et al.*<sup>[17]</sup> using a mixture of sulfa-

nilamide/N-1-naphtyl-ethylendiamine dihydrochloride, neutral Griess as reagents. Nitrotyrosine in the serum was quantified using an ELISA Kit containing Streptavidin-peroxidase conjugate which reacts with the substrate tetramethylbenzidine as per manufacturer's instructions (HyCult Biotechnology b.v., UDEN, The Netherlands) with absorbance read at 450 nm. Keratin 18 fragments (K-18) level was quantified in serum using the ELISA Kit as per manufacturer's instructions (Cusabio Biotech Co., Ltd): samples were located in microwells previously coated with a human-specific antibody and successively with one substrate and a chromogenic system. The reaction was stopped and absorbance read at 450 nm. Protein concentration was measured by using a Bio-Rad kit for the assay of proteins (Bio-Rad GmbH, Munich, Germany).

### Western blotting analysis

The expression of mitochondrial oxidative phosphorylation (OXPHOS) system components was assessed by using an antibody cocktail targeting specific subunits from complexes I (NADH-ubiquinone oxidoreductase), II (succinate dehydrogenase), III [ubiquinone-cytochrome c oxidoreductase (COX)], IV (cytochrome c oxidase), and V (ATP synthase). β-actin was used as a loading control. Frozen tissues were homogenized in a cold Ripa buffer (50 mmol/L Tris-HCl, pH 8.8; 150 mmol/L NaCl; 1% Igepal; 0.5% sodium deoxycholate; 0.1% SDS) supplemented with a protease inhibitor cocktail (Sigma) and ruptured by 30 passages through a needle. Homogenized tissues were centrifuged at 14000 rpm (4 °C, 10 min). The supernatant was collected and kept at -80 °C until used. Protein concentration of each sample was measured using BCA Protein Assay kit according to the manufacturer's protocol. After denaturation at 100 °C during 5 min in Laemmli buffer (BioRad), proteins (30 μg) were separated by electrophoresis on 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 2% of milk in TBST (50 mmol/L Tris-HCl, pH 8; 154 mmol/L NaCl, and 0.1% Tween 20) for 1 h at room temperature, membranes were incubated overnight at 4 °C with antibodies against OXPHOS components (1:2000) and β-actin (1:2500). Membranes were incubated with secondary alkaline phosphatase-

conjugated antibodies: goat anti-mouse IgG (1:5000) for 1 h at room temperature. Membranes were incubated with ECF detection system (Amersham, GE Health-Care, Piscataway, NJ) and read with the Versa Doc imaging system (Bio-Rad, Barcelona, Spain).

### Histology

Liver specimens were fixed in 10% neutral buffered formalin and paraffin embedded. Five sections of 4  $\mu\text{m}$  thickness from each sample were cut and stained with toluidin blue-periodic acid-schiff stain. Histologic features were examined on five low-power fields per specimen, and a semi-quantitative estimation of the empty vacuoles (fat) was performed by measuring the fat percentage distribution on the surface areas.

### Chemicals

Total OXPHOS Rodent Antibody Cocktail was purchased from Mitosciences Inc. (Cambridge, MA). Secondary alkaline phosphatase-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Cambridgeshire, United Kingdom). All other chemicals used were of the highest purity available and were purchased from Sigma-Aldrich Chemical Co. (Barcelona, Spain or Milan, Italy).

### Statistical analysis

All data are expressed as mean  $\pm$  SD. The Mann-Whitney rank sum test was used to compare groups. For multiple comparisons, the ANOVA on ranks analysis of variance followed by Dunn's method was used. The nonparametric Spearman rank order correlation was used to relate biochemical parameters.  $P < 0.05$  defined significance.

## RESULTS

### Effect of CD and HFD on general parameters and liver histology

Feeding CD or HFD was associated with a progressive and significant increase of liver weight but not of body weight at days 30 and 60, respectively, as compared with control rats (Table 1). In rats with fatty liver, ALT levels increased progressively. At days 30 and 60, both CD and HFD rats showed a two-to-threefold increase.

Rats on CD and HFD showed remarkable liver steatosis (Figure 1 and Table 1). There was a progressive hepatic fat infiltration ranging from large droplets at day 14 to disseminated and confluent vacuoles resulting in microvesicular steatosis at day 30 (CD) and day 60 (HFD). Single cell necrosis was rarely noted. No evidence for inflammation and/or fibrosis was present.

### Effect of CD on oxidative and nitrosative stress parameters

In the liver, CD was associated with a higher content of GSH, thioredoxin, and nitrosothiols, while GPx activity was higher at day 7 and then lower at day 30 (Table 2). MDA-TBA levels were five-fold higher from  $9.1 \pm 1.2$

nmol/g to  $75.6 \pm 5.4$  nmol/g at day 30 (Figure 2). Serum MDA-TBA and nitrosothiols levels as well as nitrotyrosine and K-18 (Table 2) were higher in CD rats.

### Effect of HFD on oxidative and nitrosative stress parameters

In the liver, HFD determined a progressive decrease in the content of GSH, thioredoxin and nitrosothiols (Table 3). GPx activity was initially unchanged, then increased and next decreased (Table 3); MDA-TBA were doubled at day 60 (Figure 2). Serum levels of MDA-TBA, K-18, nitrotyrosine and nitrosothiols were significantly higher in HFD rats (Figure 3).

### Effect of HFD on mitochondrial oxidative phosphorylation complexes

Samples from liver, heart and skeletal muscle were compared regarding the content in subunits of the mitochondrial respiratory chain. Western blotting yielded bands to all OXPHOS subunits studied, although the band corresponding to complex III was very difficult to detect in the three tissues and hence was not quantified by densitometry. Band density for each protein was normalized for the corresponding  $\beta$ -actin band.

In the liver (Figure 4), the HFD caused an increase in the amount of the Complex I subunit NDUF8 (at day 60) and Complex IV subunit I, COX I (at day 30) and a decrease of Complex II subunit 30kDa, CII-30 (days 14 and 60) and ATP synthase subunit  $\alpha$ , CV- $\alpha$  (day 14). Minor changes in the heart and skeletal muscle proteins were observed (data not shown).

### Effect of Realsil on general parameters, liver histology, stress markers, and mitochondrial proteins

Realsil administration was associated with a lower increase of liver weight and a less pronounced increase of serum transaminase levels in rats (Table 1). As shown (Figure 1 and Table 2), Realsil resulted in a lower extent of fat infiltration in both CD and HFD rats. In CD rats, Realsil determined an improvement in the number and diameter of fat droplets. In HFD rats, Realsil was associated with a lower diameter of fat droplets; the number of droplets per histological section was lower at day 30 but higher at day 60, as a likely consequence of a less confluent phenomenon.

Realsil was protective against CD and HFD induced oxidative and nitrosative changes both in the liver and in plasma. The improvement in such parameters was more evident for CD rats. In particular, the decrease of liver GSH and nitrosothiols was kept to 50% compared with untreated rats, while the changes in MDA-TBA, thioredoxin, and GPx were less marked although significant (Table 2). The same parameters were less affected by Realsil in HFD rats (Table 3 and Figure 3). In fact, the hepatic concentrations of GSH, MDA-TBA, GPx, and nitrosothiols did not differ significantly compared to untreated HFD rats. Only thioredoxin levels were maintained to a higher level by Realsil administration. In these

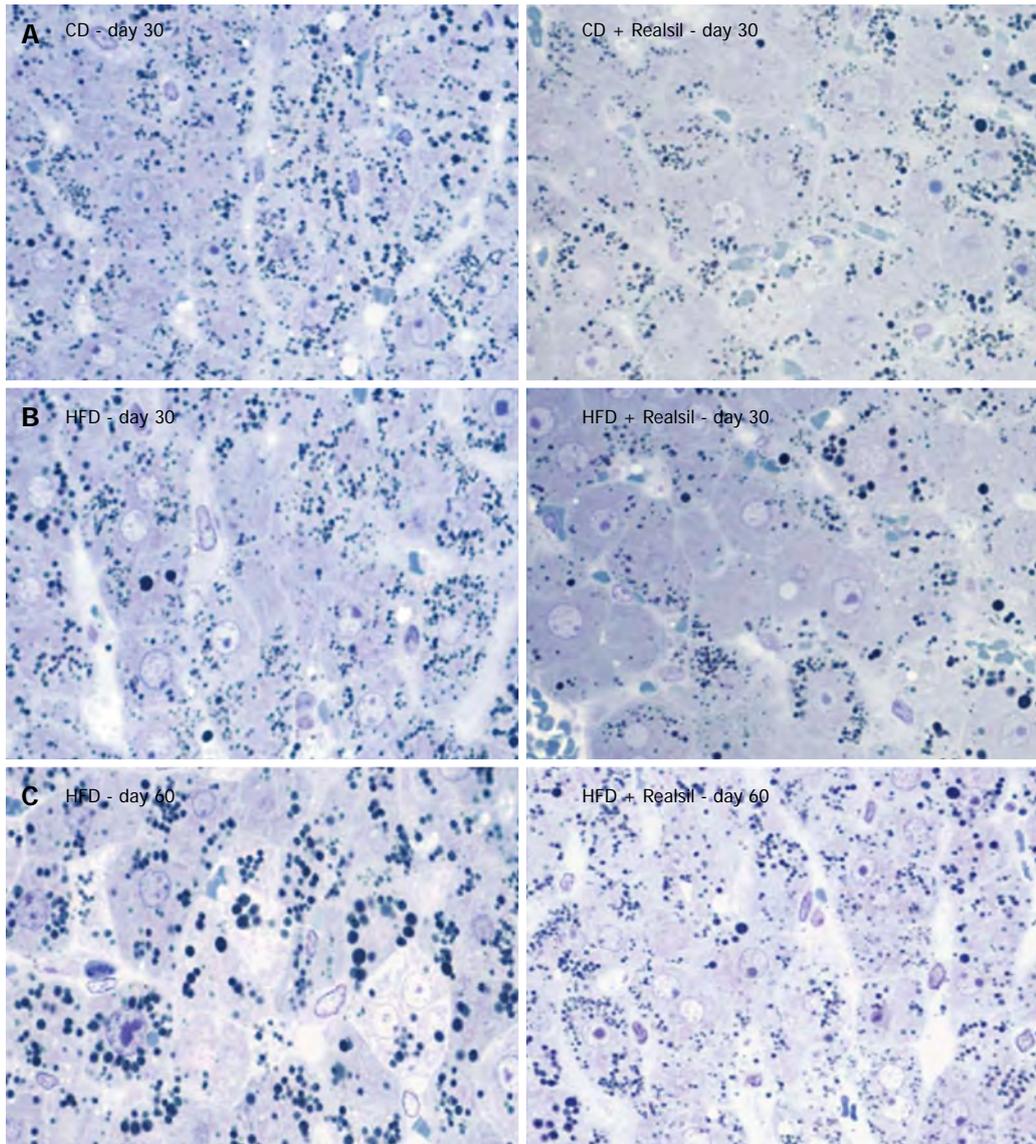


Figure 1 Light micrographs of rat liver stained with toluidin blue-periodic acid-schiff stain. A: Liver from rat fed a choline deficient diet (day 30); B: Liver from rat fed a high fat diet (day 30); C: Liver from rat fed a high fat diet (day 60). Right column without and left column with administration of Realsil (Magnification: 200 ×). CD: Choline deficiency; HFD: High fat diet.

rats, Realsil halved the variations of serum MDA-TBA and nitrotyrosine levels, while it was less effective on K-18 ( $222 \pm 15$  IU/L *vs*  $242 \pm 23$  IU/L at day 60 in treated and untreated HFD rats, respectively) and nitrosothiols ( $72 \pm 10$  nmol/L *vs*  $81 \pm 9$  nmol/L at day 60 in treated and untreated HFD rats, respectively) levels.

In HFD animals receiving also Realsil, several changes occurred in liver mitochondria (Figure 4). At day 14, administration of Realsil resulted in a significant increase in the protein levels of NDUFB8 subunit, which were slightly decreased (although not statistically significant) by the diet alone. On the other hand, Realsil in HFD animals further decreased the amount of CII-30 (day 14), NDUFB8 (day 30), and COX I (day 60). At day 60, Realsil had protective effect on the Complex II subunit CII-30 by increasing the amount of the protein that was decreased by the diet alone. Interestingly, in the muscle,

at day 60 Realsil reversed the effect induced by HFD in CII-30 subunit and in COX I subunit and, at day 30, decreased CV- $\alpha$  subunit protein amount.

## DISCUSSION

Obesity is a health problem in developed countries<sup>[18-21]</sup> and is related to insulin resistance, dyslipidemia, type 2 diabetes, hypertension, and liver steatosis (NAFLD)<sup>[22]</sup>. Simple steatosis is considered a benign condition with low risk of evolution, while its inflammatory form (NASH) is considered a risk factor for liver cirrhosis.

Mechanisms governing hepatocellular fat deposition involve metabolic pathways partly depending on the up-regulation of peroxisome proliferator-activated receptor and on the consequent activation of adipocyte differentiation programs<sup>[23]</sup>. The relationship between

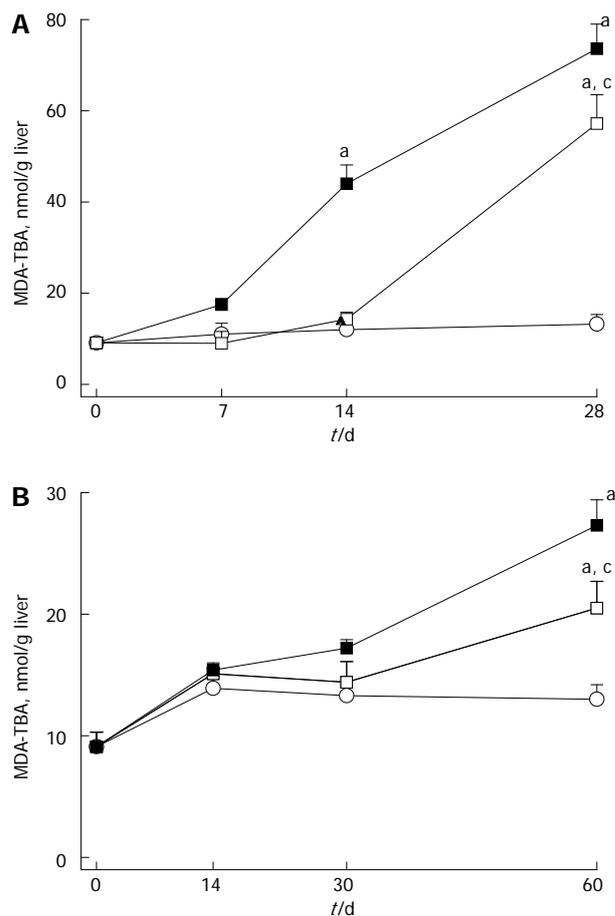
**Table 2** Concentrations of total glutathione, thioredoxin, nitrosothiols, and activity of glutathione peroxidase, malondialdehyde-thiobarbituric acid reactive substances, nitrosothiols, nitrotyrosine, and keratin-18 in the liver of rats

|                                |   | Day 7                    | Day 14                    | Day 30                    |
|--------------------------------|---|--------------------------|---------------------------|---------------------------|
| Glutathione                    | S | 34.3 ± 3.1               | 33.2 ± 1.4                | 25.3 ± 1.2 <sup>a</sup>   |
| 34.0 ± 1.3 nmol/mg prt         | R | 35.1 ± 1.2               | 33.7 ± 1.0                | 30.9 ± 1.1 <sup>a,c</sup> |
| Thioredoxin                    | S | 14.2 ± 1.0 <sup>a</sup>  | 16.3 ± 2.4 <sup>a</sup>   | 5.2 ± 0.7                 |
| 5.6 ± 0.9 nmol/mg prt          | R | 11.3 ± 1.0 <sup>a</sup>  | 14.4 ± 1.0 <sup>a</sup>   | 6.2 ± 0.7 <sup>c</sup>    |
| Nitrosothiols                  | S | 32.9 ± 3.5 <sup>a</sup>  | 30.0 ± 2.2 <sup>a</sup>   | 13.0 ± 1.2 <sup>a</sup>   |
| 16.8 ± 4.7 pmol/mg prt         | R | 35.4 ± 2.7 <sup>a</sup>  | 27.3 ± 2.2 <sup>a,c</sup> | 15.8 ± 1.5 <sup>a,c</sup> |
| MDA-TBA                        | S | 17 ± 4 <sup>a</sup>      | 39 ± 8 <sup>a</sup>       | 92 ± 10 <sup>a</sup>      |
| 12 ± 3 nmol/L                  | R | 12 ± 3                   | 19 ± 4 <sup>a,c</sup>     | 41 ± 9 <sup>a,c</sup>     |
| Nitrosothiols                  | S | 73 ± 15                  | 79 ± 12 <sup>a</sup>      | 88 ± 10 <sup>a</sup>      |
| 62 ± 10 nmol/L                 | R | 67 ± 10                  | 69 ± 10                   | 74 ± 11 <sup>a,c</sup>    |
| Nitrotyrosine                  | S | 6 ± 2                    | 10 ± 3 <sup>a</sup>       | 14 ± 3 <sup>a</sup>       |
| 6 ± 1 nmol/L                   | R | 5 ± 1                    | 5 ± 2 <sup>c</sup>        | 10 ± 2 <sup>a,c</sup>     |
| K-18                           | S | 222 ± 21                 | 233 ± 16 <sup>a</sup>     | 289 ± 20 <sup>a</sup>     |
| 198 ± 20 U/L                   | R | 210 ± 10                 | 213 ± 13 <sup>c</sup>     | 240 ± 17 <sup>a,c</sup>   |
| GPx activity                   | S | 9.3 ± 1.2 <sup>a</sup>   | 21.1 ± 3.7 <sup>a</sup>   | 13.7 ± 1.8 <sup>a</sup>   |
| 4.5 ± 0.4 nmol NADH/min/mg prt | R | 6.3 ± 0.9 <sup>a,b</sup> | 20.8 ± 1.4 <sup>a</sup>   | 16.6 ± 2.6 <sup>a,b</sup> |

The liver of rats fed a choline-deficient (CD) diet without (S)/with (R) Realsil or a normal choline-supplemented diet (controls). Values from controls are reported in the first column under the parameter. Data are mean ± SD of  $n = 5$  rats per group at each time point. <sup>a</sup> $P < 0.05$  vs control rats; <sup>c</sup> $P < 0.05$  vs rats on CD diet without Realsil at the same time point. GPx: Glutathione peroxidase; MDA-TBA: Malondialdehyde-thiobarbituric acid reactive substances; K-18: Keratin-18; prt: Protein.

intracellular metabolic processes and systemic changes occurring in patients with fatty liver are still under debate. Also, the effect of modulating compounds and antioxidant molecules on such lipid effectors and on NO metabolic changes has been poorly investigated. Mitochondria, coordinators of energy metabolism, are actively involved in such processes<sup>[18,21]</sup>. In NASH patients, mitochondria show morphological alterations and functional impairment<sup>[7,24]</sup>. Ultrastructural modifications were also observed in rats fed a steatogenic diet (HFD)<sup>[25]</sup>. Mitochondria are the most relevant source of ROS in most cells and especially in fatty hepatocytes<sup>[26,27]</sup>. ROS alter the activity of JNK enzymes, disturb insulin signaling, and enhance potassium transport across the inner mitochondrial membrane<sup>[28]</sup> leading to mitochondrial uncoupling and triggering as well adaptive response<sup>[29-31]</sup>.

In a recent study<sup>[3]</sup>, we observed that hepatocytes react to fat deposition by a very early increase of GSH and thioredoxin both in the cytosol and in the mitochondria to likely prevent lipid and protein oxidation. Prolongation of steatogenic diet, however, led to major mitochondrial redox changes, *i.e.*, increased MDA-TBA, progressive decrease of GSH and thioredoxin, and increase of mixed disulfides, nitrates, and nitrosothiols, all consistent with both oxidative damage and increased NO synthesis. In fatty livers, NO synthase (iNOS) is induced by enhanced inflow of gut-derived toxins<sup>[32]</sup> and tumor necrosis factor- $\alpha$  expression<sup>[33]</sup> and generates hyper reactive species such as NO with accumulation of nitrotyrosine<sup>[33]</sup>. This likely reflects an increased peroxynitrite formation



**Figure 2** Concentrations of malondialdehyde-thiobarbituric acid reactive substances. A: Liver of rat fed a choline-deficient diet; B: Liver of rat fed a high fat diet. Closed square: Without; open square: With Realsil administration; open circle: Control rats fed a standard chow-diet with adequate content of choline. Data are mean ± SD of  $n = 5$  rats per group at each time point. <sup>a</sup> $P < 0.05$  vs control rats; <sup>c</sup> $P < 0.05$  vs untreated rats at the same time points. MDA-TBA: Malondialdehyde-thiobarbituric.

and suggests potential NO participation to liver injury<sup>[34]</sup>. Thioredoxin, a redox-active protein induced by oxidative stress<sup>[35]</sup>, is actively involved in NO regulation through nitrosothiols cleavage<sup>[36,37]</sup>. Nitrosothiols, formed by conjugation of NO with free thiols, oppose peroxynitrite formation and act as intracellular messenger controlling cellular and mitochondrial functions<sup>[38,39]</sup>.

In the fatty liver, intracellular redox changes and protein nitrosation may represent a major factor stimulating the progression from simple steatosis to NASH<sup>[40]</sup>. Also, a critical role for such hepatic variations in the pathogenesis of systemic chronic inflammatory conditions has been recently proposed<sup>[41,42]</sup>. In our study, we show that liver steatosis is associated with high levels of circulating NO derivatives (nitrosothiols and nitrotyrosine) and with high levels of K-18, the major keratin expressed in the liver and one of the most prominent substrates of caspases during hepatocytes apoptotic death<sup>[43]</sup>. These alterations were closely dependent on the changes occurring in the liver in which GSH content and thioredoxin activity declined with ongoing steatosis. Also, the close

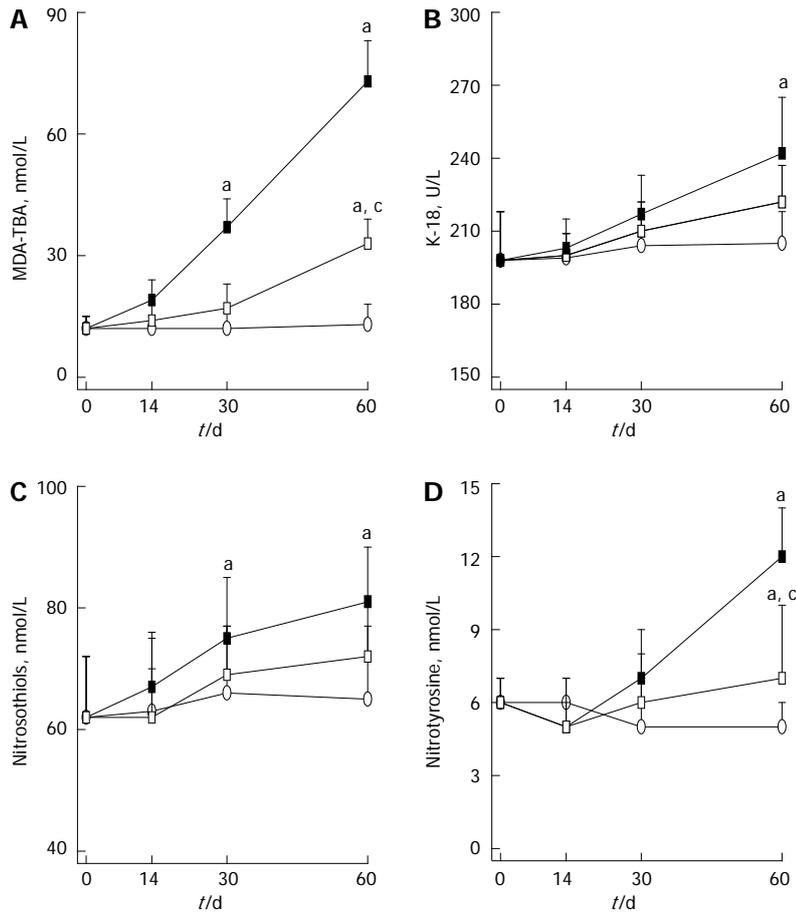


Figure 3 Plasma concentration of redox active and nitrogen regulating molecules. A: Malondialdehyde-thiobarbituric acid reactive substances (MDA-TBA); B: Keratine 18 (K-18); C: Nitrosothiols; D: Nitrotyrosine. Rats were fed a high fat diet without (closed square) or with (open square) Realsil administration; control rats fed a standard chow-diet (open circle). Data are mean  $\pm$  SD of  $n = 5$  rats per group at each time point. <sup>a</sup> $P < 0.05$  vs control rats; <sup>c</sup> $P < 0.05$  vs untreated rats at the same time points.

**Table 3** Concentrations of total glutathione, thioredoxin, nitrosothiols, and activity of glutathione peroxidase in the liver of rat

|                                    |   | Day 14                        | Day 30                      | Day 60                        |
|------------------------------------|---|-------------------------------|-----------------------------|-------------------------------|
| Glutathione                        | S | 23.7 $\pm$ 1.9 <sup>a</sup>   | 20.8 $\pm$ 2 <sup>a</sup>   | 22.4 $\pm$ 2.4 <sup>a</sup>   |
| 33.4 $\pm$ 0.8 nmol/mg prt         | R | 22.3 $\pm$ 1.8 <sup>a</sup>   | 23.9 $\pm$ 1.7 <sup>a</sup> | 23 $\pm$ 1.6 <sup>a</sup>     |
| Thioredoxin                        | S | 9.2 $\pm$ 0.9 <sup>a</sup>    | 8.9 $\pm$ 0.8 <sup>a</sup>  | 4.3 $\pm$ 1.0                 |
| 5.4 $\pm$ 0.5 nmol/mg prt          | R | 7.6 $\pm$ 0.7 <sup>a,c</sup>  | 7.8 $\pm$ 0.7 <sup>a</sup>  | 7.4 $\pm$ 1.7 <sup>a,c</sup>  |
| Nitrosothiols                      | S | 18.1 $\pm$ 1.5                | 19.6 $\pm$ 2.5              | 10 $\pm$ 2.3 <sup>a</sup>     |
| 20.4 $\pm$ 3.1 pmol/mg prt         | R | 14.4 $\pm$ 1.7 <sup>a,c</sup> | 16.1 $\pm$ 2.3 <sup>a</sup> | 14.3 $\pm$ 1.9 <sup>a,c</sup> |
| GPx                                | S | 4.7 $\pm$ 0.6 <sup>a</sup>    | 6.5 $\pm$ 1.3 <sup>a</sup>  | 4.6 $\pm$ 1.6                 |
| 3.9 $\pm$ 0.3 nmol NADH/min/mg prt | R | 3.4 $\pm$ 0.5 <sup>c</sup>    | 4.3 $\pm$ 1.0 <sup>c</sup>  | 4.1 $\pm$ 1.0                 |

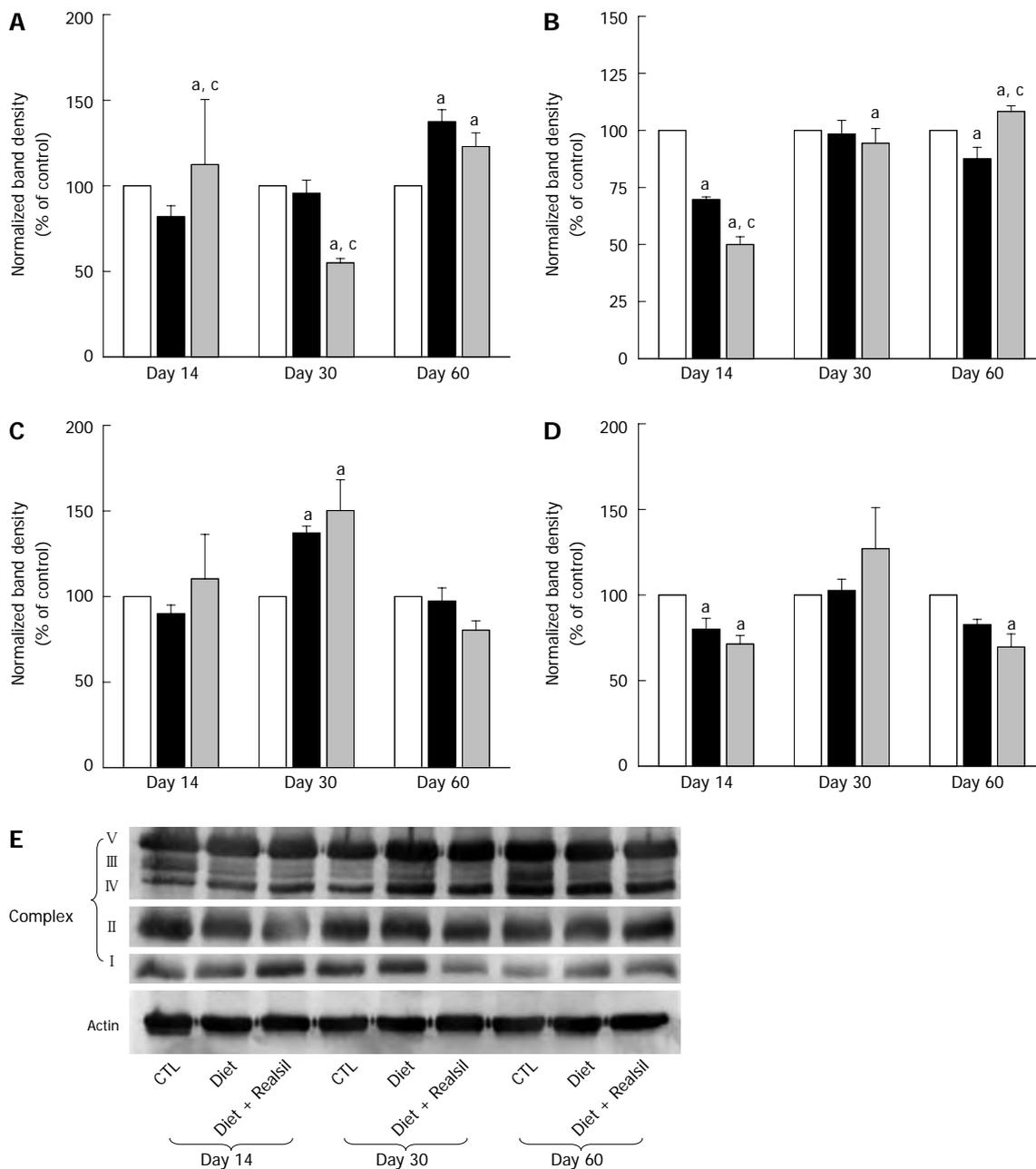
The liver of rats fed a high fat diet (HFD) diet without (S)/with (R) Realsil or a standard control diet (controls). Values from controls are reported in the first column under the parameter. Data are mean  $\pm$  SD of  $n = 5$  rats per group at each time point. <sup>a</sup> $P < 0.05$  vs control rats; <sup>c</sup> $P < 0.05$  vs rats on HFD diet without Realsil at the same time point. GPx: Glutathione peroxidase; NADH: Nicotinamide adenine dinucleotide; prt: Protein.

relation observed between the circulatory NO derivatives and K-18 levels clearly links NO with the hepatic inflammatory processes occurring under marked liver steatosis.

Previous studies have demonstrated elevation of these molecules in NAFLD<sup>[44]</sup> as a consequence of the increased apoptotic rate due to hepatic inflammation<sup>[45]</sup>.

In the present work, the HFD promoted alterations in mitochondrial complexes, although not deep enough to result in bioenergetic changes, as reported for other diet models including CD<sup>[46,47]</sup>. Interestingly, the limited array of alterations observed in the heart and skeletal muscle points to the conclusion that this model selectively affects the liver.

In this scenario, the administration of antioxidant molecules able to contrast oxidative and nitrosative phenomena improved most of the investigated stress parameters both in the liver and in serum. In fact, in our study the administration of Realsil was effective in reducing the extent of fatty infiltration of the liver and in modulating the changes in mitochondrial function and oxidative and nitrosative stress both in the liver and in the systemic circulation. This would suggest that Realsil is effective in contrasting the metabolic alterations resulting in excess fat deposition and at the same time in counteracting the increased formation of ROS and NO species.



**Figure 4** Western blotting analysis of the components of liver mitochondrial oxidative phosphorylation system in rats receiving high fat diet with (grey bar) or without (black bar) Realsil and in control (white bar) rats fed a standard chow-diet. Band density for the target protein normalized for the corresponding  $\beta$ -actin for A: Respiratory complex I; B: Respiratory complex II; C: Respiratory complex IV; D: Respiratory complex V; E: Picture representative of Western blotting analysis. Data are means  $\pm$  SE of  $n = 3-4$  independent experiments. <sup>a</sup> $P < 0.05$  vs control rats; <sup>c</sup> $P < 0.05$  vs untreated rats at the same time points.

Silybin, the major constituent of milk thistle extract, affords hepatoprotection *in vitro* and *in vivo*<sup>[48,49]</sup> by inhibiting the production of pro-inflammatory and pro-fibrogenic factors<sup>[12,50]</sup>. The conjugation with phospholipids greatly increases its intestinal absorption and the systemic bioavailability<sup>[51]</sup>. However, the mechanisms of the hepatocyte protection have not been completely defined. Some hypotheses point to a potential antioxidant cytoprotective effect of Realsil by including the modulation of protein oxidation/denitrosation and the maintenance of membrane lipid composition and function<sup>[52]</sup>. To demonstrate these effects, we investigated the protective ef-

fects of Realsil on oxidative/nitrosative changes both in the liver and in circulation.

Indeed, although the subtle mechanisms regulating the protein nitrosation/denitrosation process have not been completely identified, it has been shown that thioredoxin-deficient cells denitrosate nitrosothiols less efficiently<sup>[37]</sup> and that nitrosative stress is critically important in promoting S-nitrosylation and nitration of various mitochondrial proteins, leading to mitochondrial dysfunction, decreased energy supply, and increased hepatic injury<sup>[53]</sup>. With ongoing steatosis, the decrement in hepatic thioredoxin and GSH levels we observed may be due

also to a down-regulation process associated with excess deposition of fat and toxic molecules and may indirectly contribute to the progressive appearance of other oxidative changes.

In our study, Realsil was able to counteract most of the oxidative biochemical alterations during the early phases of steatosis while it resulted less effective later (30 and 60 d) when the extent of fat infiltration was massive. The protection promoted by Realsil is certainly exerted at different levels and particularly at mitochondrial level<sup>[54]</sup>. The changes (both morphological and biochemical) observed in the liver of rats receiving Realsil were also evident at systemic level and this more explicitly relates systemic with hepatic changes in animals with fatty liver.

## COMMENTS

### Background

Fatty degeneration represents a leading factor of hepatocyte dysfunction and is associated with excess delivery of stress molecules thus rendering the liver a major source of systemic alterations in patients with metabolic syndrome. Adaptive metabolic mechanisms have been described during the early phase of fatty infiltration including expression of intracellular sensors and signaling molecules for lipid metabolism and oxidative stress pathways. Vitamin E efforts a remarkable protection against lipid oxidation and, if vehicled within a phospholipids complex together with silybin, a *silybum marianum* extract, protects against pro-fibrotic oxidative injury.

### Research frontiers

The threshold above which fat infiltration becomes dangerous is not clear, so far. Several observations suggest that ongoing fatty degeneration indeed exposes hepatocytes to higher risk of oxidative damages.

### Innovations and breakthroughs

Little is known about the effectiveness of vitamin E-silybin-phospholipid complex in blocking or modulating reactive oxygen species/NO production and their oxidant effects. This study also gives answer to the question whether administration of an antioxidant complex is able to break off the intracellular mechanisms leading to a progressive accumulation of neutral lipids in fatty hepatocytes and mitochondrial dysfunction.

### Applications

Realsil was able to counteract most of the oxidative hepatic changes during the early phases of steatosis while it resulted less effective later when the extent of fat infiltration was massive. The changes observed in the rat fatty liver were also evident at systemic level pointing to a relationship between systemic and hepatic alterations.

### Terminology

Liver steatosis occurs when the amount of neutral fat exceeds 5% of organ weight. Realsil is a compound constituted by vitamin E and silybin complexed with phospholipids. Silybin is a *silybum marianum* extract; it is known to protect against pro-fibrotic oxidative injury.

### Peer review

In this descriptive study, the authors show that Realsil is able to reduce liver injury in two different animal models of steatosis. Realsil seems to protect by decreasing toxic free radicals species as highly delivered by fatty hepatocytes.

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**P- Reviewers** Cai SY, Wisse E **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Zhang DN



## Incidence and mortality of acute and chronic pancreatitis in the Netherlands: A nationwide record-linked cohort study for the years 1995-2005

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Author contributions: All the authors contributed equally to this manuscript.

Supported by Unrestricted Grant from Axcan Pharma Incorporate, Canada, to Spanier BMW; Dutch Society of Gastroenterology, Gastrostart Project Number 2007-7

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Received: January 9, 2013 Revised: March 5, 2013

Accepted: April 10, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To analyze trends in incidence and mortality of acute pancreatitis (AP) and chronic pancreatitis (CP) in the Netherlands and for international standard populations.

**METHODS:** A nationwide cohort is identified through record linkage of hospital data for AP and CP, accumulated from three nationwide Dutch registries: the hospital discharge register, the population register, and the death certificate register. Sex- and age-group specific incidence rates of AP and CP are defined for the period 2000-2005 and mortality rates of AP and CP for the period 1995-2005. Additionally, incidence and mortality rates over time are reported for Dutch and international (European and World Health Organization) standard populations.

**RESULTS:** Incidence of AP per 100000 persons per year increased between 2000 and 2005 from 13.2 (95%CI:

12.6-13.8) to 14.7 (95%CI: 14.1-15.3). Incidence of AP for males increased from 13.8 (95%CI: 12.9-14.7) to 15.2 (95%CI: 14.3-16.1), for females from 12.7 (95%CI: 11.9-13.5) to 14.2 (95%CI: 13.4-15.1). Irregular patterns over time emerged for CP. Overall mean incidence per 100000 persons per year was 1.77, for males 2.16, and for females 1.4. Mortality for AP fluctuated during 1995-2005 between 6.9 and 11.7 per million persons per year and was almost similar for males and females. Concerning CP, mortality for males fluctuated between 1.1 (95%CI: 0.6-2.3) and 4.0 (95%CI: 2.8-5.8), for females between 0.7 (95%CI: 0.3-1.6) and 2.0 (95%CI: 1.2-3.2). Incidence and mortality of AP and CP increased markedly with age. Standardized rates were lowest for World Health Organization standard population.

**CONCLUSION:** Incidence of AP steadily increased while incidence of CP fluctuated. Mortality for both AP and CP remained fairly stable. Patient burden and health care costs probably will increase because of an ageing Dutch population.

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**Key words:** Acute pancreatitis; Chronic pancreatitis; Epidemiology; Incidence; Mortality

**Core tip:** Large scale epidemiological studies reporting time trends of incidence and mortality of chronic pancreatitis (CP) are strikingly scarce compared to the also limited epidemiological studies on acute pancreatitis (AP). Reported are the Dutch incidence rates of AP and CP for the period 2000-2005, and mortality rates of AP and CP for the period 1995-2005. The incidence rates of AP steadily increased while the incidence of CP fluctuated. Population mortality for both AP and CP remained fairly stable. Both incidence and mortality rates increased markedly by age. So, especially in ageing populations, it is to be expected that patient burden and health care costs will increase.

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## INTRODUCTION

Over the last decades, the incidence and number of hospital admissions of both acute pancreatitis (AP)<sup>[1-10]</sup> and chronic pancreatitis (CP) have consistently increased in the Western countries<sup>[11-14]</sup>. Increasing alcohol intake, more gallstone-related pancreatitis, increased pancreatic enzyme testing and improvements of diagnostic tests and interventional techniques have all been suggested as possible explanations<sup>[4,5,6,8,15-17]</sup>.

The disease spectrum of AP ranges from mild and self-limiting (approximately 85%) to a life-threatening illness resulting in significant morbidity and mortality<sup>[18-21]</sup>. At onset, AP regularly results in hospitalization<sup>[22,23]</sup>. Generally, in the Western countries the case fatality proportion of AP decreased over time, but the overall population mortality did not change<sup>[2,16]</sup>.

CP is characterized by ongoing or recurrent episodes of abdominal pain accompanied by progressive pancreatic exocrine and endocrine insufficiency. Hospitalization is required in case of an exacerbation to control pain (*e.g.*, opioid medication, endoscopic duct drainage, pancreaticojejunostomy and/or resection) and for the treatment of complications such as pseudocysts<sup>[15,16,24,25]</sup>. The overall survival for CP patients is reduced compared with the general population. Most notably because of the impact of non-pancreatic effects of excess alcohol consumption and/or smoking, independent of CP itself<sup>[15,16]</sup>.

Epidemiological studies from the Netherlands for AP are scarce and this is even more the case for CP<sup>[2,11,22]</sup>. Eland *et al*<sup>[2]</sup> reported the latest incidence rates and mortality of AP from 1985 to 1995, both based on hospital discharge data. Our group reported about the trend in hospital admissions in the Netherlands for AP and CP<sup>[11]</sup>. For both groups, the hospital admissions have increased substantially from 1992 to 2004.

In this study we report the incidence rates of AP and CP for the period 2000-2005 and mortality rates of AP and CP for the period 1995-2005 following linkage of three distinct nation-wide Dutch registries. Additionally, data on incidence and mortality rates over time are reported for Dutch and international standard populations.

## MATERIALS AND METHODS

### Case finding

Cases of AP and CP were identified by linkage of three distinct nation-wide Dutch registries: the hospital discharge register [HDR: formerly also known as the National Information System on Hospital Care (NISHC)],

the population register (PR), and the death certificate register (DCR). The HDR contains discharge data from academic and general hospitals in the Netherlands (<http://www.dutchhospitaldata.nl/>). Since 1992 almost all (> 97%) Dutch hospitals are linked to the HDR with 99% coverage of all hospital admissions. For each hospital discharge the dates of admission and discharge, type of admission, and diagnoses at discharge (primary and secondary) are recorded along with anonymous patient characteristics like: sex, date of birth, postal code of the living address, and country of origin. Hospital discharge diagnoses in the HDR are coded according to the International Classification of Diseases, 9<sup>th</sup> revision, Clinical Modification (ICD-9CM). We retrospectively retrieved all hospital admissions (> 1 d of hospital stay) for the period 1 January 1995-31 December 2005 from the HDR with acute (code 577.0) or chronic (code 577.1) pancreatitis as the primary discharge diagnoses.

A single patient with multiple admissions will have several records in the HDR. Because of its anonymous nature however, only partially identifiable information is provided at the record level. For the accurate count of pancreatitis cases, it is necessary to identify different admissions, potentially in different hospitals, by the same patient. To identify these unique cases in the HDR the records were linked to the PR which is maintained by Statistics Netherlands of the Dutch Ministry of Economy Affairs. The PR contains continuously updated demographic data on all citizens residing in the Netherlands like name, date of birth, sex, nationality, living address, dates of immigration and/or emigration, and date of death.

HDR records can be linked to the PR by a combination of three identifiers: sex, date of birth, and (the numeric part of) the postal code at the time of admission. Linkage fails if several citizens are of the same sex, are born on the same date, and live in the same postal code area at the time at least one of them is admitted (“administrative siblings”). Also, linkage fails if any key data are lacking in the HDR or in the PR.

Statistics Netherlands enables the unique linkage of about 86% of all yearly hospital admissions to the PR, suggesting that counts at the person level should be multiplied by 100/86 to derive national estimates. However, the probability of unique linkage is not equal across subgroups in the general population and over time. For instance, persons in high-density population areas, persons born in countries with inaccurate birth dates, and moving persons have a lower probability of unique linkage. To compensate for linkage failure, stratum-specific multipliers were calculated for sex, 5-yearly age cohorts, and country of origin (available from the corresponding author upon request). While doing so, we took into account that our aim was to estimate the incidence rather than the prevalence of AP and CP.

### From case finding to incident cases

We defined an incident case during any specific year as

a person with an admission for AP or CP during that year which is not preceded by another admission of the same person with the same discharge diagnosis during at least five years prior to the specified year. To identify incident cases, it is mandatory that all admissions during the observation period have been identified for each person. Hence, a person known in the PR should be always uniquely identifiable during the observation period 1995-2005 based on the linkage keys sex, age, and the postal code.

### **Incidence rates and standardized incidence rates**

Sex- and age- specific incidence rates for the years 2000-2005 were defined as the yearly number of new pancreatitis cases per 100000 members of the Dutch reference population alive during the year, excluding immigrated or emigrated citizens and after adjustment for stratum-specific linkage failure between the HDR and the PR<sup>[26]</sup>. The yearly incidence rates were standardized with reference to the age and sex distribution of the Dutch reference population in 2000 to identify the possible impact of a Dutch ageing population on the observed trends in incidence rates over time. Standardizations were also performed with reference to the age distribution (assuming evenly distributed male and females) of the European and World Health Organization (WHO) standard populations to enable direct comparisons among trends in incidence and mortality rates across countries that differ by age (and sex) distributions of their respective populations<sup>[27]</sup>.

### **Mortality rates and standardized mortality rates**

Deaths associated with AP and CP were identified by linkage of the PR and the DCR which is also maintained by Statistics Netherlands.

General practitioners and hospitals are obliged to complete a death certificate for each deceased person, and notify the primary and up to three secondary causes of death. Causes of death are coded according to the International Classification of Diseases coding system with acute and chronic pancreatic related death coded as 577.0 and 577.1 respectively for the year 1995 (ICD-9CM) or K850 and K860/K861 respectively for the years 1996 and later (ICD-10CM). The register is nearly complete (99.7%) with regard to persons deceased in the Netherlands, meaning that deaths after emigration are lacking. For their completeness and reliability, only the primary causes of death were analyzed. The mortality rate was expressed per 1000000 general population members excluding the ones that emigrated any time between 1995 and 2005. Again, yearly mortality rates were recalculated with reference to the age and sex distribution of the Dutch reference population in 2000 as well as with reference to the age distribution of the European and WHO standard populations.

### **Statistical analysis**

We assumed the AP and CP incidence and mortality rates to follow Poisson distributions when calculating 95%CI. The Statistical Package for the Social Sciences versions

14.0 and 18.0 (SPSS, Chicago, IL, United States) were used for statistical analysis.

## **RESULTS**

In 1995-2005, 18819221 citizens were known in the population register with 49.75% being male. Slightly under 10.4 percent immigrated or emigrated, leaving 16866819 patients in the Dutch reference population for the incidence estimates, 12163581 of whom were always uniquely identifiable during 1995-2005. Hence, the average multiplier for the incidence estimates amounted to 1.387.

### **Incidence rates acute pancreatitis per 100000 persons per year**

The overall incidence rate of AP increased during the 2000-2005 period from 13.2 (95%CI: 12.6-13.8) in 2000 to 14.7 (95%CI: 14.1-15.3) in 2005. The incidence rate for males rose from 13.8 (95%CI: 12.9-14.7) to 15.2 (95%CI: 14.3-16.1), for females from 12.7 (95%CI: 11.9-13.5) to 14.2 (95%CI: 13.4-15.1), reflecting yearly increases of 1.6% and 1.9% respectively (Figure 1A).

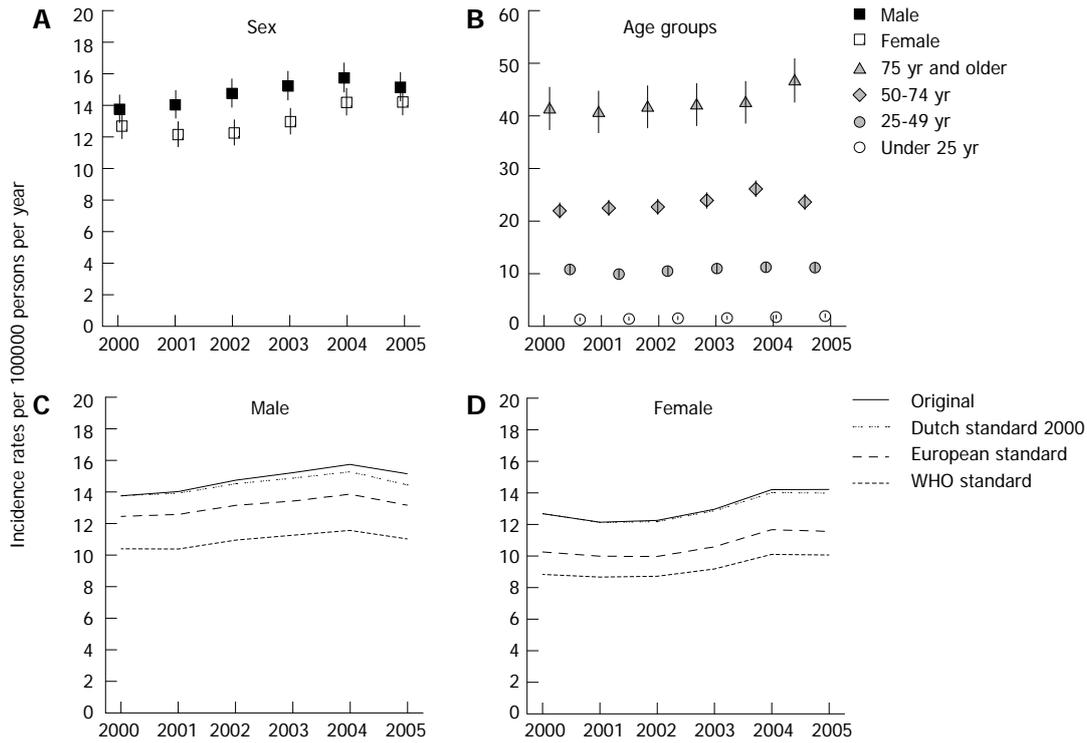
Incidence rates ranged from below 1 in the younger age groups (< age 15 years) to as high as 50.7 in 2005 for the patients of 85 years and above. Figure 1B shows the incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The incidence rate in the 50-74 age group was highest in 2004 (26.1; 95%CI: 24.6-27.7); in the eldest age group the incidence rate was increased in 2005 (46.5; 95%CI: 42.5-50.9) compared to the preceding years.

The mean yearly incidence rates of AP by 5-yearly age groups and by sex peaked at the ages of 80-84 for both, males (55.4; 95%CI: 42.8-71.6) and females (41.9; 95%CI: 33.8-52.0). The mean yearly incidence rate was higher for females (7.1; 95%CI: 4.7-10.7) than males (3.4; 95%CI: 1.9-6.0) for persons in their early twenties. However, between the ages of 44 and 84, the opposite was observed with 30%-63% higher mean yearly incidence rates for males than for females.

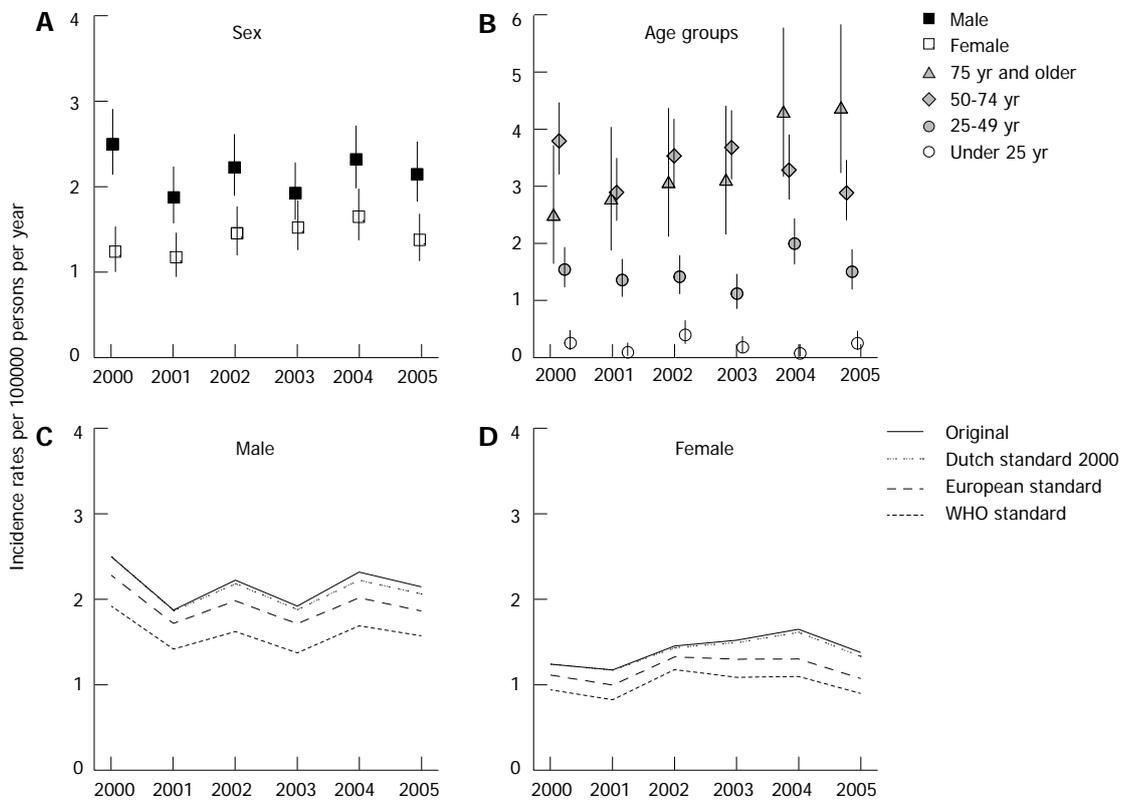
Figure 1C and D (males and females) show the original as well as the Dutch 2000, the European and the WHO standardized incidence rates of AP. Both figures show lowest incidence rates for the WHO standard population (range males: 10.4-11.6; range females: 8.6-10.1), followed by the European standard population (range males: 12.5-13.9; range females: 10.0-11.7), and the Dutch standard population (range males: 13.8-15.3; range females: 12.1-14.0). The figures show that the discrepancy between the original incidence rate and the standardized rates increases over time for males, indicating a small effect of an ageing Dutch population.

### **Incidence rates chronic pancreatitis per 100000 persons per year**

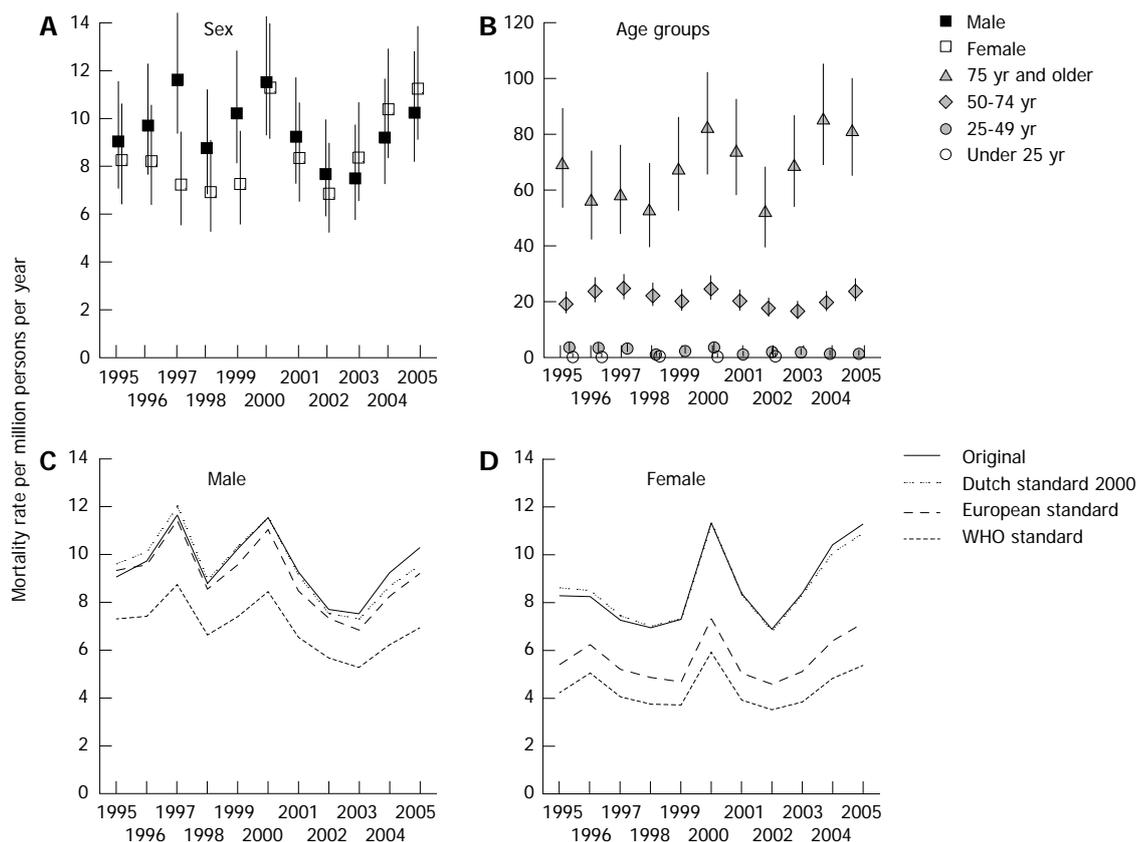
The overall incidence rate of CP during the 2000-2005 period averaged 1.77, fluctuating between 1.52 (95%CI: 1.32-1.74) in 2001 and 1.98 (95%CI: 1.76-2.22) in 2004. Figure 2A shows the incidence rates of male and female



**Figure 1** Incidence rates acute pancreatitis per 100000 persons per year. A: The incidence rates of male and female acute pancreatitis (AP) cases; B: The incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized incidence rates of AP.



**Figure 2** Incidence rates chronic pancreatitis per 100000 persons per year. A: The incidence rates of male and female chronic pancreatitis (CP) cases; B: The incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized incidence rates of CP.



**Figure 3** Mortality rates acute pancreatitis per million persons per year. A: The male and female mortality rates of acute pancreatitis (AP) for 1995-2005; B: The mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized mortality rates of AP.

CP cases. The incidence rates for males averaged 2.16, 50% higher than the incidence rates for females (mean of 1.4). The incidence rates for males fluctuated over time with lower rates in the years 2001 and 2003 (both 1.9; 95%CI: 1.6-2.3) compared with the year 2000 (2.5; 95%CI: 2.1-2.9). Among females, the incidence rates during the years 2003 (1.5; 95%CI: 1.3-1.8) and 2004 (1.6; 95%CI: 1.4-2.0) were higher than during the year 2001 (1.2; 95%CI: 0.9-1.5).

Incidence rates ranged from below 0.5 in the younger age groups (< age 20 years) to as high as 5.2 (95%CI: 3.5-7.8) in 2005 for the patients between 75 and 79 years of age. Figure 2B shows the incidence rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). While the incidence rate fluctuated in the 50-74 years age group between 3.8 (95%CI: 3.2-4.5) in 2000 and 2.9 (95%CI: 2.4-3.5) in 2001 and 2005, the incidence rate in the age group of 75 and older increased steadily by 75%, from 2.5 (95%CI: 1.7-3.7) in 2000 to 4.4 (95%CI: 3.2-5.8) in 2005.

The mean yearly incidence rates of CP for males between 65 and 74 years (65-69 years: 6.2, 95%CI: 3.9-9.8; 70-74 years: 5.1, 95%CI: 3.0-8.9) more than doubled the rates for females (65-69 years: 2.3, 95%CI: 1.2-4.7; 70-74 years: 2.4, 95%CI: 1.2-5.0).

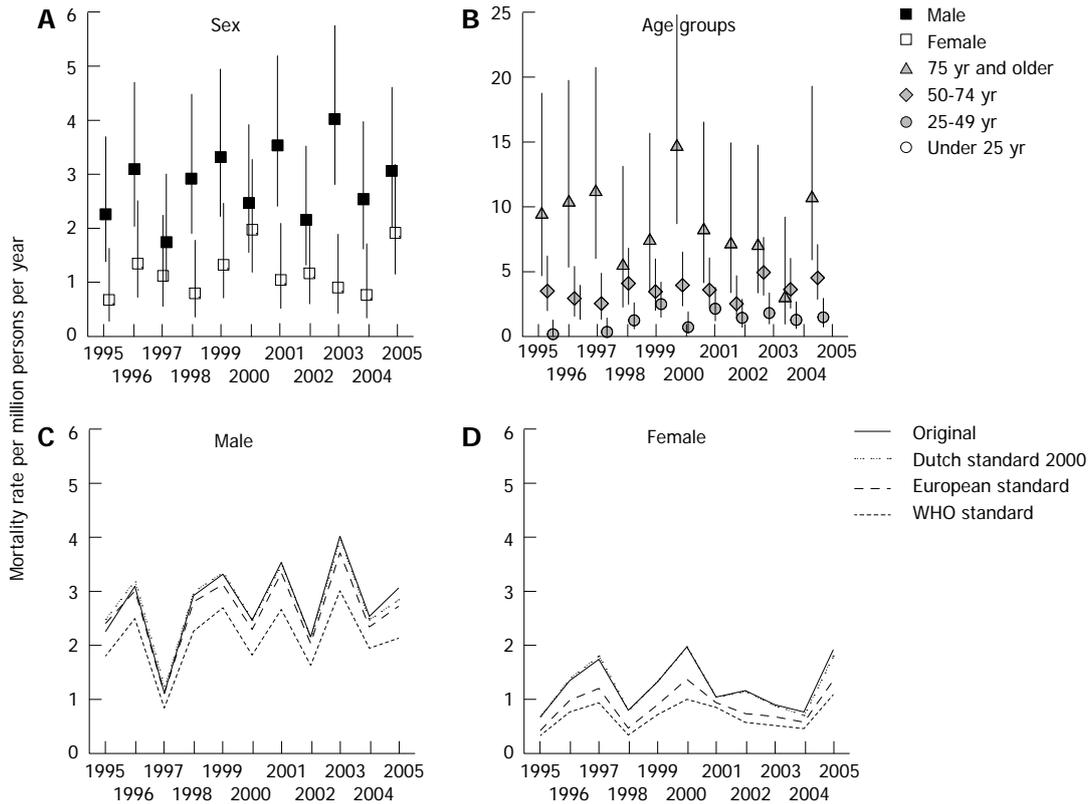
Figure 2C and D (males and females) show the original as well as the Dutch 2000, the European and the WHO standardized incidence rates of CP. The incidence

rates for the WHO standard population ranged from 1.4 to 1.9 for males and from 0.8 to 1.2 for females. For the European standard population, the ranges were 1.7-2.3 and 1.0-1.3 for males and females respectively. The ranges for the Dutch standard population in 2000 (range males: 1.9-2.5; range females: 1.2-1.6) were nearly identical to the non-standardized data.

#### **Mortality rates acute pancreatitis per million persons per year**

Between 1995 and 2005, 1524492 persons died in the Netherlands. In 2264 cases AP was notified as a cause of death. Of these, 65.6% or 1484 cases (0.97 pro mille of all deceased persons) died with AP as the primary cause of death, including 764 males and 720 females.

Figure 3A shows the male and female mortality rates of AP for 1995-2005. The mortality rates fluctuated between 6.9 and 11.7 were similar for males and females except for the years 1997 and 1999 when more males than females died (1997: 11.7; 95%CI: 9.4-14.5 *vs* 7.26; 95%CI: 5.56-9.48); 1999: 10.3; 95%CI: 8.2-12.9 *vs* 7.3; 95%CI 5.61-9.51). Figure 3B shows the mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The mortality rate for patients younger than 50 years of age stayed below 3.6 (95%CI: 2.3-5.6) and for the 50-74 years age group below 18.6 (95%CI: 14.8-23.5), whereas the mortality rate more than tripled up to 85.3 (95%CI:



**Figure 4** Mortality rates chronic pancreatitis per million persons per year. A: The male and female mortality rates of chronic pancreatitis (CP) for 1995-2005; B: The mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years); C (males) and D (females): The original as well as the Dutch 2000, the European and the World Health Organization (WHO) standardized mortality rates of CP for 1995-2005.

69.1-105.4) for the oldest age group.

Figure 3C and D (males and females) show the original as well as the Dutch 2000, the European, and the WHO standardized mortality rates of acute pancreatitis. An ageing effect for males is present with in 1995 a 6.1% higher and in 2005 a 7.1% lower Dutch standardized rate compared to the original rate. On average, the mortality rates for the European and Dutch standard populations are 31.6%, respectively 36.8% higher than the WHO standard. Although the overall pattern is irregular, a gradual decline over time in mortality rates for males can be noted.

Among the females a smaller ageing effects emerges with in 1995 a 4% higher and in 2005 a 3.4% lower Dutch standardized rate compared to the non-standardized rate. On average, the mortality rates for the European and WHO standard populations are 52.7%, respectively 96.8% below the Dutch standard.

#### **Mortality rates chronic pancreatitis per million persons per year**

Between 1995 and 2005 745 patients died in the Netherlands with CP notified as a cause of death. Of these, 43.9% or 327 cases (0.21 pro mille of all deceased Dutch patients in the same period) died with CP as the primary cause of death, including 223 males and 104 females.

Figure 4A shows the male and female mortality rates of CP for 1995-2005. The mortality rates for males fluctuated between 1.1 (95%CI: 0.6-2.3) in 1997 and 4.0

(95%CI: 2.8-5.8) in 2003, for females between 0.7 (95%CI: 0.3-1.6) in 1995 and 2.0 (95%CI: 1.2-3.2) in 2000. Except for 1997, 2000, 2002 and 2005 the male mortality rate exceeded the female mortality rate.

Figure 4B shows the mortality rates for four major age groups (< 25, 25-49, 50-74 and > 74 years). The mortality rate for patients younger than 50 years of age stayed below 2.3 (95%CI: 1.3-4.0) and for the 50-74 age group below 4.5 (95%CI: 2.9-7.1), whereas for the oldest age group the mortality rate went up to as high as 14.7 (95%CI: 8.7-24.9).

Figure 4C and D (males and females) show the original as well as the Dutch 2000, the European, and the WHO standardized mortality rates of CP for 1995-2005. Again, an ageing effect for males is present with in 1995 a 9.7% higher and in 2005 a 7.1% lower Dutch standardized rate compared to the original rate. On average, the European and WHO standardized rates are 5.9% and 32.4% lower than the Dutch rate. No clear ageing effect is observed for females. On average, the European and WHO standardized rates are 42% and 83.5% lower than the Dutch rate.

## **DISCUSSION**

We performed a nationwide record-linked study to analyze the time trends of the incidence and mortality rates of AP and of CP in the Netherlands. We show that

between 2000-2005 the incidence of AP per 100000 persons per year increased over time for both, males (from 13.8 to 15.2) and females (from 12.7 to 14.2). Relatively stable patterns over time emerged for the incidence rate of AP per 100000 persons per year by different age groups. The steady increase of the incidence of AP over time corresponds to the results of a former retrospective study performed in the Netherlands between 1985-1995<sup>[2]</sup>. However, the similar growth pattern in our study was observed at a lower level. Eland *et al*<sup>[2]</sup> observed incidence rates for the year 1995 of 17.0 males and 14.8 females per 100000 person-years. If we take for granted that no decrease in incidence rates took place during the unobserved years 1996-1999, then several decisions concerning study design may have contributed to the difference in incidence rate level. Eland *et al*<sup>[2]</sup> retrieved primary as well as secondary discharge diagnoses from the HDR, whereas we only included AP as a primary discharge diagnosis in order to reduce the risk of misclassification of cases. Further, we excluded single-day admissions for AP. This seems reasonable, because patients with a first attack of AP usually get admitted for several days<sup>[2,9]</sup>. Moreover, by identifying unique cases following the linkage of two nationwide registries, double counting of cases is a circumvented issue in our study. Considering these reasons for differences in level despite a similar growth pattern, the former reported incidence rates in the Netherlands may be somewhat overestimated.

Other recent population-based studies in Western countries too report increasing incidence rates of AP<sup>[3,4,6,7,10,28-31]</sup>. The studies - although somewhat heterogeneous by design - indicate that the reported incidence rates of AP in the Netherlands are low and even far lower compared to reported rates from several Scandinavian countries and the United States<sup>[16]</sup>. The observed differences in incidence rates between these geographical locations are not clearly understood and presumably reflects differences in risk factor prevalence. It has been suggested that the increase of the incidence of AP in the Western countries could be explained by an increase in alcohol intake<sup>[16]</sup>. According to the registry data of Statistics Netherlands the general self-reported alcohol consumption slightly decreased in the period 2000-2005 ([www.cbs.nl](http://www.cbs.nl)). Therefore, the incidence increase of acute pancreatitis seems not to be explained by a change in alcohol consumption, at least not in the Netherlands. We observed that the incidence rates of AP increased considerably with age. This is in accordance with observations elsewhere<sup>[2-4,7,29]</sup>.

The incidence rates of CP in the Netherlands have now been reported for the first time. In contrast to AP, irregular patterns over time emerged for the incidence rates of CP per 100000 persons per year for males (mean: 2.16) and females (mean: 1.4). The incidence rate increased with age, with a top in 2005 at 4.4 in the age group of 75 or older. Recent large scale epidemiological studies reporting the time trends of incidence of CP are strikingly scarce<sup>[15,16]</sup>. The latest reported incidence rates of CP vary between 5.9 and 7.9 per 100000 per-

sons<sup>[11,31,32]</sup>. In comparison to those studies, our observed incidence rates of CP are somewhat lower. This may in part be explained by our study limitations (see below). In accordance to other studies, we show that CP is predominantly a disease of males<sup>[11-13,15,16,31]</sup>. The average incidence rate for males is 50% higher than for females. This is even more pronounced in the older age groups.

The mortality rate for AP per million persons per year fluctuated between 6.9 to 11.7 during the 1995-2005 period. Others too reported fairly stable overall annual population mortality rates<sup>[2,10,13]</sup>. The mortality rate increased rapidly for patients of 50 years of age and above, which too is in accordance with observations elsewhere<sup>[2,4,10,29]</sup>. Advanced age may be an independent risk factor for severe AP<sup>[33]</sup>. Mortality rates were almost similar for males and females.

Concerning CP, the mortality rate per million persons per year for both males and females fluctuated within a stable bandwidth. Tinto *et al*<sup>[13]</sup> also reported that between 1979 and 1999 the age-standardized mortality rate broadly remained unchanged in England. Merely, the mortality rate for males exceeded the female rate. The mortality rate for persons of 75 years of age and older increased most promptly. Generally, the survival rate for patients with CP is poor<sup>[15,16,34,35]</sup>. CP patients tend to die of other causes such as smoking related cancers, cardiovascular disease and alcoholic liver cirrhosis.

Both incidence and mortality rates over time are reported for Dutch and international standard populations. On average, the reported rates are the lowest for the WHO standard population, followed by the European and the Dutch standard populations, which clearly reflects their different age distributions. In addition, an ageing effect was observed during the study period, particularly for male incidence rates. The ageing of the Dutch population will most likely continue for another decade, for which reason an increase in patient burden and a rise in health care costs can be anticipated.

Our study has several potential limitations. We defined an incident case during any specific year as a person with an admission that was not preceded by another admission of the same person with the same discharge diagnosis during at least five years prior to the specified year. So, an admitted, incident case in 2000 had no previous admission during 1995-1999 (five years). Further, an admitted, incident case in 2002 had no previous admission in 1995-2001 (seven years). It would have been a contradiction to allow a person to be classified as an incident case in 2002, if he had already been admitted once before in 1996 for the same reason. This analytical approach however may have led to a downward pressure on the incidence rates in later years (near 2005) compared to the earlier years (near 2000). Hence, growth patterns may be slightly underestimated.

Another limitation is that we did not identify incident CP cases among the persons already identified as incident AP cases. Presumably, this resulted in only a minimal underestimation of the incidence rate of CP. A recent study showed that CP developed in alcoholic AP cases with a

cumulative incidence of just 13% in 10 years<sup>[36]</sup>. Only, in patients with a recurrent alcoholic AP, the incidence of CP at 2 years after initial relapse was 38%. Unfortunately, we do not have sufficient data about the etiology of AP and CP in our study population.

Another issue concerns the reliability of the reported incidence rates which depends heavily on the HDR as a reliable source for hospital discharge data concerning AP and CP.

Eland *et al*<sup>[2]</sup> performed, as a part of a retrospective study in which incidence rates of AP in the Netherlands were assessed, a restricted validation analysis. They concluded that the overreporting due to miscoding and the underreporting due to non-coding were comparable and that observed incidence rates seem to reflect true rates.

Previously, we retrospectively analyzed the reliability of hospital discharge data of in total 483 admissions for both AP and CP collected in the HDR. We observed a substantial miscoding and non-coding of discharge diagnoses of AP and CP on the level of individual hospital admissions, ultimately leading to a limited underestimation at group level of the total number of AP and CP diagnoses of 15.8% and 6% respectively<sup>[37]</sup>.

Furthermore, there is a potential underestimation of the incidence rates due to non-referral of AP and CP patients. For the present study, we retrieved the AP and CP hospital admissions (> 1 d of hospital stay) from the HDR. Underestimation due to non-referral is probably limited for AP, because in the Netherlands almost all AP patients are admitted to a hospital for more than one day<sup>[22]</sup>. Whether or not this also holds for CP patients in the Netherlands is unknown. Generally, some CP patients, especially in the early stage of the disease, are only treated in an outpatient clinic setting and treated in a single-day hospital admission. So, by excluding the single-day admissions in our study, the underestimation will be of greater importance for CP compared to the AP. Historical volume data from the forerunner of the HDR (the earlier mentioned NISHC) on the use of hospital resources by CP patients suggest that approximately 6% of all admissions are single-day admissions. Considering that individual CP patients frequently need multiple single-day admissions, once under such treatment, it is likely that at the person count level, the degree of underestimation is even less than 6%.

In conclusion, we observed an increase in the incidence rate of AP and a fluctuating incidence rate of CP between 2000 and 2005 in the Netherlands. On average, the mortality rate for both AP and CP remained fairly stable between 1995-2005. Both incidence and mortality rates increase markedly by age and are lower for international standard populations. Therefore, in light of the continuing ageing of the Dutch population, patient burden and health care costs will most probably increase.

## COMMENTS

### Background

Over the last decades, the incidence and number of hospital admissions of

both acute pancreatitis (AP) and chronic pancreatitis (CP) have consistently increased in the Western countries. AP and CP are associated with significant morbidity and mortality and a substantial use of health care resources.

### Research frontiers

Large scale epidemiological studies reporting time trends of incidence and mortality of CP are strikingly scarce compared to the also limited epidemiological studies on AP. Mostly, national registries are used in isolation for epidemiological studies on AP and CP. Joint application of these registries by record linkage at the level of the individual patients provides a unique opportunity for improving the accuracy of epidemiological data.

### Innovations and breakthroughs

By following linkage of three distinct nationwide Dutch registries, the authors report in a more valid way the incidence and mortality rates of AP and CP (now for the first time) in the Netherlands. Standardizations were performed with reference to age distribution of the European and World Health Organization standard populations to enable direct comparisons among trends in incidence and mortality rates across countries that differ by age and sex distributions of their respective populations.

### Applications

The incidence rates of AP steadily increased while the incidence of CP fluctuated. Population mortality for both AP and CP remained fairly stable. Both, incidence and mortality rates increased markedly with age. In ageing populations, it is to be expected that patient burden and health care costs will increase.

### Terminology

The disease spectrum of AP ranges from mild and self-limiting to a life-threatening illness. CP is characterized by ongoing or recurrent episodes of abdominal pain accompanied by progressive pancreatic exocrine and endocrine insufficiency. Main etiological factors are gallstones and alcohol abuse.

### Peer review

This is a descriptive study of the trends in incidence and mortality of AP and CP from three linked nationwide Dutch registries for the years 1995-2005. The databases included the hospital discharge registry, the population registry, and the death certificate registry. The authors discuss the limitations of each database and the methods to link them. Given these limitations, major findings included the observation that the incidence of AP increased in both males and females, but the mortality rates remained stable. The incidence and mortality of AP and CP increased with age, which has implications for future incidence in an aging population.

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P- Reviewers Ho SB, Kamisawa T S- Editor Gou SX  
L- Editor A E- Editor Li JY



## Matrix metalloproteinase-9 in the initial injury after hepatectomy in mice

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Supported by Partially by Grants to Nguyen JH from the Deason Foundation (Sandra and Eugene Davenport, Mayo Clinic CD CRT-II), the AHA (0655589B) and NIH (R01NS051646-01A2); and the Grant to Hori T from the Uehara Memorial Foundation (200940051)

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Received: December 17, 2012 Revised: January 7, 2013

Accepted: February 5, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To investigate the role of matrix metalloproteinase (MMP)-9 in the pathogenesis of postoperative liver failure (PLF) after extended hepatectomy (EH).

**METHODS:** An insufficient volume of the remnant

liver (RL) results in higher morbidity and mortality, and a murine model with 80%-hepatectomy was used. All investigations were performed 6 h after EH. Mice were first divided into two groups based on the postoperative course (*i.e.*, the PLF caused or did not), and MMP-9 expression was measured by Western blotting. The source of MMP-9 was then determined by immunohistological stainings. Tissue inhibitor of metalloproteinase (TIMP)-1 is the endogenous inhibitor of MMP-9, and MMP-9 behavior was assessed by the experiments in wild-type, MMP-9(-/-) and TIMP-1(-/-) mice by Western blotting and gelatin zymography. The behavior of neutrophils was also assessed by immunohistological stainings. An anti-MMP-9 monoclonal antibody and a broad-spectrum MMP inhibitor were used to examine the role of MMP-9.

**RESULTS:** Symptomatic mice showed more severe PLF (histopathological assessments:  $2.97 \pm 0.92$  vs  $0.11 \pm 0.08$ ,  $P < 0.05$ ) and a higher expression of MMP-9 ( $71085 \pm 18274$  vs  $192856 \pm 22263$ ,  $P < 0.01$ ). Non-native leukocytes appeared to be the main source of MMP-9, because MMP-9 expression corresponding with CD11b positive-cell was observed in the findings of immunohistological stainings. In the histopathological findings, the PLF was improved in MMP-9(-/-) mice ( $1.65\% \pm 0.23\%$  vs  $0.65\% \pm 0.19\%$ ,  $P < 0.01$ ) and it was worse in TIMP-1(-/-) mice ( $1.65\% \pm 0.23\%$  vs  $1.78\% \pm 0.31\%$ ,  $P < 0.01$ ). Moreover, neutrophil migration was disturbed in MMP-9(-/-) mice in the immunohistological stainings. Two methods of MMP-9 inhibition revealed reduced PLF, and neutrophil migration was strongly disturbed in MMP-9-blocked mice in the histopathological assessments ( $9.6 \pm 1.9$  vs  $4.2 \pm 1.2$ ,  $P < 0.05$ , and  $9.9 \pm 1.5$  vs  $5.7 \pm 1.1$ ,  $P < 0.05$ ).

**CONCLUSION:** MMP-9 is important for the process of PLF. The initial injury is associated with MMP-9 derived from neutrophils, and MMP-9 blockade reduces PLF. MMP-9 may be a potential target to prevent PLF after

EH and to overcome an insufficient RL.

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**Key words:** Matrix metalloproteinase; Shear stress; Sinusoidal injury; Hepatectomy; Portal hypertension

Ohashi N, Hori T, Chen F, Jermanus S, Nakao A, Uemoto S, Nguyen JH. Matrix metalloproteinase-9 in the initial injury after hepatectomy in mice. *World J Gastroenterol* 2013; 19(20): 3027-3042 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3027.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3027>

## INTRODUCTION

Liver resection is considered the standard treatment for primary malignant tumors and liver metastases. Currently, advanced surgical techniques for hepatectomy, development of preoperative evaluation and improvements in intensive postoperative care have resulted in a decline in perioperative morbidity and mortality. However, postoperative liver failure (PLF) still occurs despite these developments. Extended hepatectomy (EH) has the advantage of high curability, but increases morbidity and mortality compared with more limited resections<sup>[1]</sup>. The volume of the remnant liver (RL) is correlated with perioperative morbidity and mortality<sup>[1]</sup>. PLF is also related to the patient's condition<sup>[2]</sup>. The rationale for PLF is the overall recovery course after EH. Prognosis of PLF in insufficient RL after EH is poor<sup>[1,2]</sup>.

The mechanism of progressive PLF after EH is not fully understood. The main pathway of failure of liver regeneration is still controversial, and it is unclear how excessive apoptosis or progressive necrosis is attributable to insufficient RL after EH. Matrix metalloproteinases (MMPs) are a family of zinc-containing neutral proteases that are capable of degrading the extracellular matrix and basement membrane. Among MMPs, MMP-9, also known as gelatinase B, is well characterized<sup>[3-5]</sup>. Expression of MMP-9 is reported to be associated with several pathophysiological conditions, such as rheumatoid arthritis<sup>[6]</sup>, atherosclerosis<sup>[6]</sup>, encephalopathy<sup>[7]</sup> and tumor invasion<sup>[8,9]</sup>. Recent studies have demonstrated that MMP-9 plays a pivotal role in ischemia/reperfusion injury in the liver transplantation field<sup>[4,10]</sup>. We hypothesized that MMP-9 plays a role in the pathogenesis of PLF after EH accompanied by postoperative shear stress due to portal hypertension and insufficient RL, and we previously reported the results of our preliminary study<sup>[11]</sup>. In this study, we examined the initial pathway of necrosis in the PLF process and MMP-9 expression in the early phase after EH by using an experimental mice model. In addition, we evaluated the efficacy of MMP-9 blockade on initial injury in the liver by deleting MMP-9 in the mouse, using a monoclonal antibody and a broad spectrum MMP inhibitor.

## MATERIALS AND METHODS

### Animals

Male C57BL/6 mice [wild-type (WT), 10-14 wk old] purchased from Jackson Laboratory (Bar Harbor, ME), were housed in a conventional mouse room with a 12-h light/dark cycle, food, and water. MMP-9(-/-) mice, which were a gift from Dr. Roberts Senior (Washington University, St. Louis, MO), and tissue inhibitor of metalloproteinase (TIMP)-1(-/-) mice, which were purchased from Jackson Laboratory were used. Both knock-out strains had the background of C57/BL/6, and all mice were bred in our secure animal facility. All experimental protocols were approved by the ethical committee of the Mayo Clinic (Protocol No. IACUC 24907) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### Eighty percent-partial hepatectomy in mice

Surgical procedures for the murine hepatectomy model are well established, and our method has been described in detail elsewhere<sup>[12]</sup>. For 80%-partial hepatectomy (PH), the left posterior, left and right anterior, and right posterior lobes were resected. In brief, under general anesthesia using isoflurane, liver lobes were mobilized after laparotomy. A hemostatic clip (Teleflex Medical, Triangle Park, NC) was applied across the pedicle at the base of the liver lobes instead of ligation, and liver lobes were cut distal to the applied clip. Only laparotomy was performed as the sham control group. Before abdominal closure, 2 mL of warm saline was administered intraperitoneally. Cephalexin (30 mg/kg) and buprenorphine (0.1 mg/kg) were given subcutaneously. To preserve the same quality throughout the series, all procedures for presented data were performed only by Ohashi N. To confirm the presented data, Hori T repeated this study including 90%-PH. Postoperatively, mice were housed under a controlled temperature, humidity, and light with free access to food and water.

First, we performed 90%-PH in 50 WT mice, and all mice died at the early postoperative period. In this study, we employed 80%-PH as the relevant clinical model. Ninety-percent-PH showed greater damage in the preliminary study, but we considered that 10%-RL was clinically irrelevant. Paradoxically, some survivors without any symptoms were observed after 80%-PH.

We then performed 80%-PH in 38 WT mice and sham surgery in 12 WT mice, to evaluate the postoperative course. In the following experiments, 80%-PH was performed with 20 age-matched WT mice, 19 MMP-9(-/-) mice and 20 TIMP-1(-/-) mice.

Focal and/or patchy necrosis is an important finding after PH<sup>[13-16]</sup>, and progressive necrosis is found from the early postoperative period after EH<sup>[13,14,16]</sup>. In this study, all of the mice were sacrificed at 6 h after 80%-PH. The RL was harvested and divided for histological analysis (formalin and frozen fixation) and protein analysis (snap frozen at -80 °C). Serum samples were collected and sep-

arated by using Microtainer tubes (BD, Franklin Lakes, NJ).

### **MMP-9 inhibition experiment using anti-MMP-9 monoclonal antibody and the broad-spectrum MMP inhibitor GM6001**

The WT mice were treated with 3 mg/kg of anti-MMP-9 neutralizing monoclonal antibody (clone 6-6B, EMD, Gibbstown, NJ) by intravenous injection 1 h before 80%-PH (MMP-9 mAb group,  $n = 6$ ). In the control mice, the same volume of non-immunized murine IgG of the same isotype (EMD, Gibbstown, NJ) was injected in the same manner (control IgG group,  $n = 6$ ). In another experiment, a broad spectrum MMP-inhibitor, GM6001 (Millipore, Billerica, MA) (100 mg/kg), diluted in 10% dimethyl sulfoxide (DMSO) was administrated intraperitoneally 2 h before 80%-PH (GM6001 group,  $n = 10$ ). Ten percent DMSO was injected in the control mice in the same manner as for GM6001 (vehicle group,  $n = 10$ ).

### **Biochemical analysis**

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined by a commercially available kinetic detection kit (Pointe Scientific, INC, Canton, MI), and total bilirubin (T-Bil) levels were determined by the QuantiChrom™ Bilirubin Assay Kit (BioAssay Systems, Hayward, CA).

### **Western blotting analysis**

Liver samples were homogenized in a buffer containing 10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton-X, 0.1% sodium dodecyl sulfate (SDS), 1 mmol/L ethylene diamine tetra-acetic acid (EDTA), 1 mmol/L ethylene glycol tetra-acetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, and protease and phosphatase inhibitors. Homogenates were centrifuged at 105000  $g$  for 1 h at 4 °C. Supernatants were collected and protein concentration was determined by BCA assay (Pierce, Rockford, IL). Forty micrograms of protein was separated *via* SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in TBS-T [20 mmol/L Tris (pH 7.4), 500 mmol/L NaCl, and 0.05% Tween-20] and probed using an antibody for MMP-9 (R and D, Minneapolis, MN), and then they were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by enhanced chemi-luminescence (ECL) or ECL-plus reagent (Amersham Biosciences, Piscataway, NJ). Equal loading was confirmed by immunoblotting using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (IMGENEX, San Diego, CA) on the same membrane. Signals were quantified using the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

### **Gelatin zymography**

The RL extracts were analyzed by gelatin zymography

with affinity chromatography to characterize gelatinase activity. In brief, 400  $\mu$ g of extract samples were incubated with 100  $\mu$ L of Gelatin-Sepharose 4B (GE Healthcare) and equilibrated buffer containing 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 5 mmol/L CaCl<sub>2</sub>, 0.02% Tween-20, and 10 mmol/L EDTA for 2 h at 4 °C. After multiple washing, gelatin-Sepharose beads were resuspended in the same volume of 2X zymography sample buffer (Bio-Rad Laboratories, Hercules, CA) and loaded on 10% SDS-PAGE gels containing 1 mg/mL of gelatin (Bio-Rad Laboratories). After electrophoresis, the gel was washed with 2.5% Triton X-100 for renaturing twice for 30 min, and it was then incubated in development buffer (Bio-Rad Laboratories) for 20 h at 37 °C. After incubation, the gel was fixed and stained with 0.5% Coomassie Blue R-250 (Bio-Rad Laboratories) for 1 h and destained with 10% acetic acid in 40%-methanol solution. Gelatinase zymography standards (Millipore, Billerica, MA) were used for the positive control.

### **Histology and immunohistochemical staining**

Formalin-fixed liver specimens were embedded in paraffin, and 5- $\mu$ m sections were stained with hematoxylin and eosin (HE). Immunohistochemical (IHC) staining for CD11b (MAC-1) and CD68 was performed on frozen sections (5  $\mu$ m), while paraffin sections were used for desmin, myeloperoxidase (MPO) and MMP-9 single staining. Antigen retrieval heating with citric acid (pH 6.0) was performed after deparaffinization with paraffin sections. For horseradish peroxidase based staining with 3,3' diaminobenzidine tetrahydrochloride (DAB) (DAKO, Carpinteria, CA), 0.3% H<sub>2</sub>O<sub>2</sub> was added to quench endogenous peroxidase activity, and an ABC kit (Vector Laboratories Inc, Burlingame, CA) was used during the staining procedure according to the manufacturer's instructions. For dual staining, sections were blocked with 5% bovine serum albumin (BSA) in PBS. The antibodies for MMP-9 (AF909), intercellular adhesion molecule 1 (ICAM-1) (AF796), CD11b (clone M1/70) (R and D, Minneapolis, MN), CD68 (clone FA-11), desmin (Abcam, Cambridge, MA), and MPO (Ab-1) (Thermo scientific, Fremont, CA) were incubated for the primary reaction. Alexa Fluor 568 donkey anti-goat IgG (H + L), Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 488 donkey anti-rat antibody (Invitrogen, Carlsbad, CA) were incubated for the secondary reaction. In each experiment, BSA solutions without antibodies were applied for a negative control.

### **Histological analysis**

The HE sections were digitally scanned with the Scanscope XT system and analyzed with Aperio Imagescope software (Aperio Technologies, Inc., Vista, CA). Histological areas were blindly counted and measured with three randomly selected fields approximately 3 mm<sup>2</sup> in each section. The positivity of DAB staining for MMP-9 was automatically calculated using the positive pixel count function with Imagescope software with 10 ran-

domly selected fields with the reference to the presence of necrosis, respectively. The value was indicated by the percentage of positive pixels out of the total pixels. The number of infiltrated granulocytes was evaluated in five randomly-captured 40 high power fields in each section with an Olympus BX50 fluorescence microscope (Olympus Optical, Tokyo, Japan).

### *In situ* zymography

*In situ* gelatinolytic activity was performed on cryostat sections (20- $\mu$ m thickness) using the EnzChek Gelatinase assay kit (Molecular Probes, Eugene, Oregon, United States). Sections were incubated with 20  $\mu$ g/mL fluorescent conjugated gelatin (DQ gelatin) in reaction buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L CaCl<sub>2</sub>, and 0.2 mmol/L sodium azide) for 2 h at 37 °C. After being washed with PBS three times, they were fixed in 4% paraformaldehyde in PBS. We incubated sections with GM6001 (100  $\mu$ mol/L) prior to DQ gelatin incubation as a negative control in each experiment. Sections were mounted with Vectashield (Vector Laboratories Inc, Burlingame, CA). Gelatinase activity was visualized using fluorescent microscopy (Olympus BX50, Japan).

### Statistical analysis

Data are presented as the mean  $\pm$  SE. Statistical comparisons were performed using ANOVA followed by the two-sample *t*-test with Bonferroni adjustment. A *P* value  $<$  0.05 was considered statistically significant.

## RESULTS

### *Necrosis is a pivotal event for PLF in the RL after 80%-PH*

Murine behavior in hepatic failure has been well described<sup>[17,18]</sup>. Mice with intrahepatic-hemorrhage and necrosis were observed to be sick and inactive, whereas mice without such injury were asymptomatic and active. We classified these mice into groups based on these findings as follows below. Mice were divided into two groups; asymptomatic (Group I) and symptomatic (Group II).

We performed 80%-PH in 38 WT mice and sham surgery in 12 WT mice to compare the difference in the RL with or without PLF at several time points after 80%-PH. Among these mice, we randomly selected five asymptomatic mice which were active, nimble and reactive to stimulation, and six symptomatic mice which were sick and inactive, slow-moving and sluggish 6 h after 80%-PH. As shown in Figure 1A and B, multiple necrotic foci with microhemorrhage in the middle zone were observed in the symptomatic mice, while a small amount of necrosis was observed in the asymptomatic mice. These HE findings are consistent with a previous report<sup>[19]</sup>. We measured the area of necrosis as an objective marker of liver injury. The area of necrosis ( $2.97\% \pm 0.92\%$  vs  $0.11\% \pm 0.08\%$ ,  $P < 0.05$ ) (Figure 1C) and the number of necrotic foci ( $3.60 \pm 0.87/\text{mm}^2$  vs  $0.26 \pm 0.17/\text{mm}^2$ ,  $P < 0.01$ ) in the RL were significantly larger in symptomatic mice than those in asymptomatic mice (Figure 1D). Serum levels of AST

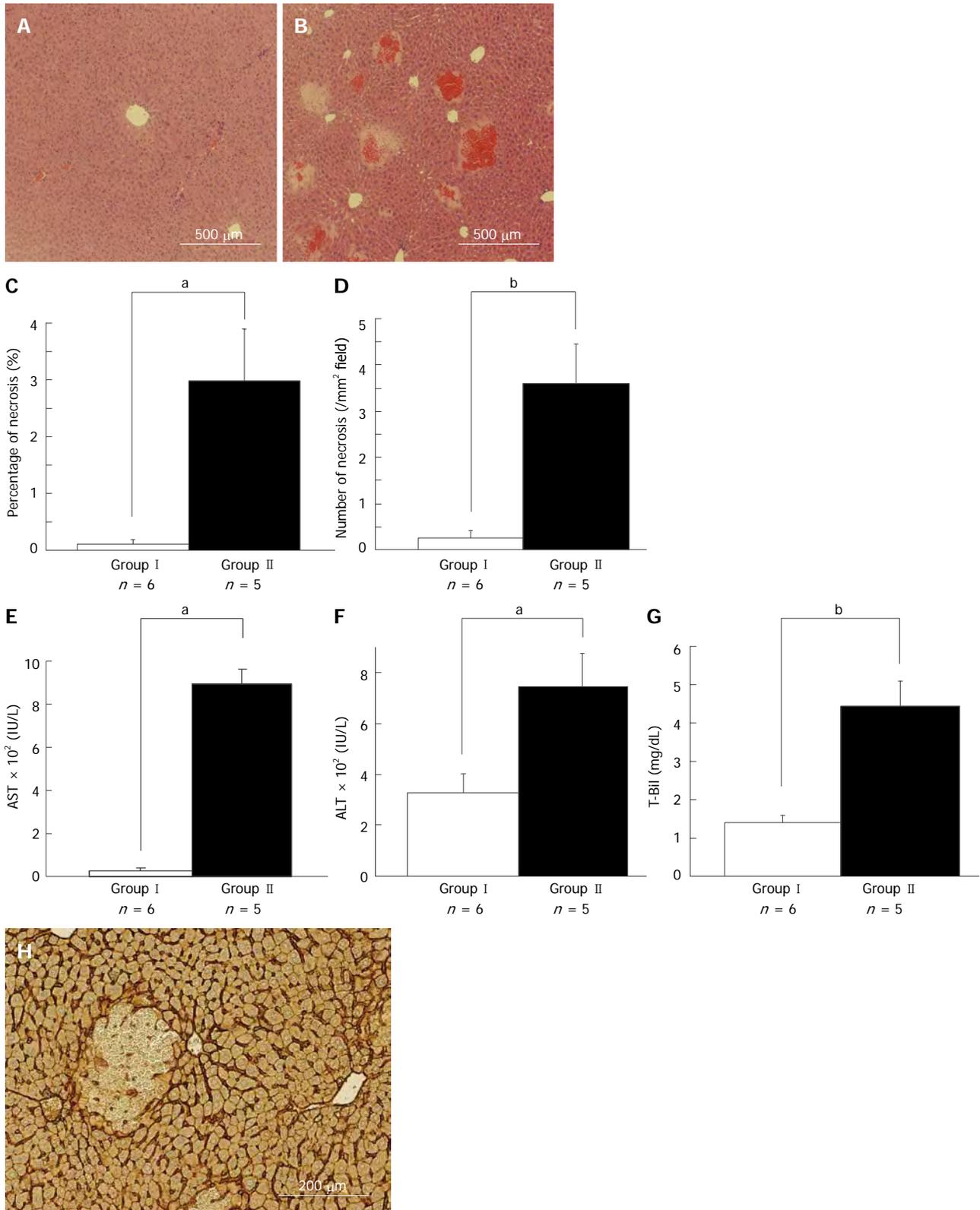
( $785 \pm 15$  IU/L vs  $452 \pm 39$  IU/L,  $P < 0.01$ ) (Figure 1E), ALT ( $744 \pm 135$  IU/L vs  $328 \pm 77$  IU/L,  $P < 0.05$ ) (Figure 1F), and T-Bil ( $4.45 \pm 0.63$  mg/dL vs  $1.41 \pm 0.19$  mg/dL,  $P < 0.01$ ) (Figure 1G) were significantly higher in symptomatic mice than those in asymptomatic mice. These results clearly indicated that symptomatic mice were in the process of PLF as shown by the increase in AST and ALT levels and hyperbilirubinemia. In some mice, which failed to recover even 6 h after 80%-PH, multiple necroses might have been responsible for the process of PSL in this model. Enhanced necrosis was observed in the RL at the late phase (12 h or later, data not shown), and these preliminary data confirmed that the necrotic pathway is progressive. In addition, intrahepatic-hemorrhage was observed in most of the necrotic lesions, which suggested the possibility that the initial sinusoid endothelial cell injury leading to sinusoidal breakdown is attributable to necrosis in the RL. In our model, dehiscence of sinusoids and loss of continuity of endothelial cells were observed in ICAM-1 immunostaining, which was strongly positive in endothelial cells (Figure 1H).

We performed retrospective analysis of perioperative factors including the resected liver weight/body weight and surgery time with 11 mice whose prognosis was predictable. Perioperative factors were not different between asymptomatic and symptomatic mice, but the resected liver weight/body weight ratio in the symptomatic mice tended to be larger than that in the asymptomatic mice ( $3.8\% \pm 0.1\%$  vs  $3.5\% \pm 0.1\%$ ,  $P = 0.06$ ). It is possible that the extent of hepatectomy is an important factor for postoperative liver injury after EH.

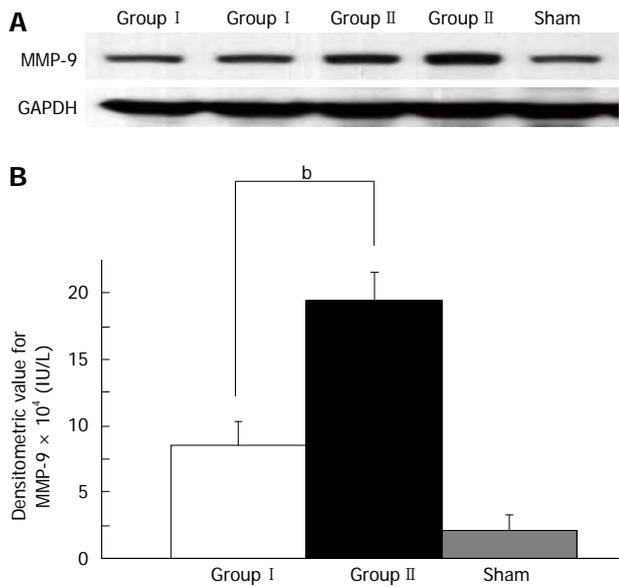
Macro- and microscopically, we confirmed the preservation of vascularization in the RL, immediately after 80%-PH by sodium fluorescent perfusion via the portal vein and abdominal aorta, to exclude model specific effects including surgical issues. In addition, we observed similar histological findings after 80%-PH with the traditional suture ligation method<sup>[13]</sup> (data not shown). These results demonstrated that the process of PSL after EH was widely and histologically characterized by RL necrosis, and was probably initialized as early onset sinusoid breakdown following progressive necrosis of hepatocytes.

### *MMP-9 expression is enhanced in RL dysfunction 6 h after 80%-PH and is associated with liver necrosis*

We compared MMP-9 expression in the RL between asymptomatic mice and symptomatic mice at 6 h. Enhanced MMP-9 expression was confirmed in symptomatic mice after EH compared with that in the asymptomatic mice after EH and sham surgery mice as shown by Western blotting analysis ( $P < 0.01$ ) (Figure 2). IHC showed that MMP-9 expression was mainly observed in round-shaped cells in the liver parenchyma and necrotic areas, and was less common in stellate-shaped cells. A small amount of MMP-9 expression was observed in hepatocytes compared with the above-mentioned cells (Figure 3A and B). To evaluate the association of necrosis and MMP-9 in liver tissue, we compared the expression level of MMP-9 between 10 randomly selected fields (3 mm<sup>2</sup>)



**Figure 1** Necrosis is a pivotal event for postoperative liver failure in the remnant liver after 80%-partial hepatectomy. There were histological and hematochemical differences between asymptomatic and symptomatic mice suspected as having postoperative liver failure 6 h after 80%-partial hepatectomy (PH) (A, B). Representative images of the remnant liver (RL) 6 h after 80%-PH in asymptomatic mice (A) and symptomatic mice (B) are shown. Significant multiple necroses with microhemorrhage were observed in the middle zone in symptomatic mice (B), while no obvious liver damage was observed in asymptomatic mice (A). The percentage area of necrosis (C) and the number of necrosis in each mm<sup>2</sup> (D) in the RL were confirmed. Necrotic areas were significantly larger and more prevalent in symptomatic mice liver compared with asymptomatic mice. Serum levels of aspartate aminotransferase (AST) (E), alanine aminotransferase (ALT) (F) and total bilirubin (T-Bil) (G) 6 h after 80%-PH are shown. AST, ALT and T-Bil levels were significantly elevated in symptomatic mice compared with those in asymptomatic mice. Representative image of immunohistochemistry of intercellular adhesion molecule-1 (ICAM-1) in the RL 6 h after 80%-PH is shown (H). ICAM-1 expression was clearly observed, particularly in the sinusoid lining in areas without necrosis. We observed a breakdown in sinusoid structure with hemorrhage in necrotic areas after 80%-PH in mice, <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs asymptomatic mice.



**Figure 2** Western blotting analyses for matrix metalloproteinase-9 were performed in the remnant liver in asymptomatic mice and symptomatic mice after 80%-partial hepatectomy. Representative image (A) and histograms (B) of Western blotting analyses for matrix metalloproteinase-9 (MMP-9) are shown. MMP-9 protein expression in the liver was enhanced in symptomatic mice in the process of postoperative liver failure compared with that in asymptomatic mice after extended hepatectomy and sham-treated mice (<sup>b</sup>*P* < 0.01 between Groups I and II).

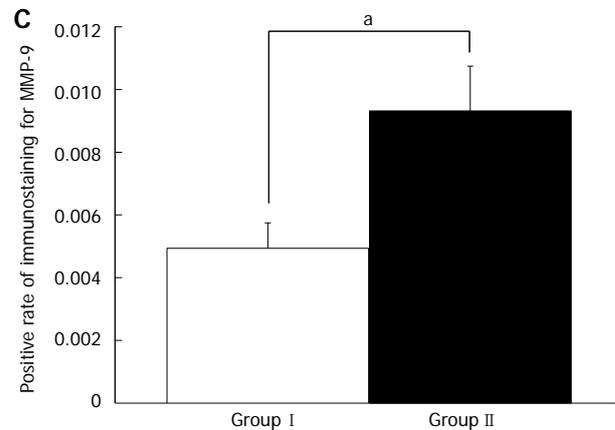
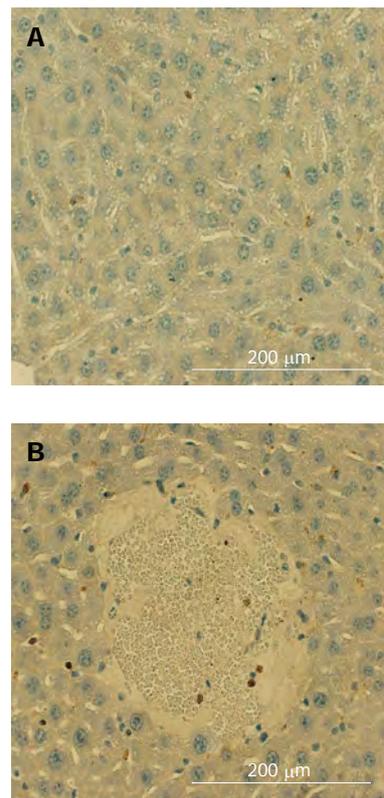
of areas with necrosis and those without necrosis in IHC sections. Enhanced MMP-9 expression was observed in the areas including necrosis compared with those without necrosis (*P* < 0.05, Figure 3C). This result suggested that MMP-9 expression is associated with necrosis in the RL after EH.

**Main source of MMP-9 is CD11b-positive nonnative leukocytes in the RL 6 h after 80%-PH**

To determine the source of MMP-9, we performed dual immunofluorescent analysis with MMP-9 and various cell marker antibodies in the liver. CD68 and desmin were used as specific markers for Kupffer cells and hepatic stellate cells in immunostaining, respectively. MMP-9 expression was not observed in CD68-positive Kupffer cells, while MMP-9 expression was partially confirmed in desmin-positive hepatic stellate cells (Figure 4A-F). In addition, we clearly detected MMP-9 expression corresponding with CD11b positive-cells, which are generally expressed in nonnative leukocytes in the liver including monocytes, granulocytes and macrophages (Figure 4G-I). These results suggested that MMP-9 was expressed in the RL 6 h after 80%-PH, and it appeared to be mainly produced by leukocytes that had migrated from the outside of the liver, similar to that described in a liver ischemia/reperfusion injury model<sup>[20]</sup>.

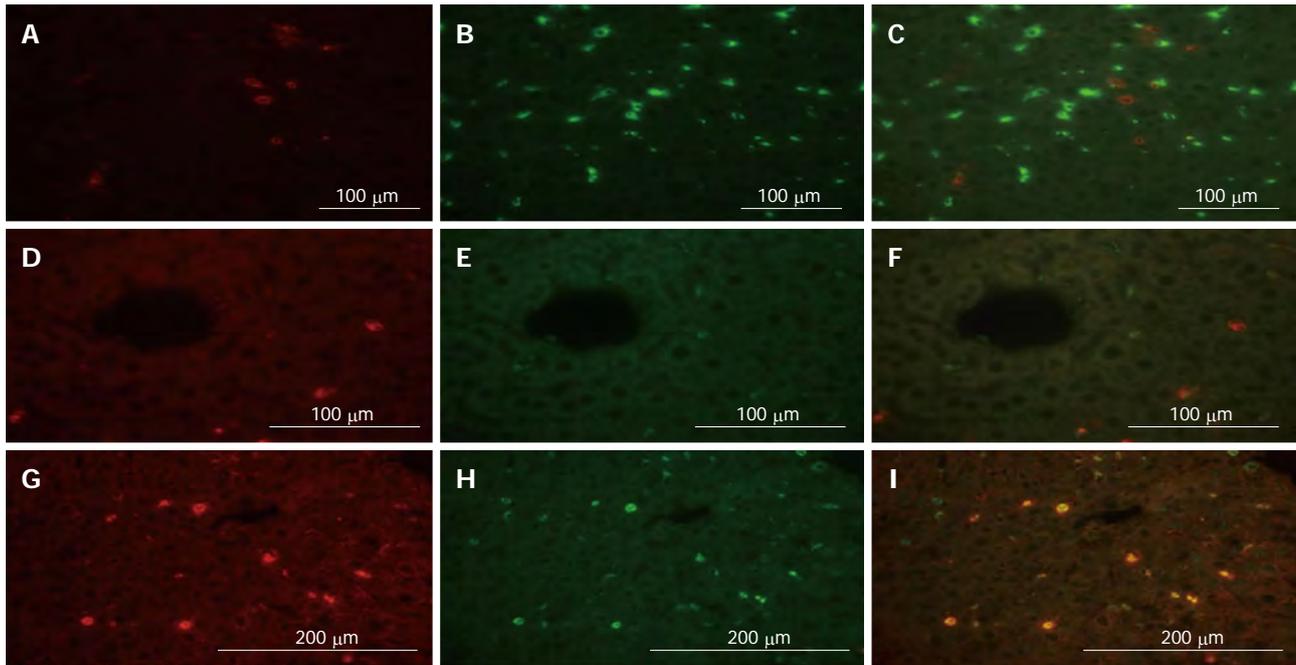
**MMP-9 deletion ameliorates initial injury in the RL 6 h after 80%-PH**

TIMP-1 is an endogenous inhibitor of MMP-9. We performed 80%-PH in 20 WT, 19 MMP-9(-/-) and 20



**Figure 3** Immunohistochemical analysis for matrix metalloproteinase-9 in the remnant liver in symptomatic mice after 80%-partial hepatectomy was performed. A: Representative images of matrix metalloproteinase-9 (MMP-9) staining in an area without necrosis; B: A necrotic area in the same sample are shown. Histograms of the results of distribution analysis show MMP-9 expression; C: Enhanced expression was observed in the areas close to necrosis compared with areas without necrosis in damaged RL (<sup>a</sup>*P* < 0.05 between Groups I and II).

TIMP-1(-/-) mice to compare the difference in the RL 6 h after 80%-PH. Among these mice, we randomly selected samples from 15 WT, 15 MMP-9(-/-) and 14 TIMP-1(-/-) mice. To evaluate the effects of MMP-9 on the initial injury in the RL, we compared the area (percentage) of necrosis and the number of necrotic foci in the RL among these samples. There were significantly fewer and smaller necrotic lesions in MMP-9(-/-) mice compared with WT mice and TIMP-1(-/-) mice (area, WT: 1.65% ± 0.23%; MMP-9(-/-): 0.65% ± 0.19%;



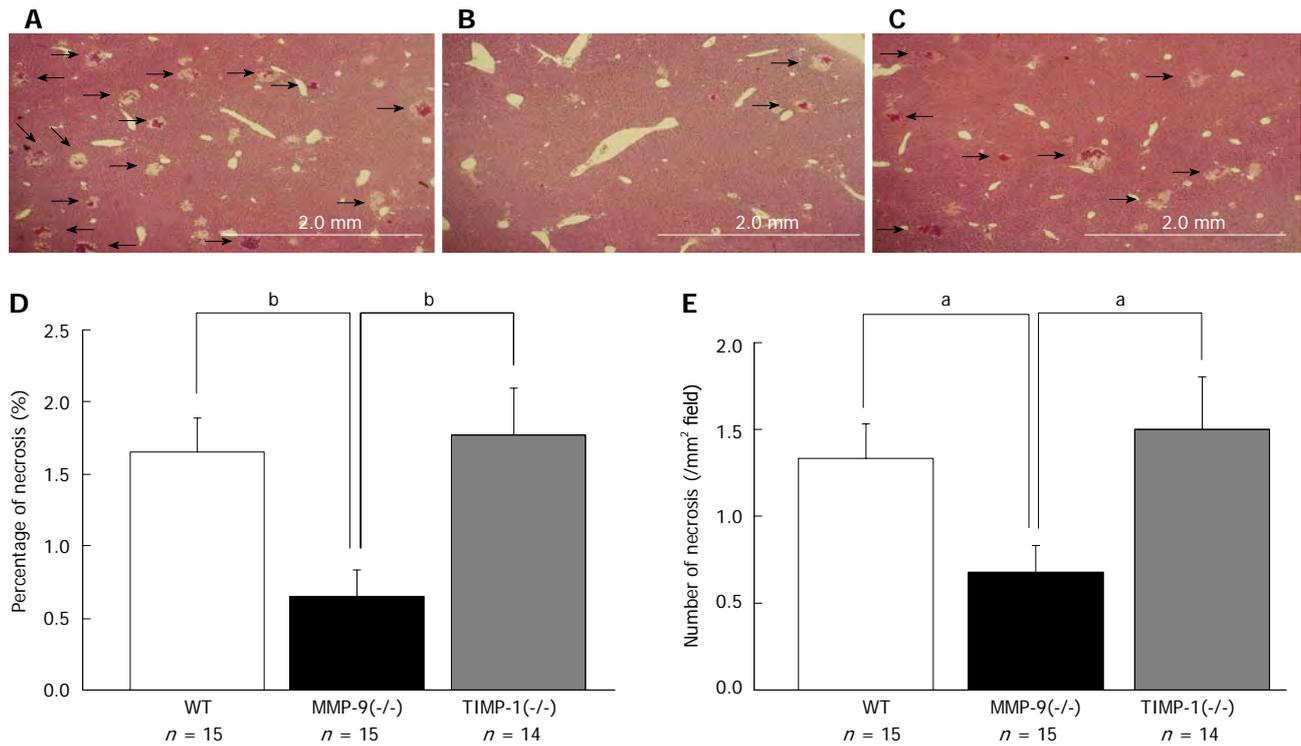
**Figure 4** Localization analysis of matrix metalloproteinase-9 by dual immune-fluorescence in the remnant liver after 80%-partial hepatectomy is shown. A: Fluorescent microscopy images of matrix metalloproteinase-9 (MMP-9) labeled in red (Alexa Fluor 568); B: CD68 labeled in green (Alexa Fluor 488); C: A merged image of MMP-9 and CD68 staining are shown; D: MMP-9 labeled in red (Alexa Fluor 568); E: Desmin labeled in green (Alexa Fluor 488); and F: A merged image of MMP-9 and desmin are shown; G: MMP-9 labeled in red (Alexa Fluor 568); H: CD11b labeled in green (Alexa Fluor 488); I: A merged image of MMP-9 and CD11b are shown. Protein expression of MMP-9 was mainly localized in CD11b-positive cells and desmin-positive cells to some degree.

TIMP-1(-/-):  $1.78\% \pm 0.31\%$ ; MMP-9(-/-) *vs* WT:  $P < 0.01$ ; MMP-9(-/-) *vs* TIMP-1(-/-):  $P < 0.01$ , (number of necrotic foci, WT:  $1.34 \pm 0.20/\text{mm}^2$ ; MMP-9(-/-):  $0.68 \pm 0.16/\text{mm}^2$ ; TIMP-1(-/-):  $1.50 \pm 0.30/\text{mm}^2$ ; MMP-9(-/-) *vs* WT:  $P < 0.05$ ; MMP-9(-/-) *vs* TIMP-1(-/-):  $P < 0.05$ ) (Figure 5). There was no significant difference in necrosis between WT and TIMP-1(-/-) mice. The levels of AST (WT:  $520 \pm 69$  IU/L; MMP-9(-/-):  $482 \pm 76$  IU/L; TIMP-1(-/-):  $636 \pm 58$  IU/L) and ALT (WT:  $544 \pm 73$  IU/L; MMP-9(-/-):  $466 \pm 92$  IU/L; TIMP-1(-/-):  $566 \pm 54$  IU/L) were lowest in MMP-9(-/-) mice, but this was not statistically significant among the groups. Additionally, T-Bil levels showed a similar tendency (WT:  $2.72 \pm 0.36$  mg/dL; MMP-9(-/-):  $2.23 \pm 0.27$  mg/dL; TIMP-1(-/-):  $2.76 \pm 0.28$  mg/dL). In Western blotting and gelatin zymography analysis, MMP-9 deletion of protein expression and activity in MMP-9(-/-) mice were confirmed. Protein expression of MMP-9 in WT mice was similar to that in TIMP-1(-/-) mice (Figure 6A). In gelatin zymography analysis, a distinct pro-form of MMP-9 and a slightly active form of MMP-9 were detected in WT and TIMP-1(-/-) mice (Figure 6B). No difference in MMP-9 activity was detected in the genotypes. Since there was no change in MMP bands among the groups because of the principle of protein separation by SDS-PAGE in zymography, we performed *in situ* gelatin zymography to examine the gelatinolytic activity *in situ*. We observed reduced gelatinolytic activity in MMP-9(-/-) mice and enhanced activity in TIMP-1(-/-) mice compared with that in WT mice (Figure 6C-E). MMP-9 deletion, which suppresses gelatinolytic activity, was associated with the amelioration

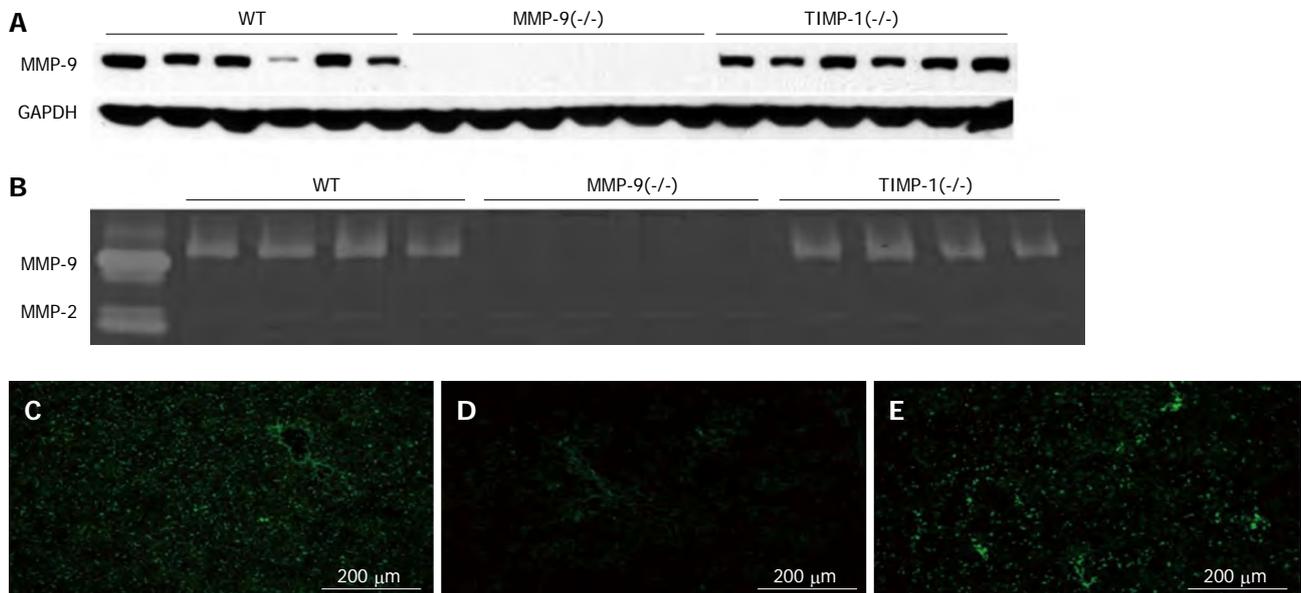
of formation of necrosis and necrotic progression in the RL after 80%-PH. However, TIMP-1 deletion, which induces the enhancement of gelatinolytic activity, did not exacerbate initial injury of the RL after EH. These results suggested that the involvement of TIMP-1 in inhibition of MMP activity appeared to be limited to PLF 6 h after EH. We investigated survival after 80%-PH was performed in WT, MMP-9(-/-) and TIMP-1(-/-) mice, but a significant survival benefit was not observed by MMP-9 deletion ( $P \geq 0.05$ ).

#### **MMP-9 deletion inhibits the accumulation of neutrophils in the RL 6 h after 80%-PH**

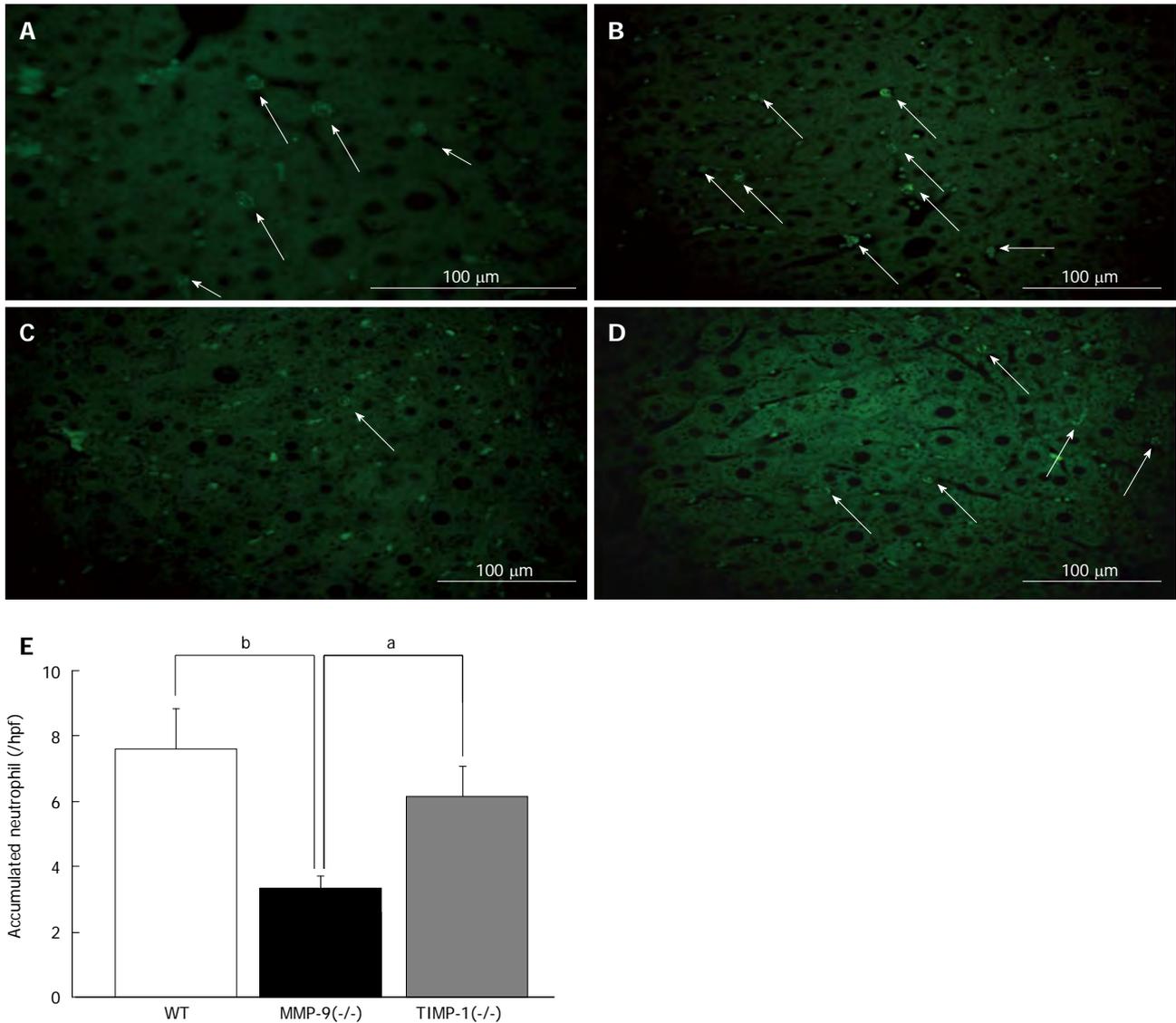
To evaluate the effect of MMP-9 deletion on cell kinetics, we investigated whether neutrophil migration after 80%-PH has an effect on the RL in WT, MMP-9(-/-) and TIMP-1(-/-) mice. We performed immunofluorescence staining for myeloperoxidase (MPO) to investigate this issue. As shown in Figure 7A, MPO-positive neutrophils could be easily recognized by characteristic staining patterns in cytoplasmic azurophilic granules, and MPO was also positive in liver tissue macrophages (Kupffer cells)<sup>[21]</sup>. Because of the difficulty in distinguishing between infiltrated neutrophils in the liver parenchyma and those that were adhered to the sinusoidal wall, accumulated neutrophils were counted in liver tissue. We observed significantly fewer neutrophils in MMP-9(-/-) mice ( $3.3 \pm 0.4$ ) than in WT ( $7.6 \pm 1.3$ ) and TIMP-1(-/-) mice ( $6.1 \pm 1.0$ ; each value per 40 high-power field; WT *vs* MMP-9(-/-),  $P < 0.01$ ; MMP-9(-/-) *vs* TIMP-1(-/-),  $P < 0.05$ ) (Figure 7B-E). There were no differences in ICAM-1 expression



**Figure 5** Histological analysis between wild-type, matrix metalloproteinase-9(-/-), and tissue inhibitor of metalloproteinase-1(-/-) mice 6 h after 80%-partial hepatectomy were performed. Representative images of the remnant liver (RL) 6 h after 80%-PH in wild-type (WT) (A), matrix metalloproteinase-9 (MMP-9)(-/-) (B), and tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice (C) are shown. Samples that included necrosis were selected for this figure. The percentage (D) and the number of necrotic foci per mm<sup>2</sup> (E) of the necrotic area in the RL are shown. We observed significantly smaller and less necrotic foci in MMP-9(-/-) mice compared with WT and TIMP-1(-/-) mice (<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs WT and TIMP-1).



**Figure 6** Matrix metalloproteinase-9 activity in the remnant liver with wild-type, matrix metalloproteinase-9(-/-), and tissue inhibitor of metalloproteinase-1(-/-) mice 6 h after 80%-partial hepatectomy is shown. A: Western blotting analysis for matrix metalloproteinase-9 (MMP-9) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for a loading control was performed. The expression of MMP-9 in wild-type (WT) and tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice and deletion in MMP-9(-/-) mice were observed at the protein level; B: Gelatin zymography analysis is shown; C-E: Pro-form MMP-9 activity and a slightly active form of MMP-9 were detected in WT and TIMP-1(-/-) mice. The activity of MMP-2 was the same depending on its genotype. *In situ* gelatin zymography analysis using DQ gelatin for gelatinolytic activity in the liver tissue is shown. A reduction in gelatinolytic activity in MMP-9(-/-) mice and enhancement in TIMP-1(-/-) mice are observed.



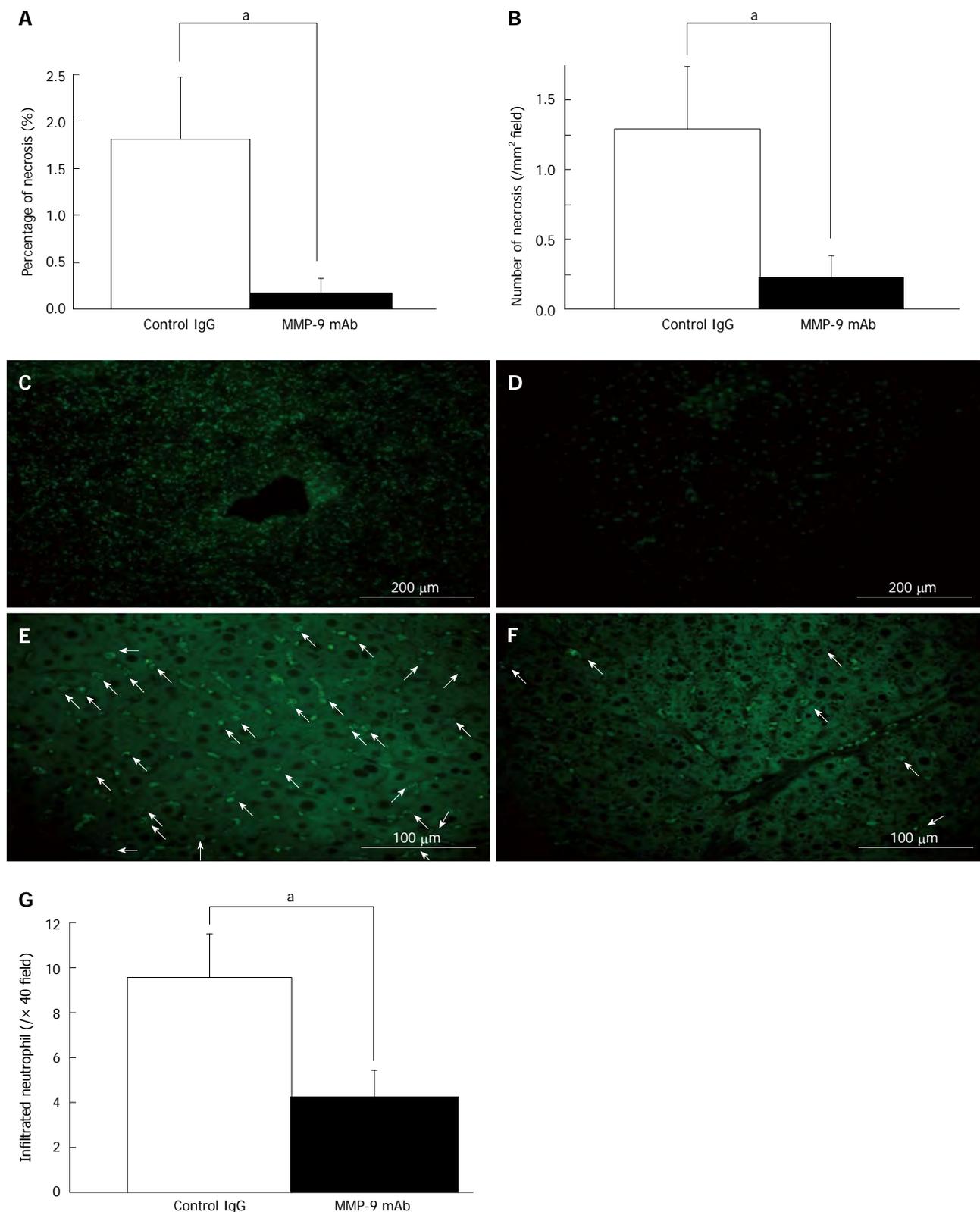
**Figure 7** Immunohistochemistry analysis for accumulated neutrophils with myeloperoxidase staining between wild-type, matrix metalloproteinase-9(-/-) and tissue inhibitor of metalloproteinase-1(-/-) mice 6 h after 80%-partial hepatectomy was performed. Myeloperoxidase (MPO) staining labeled in green (Alexa Fluor 488) is shown (A). Cytoplasmic azurophilic granules were characteristically stained in neutrophils. Representative images of MPO staining on a remnant liver (RL) section in green in wild-type (WT) (B), matrix metalloproteinase-9 (MMP-9)(-/-) (C), tissue inhibitor of metalloproteinase-1 (TIMP-1)(-/-) mice (D) are shown. A histogram of the number of accumulated neutrophils in the liver is shown. We observed significantly fewer neutrophils in the RL in MMP-9(-/-) mice than in WT and TIMP-1(-/-) mice (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs WT and TIMP-1(-/-) (E).

among WT, MMP-9(-/-) and TIMP-1(-/-) mice in Western blotting and IHC analysis (data not shown). These results suggested that MMP-9 deletion was associated with a decrease in migrated neutrophils in the liver, which was related to an amelioration of the initial injury in the RL after EH, and probably an infiltrating process seemed to be influenced. Therefore, MMP-9 might play a supportive role for infiltration of neutrophils into the RL at the early phase after EH.

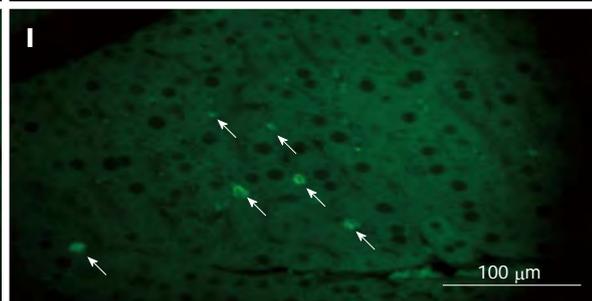
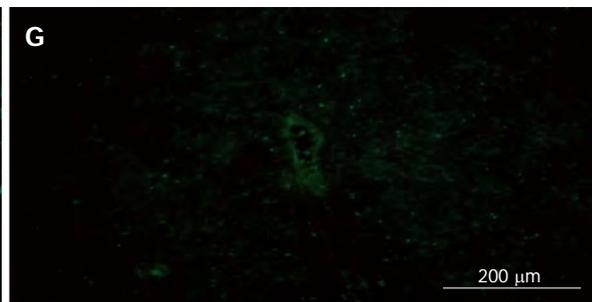
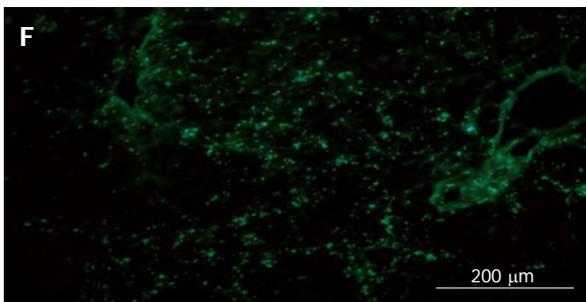
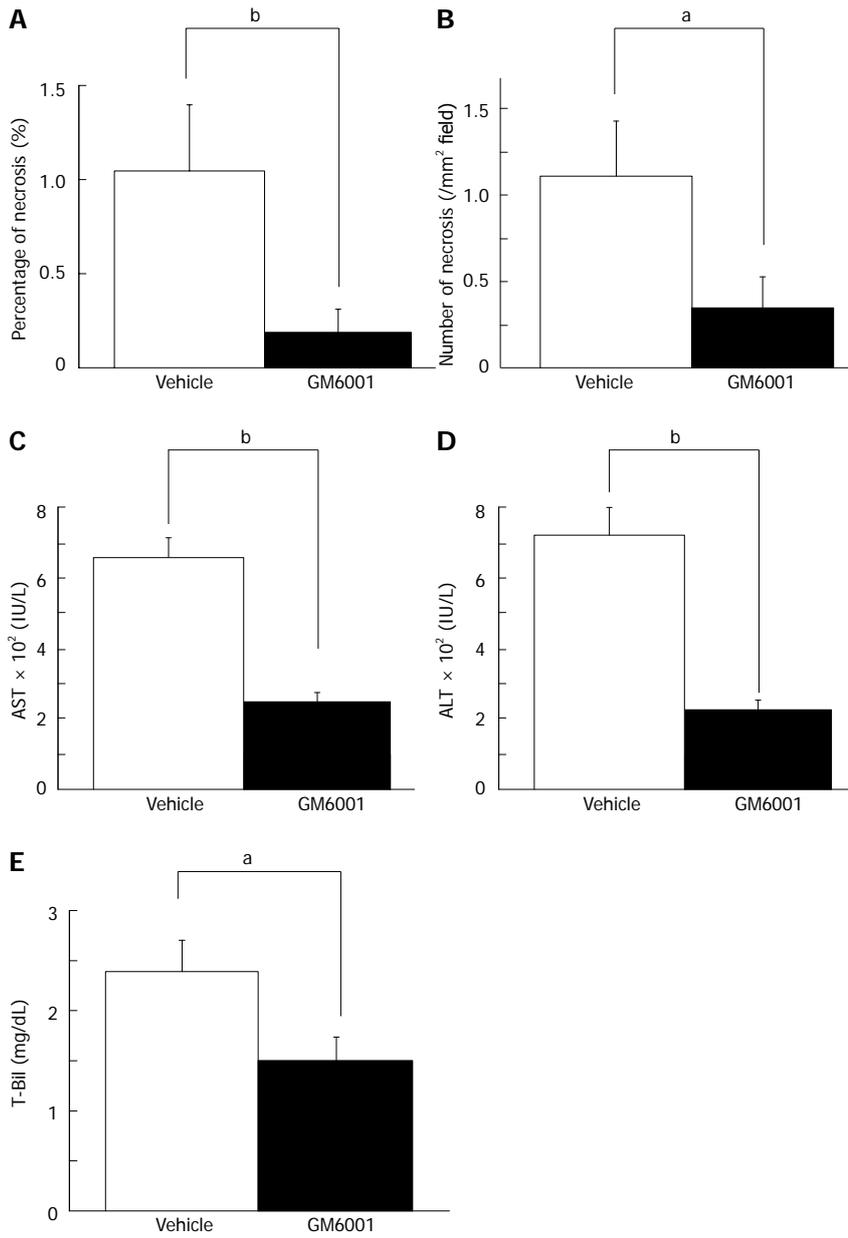
**Inhibition of MMP-9 by a monoclonal antibody of MMP-9 and the broad-spectrum MMP inhibitor GM6001 ameliorate initial injury of the liver 6 h after 80%-PH**

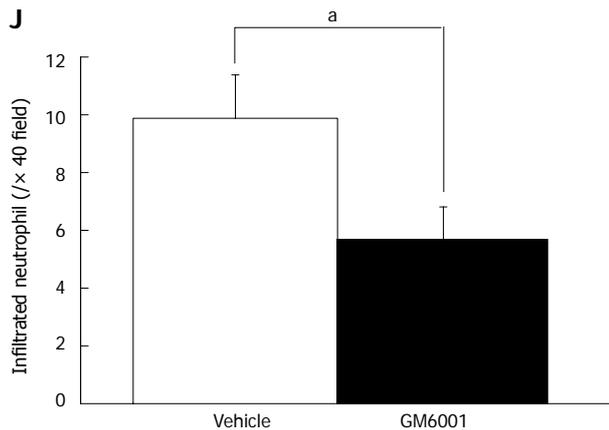
Investigation of inhibition of MMP-9 *in vivo* was performed by two different methods using a blocking

monoclonal antibody and a broad-spectrum MMP inhibitor (GM6001). Each treatment clearly improved survival curves after 80%-PH ( $P < 0.01$ ). In the RL, there were significantly smaller and fewer necrotic lesions in the monoclonal antibody-injected mice (mAb group) than in the control IgG-injected mice (control IgG group) (area:  $0.17\% \pm 0.15\%$  vs  $1.81\% \pm 0.66\%$ ,  $P < 0.05$ , Figure 8A; number of foci:  $0.23 \pm 0.16/\text{mm}^2$  vs  $1.23 \pm 0.44/\text{mm}^2$ ,  $P < 0.05$ , Figure 8B). Serum levels of AST, ALT and T-Bil were lower in the monoclonal antibody-injected mice than those in the control IgG-injected mice, but this not statistically significant ( $P \geq 0.05$ ). We confirmed suppression of gelatinolytic activity in the mAb group by *in situ* gelatin zymography compared with that in the control IgG group (Figure 8C and D). There were significantly



**Figure 8** Inhibition of matrix metalloproteinase-9 by a monoclonal antibody of matrix metalloproteinase-9. We investigated the outcome of inhibition of matrix metalloproteinase-9 (MMP-9) by monoclonal antibody (mAb) in 80%-partial hepatectomy (PH) in mice, and performed histological analysis of liver necrosis 6 h after 80%-PH (A, B). Liver damage was significantly reduced in the group with mAb compared with that in the control IgG group. Serum levels of aspartate aminotransferase, alanine aminotransferase and total bilirubin were lower in the mAb-treated mice, but this was not significant. Representative images of *in situ* gelatin zymography analysis with DQ gelatin revealed that gelatinolytic activity was suppressed in the mAb-treated mice (D) compared with that in control IgG-treated mice (C). Representative images of myeloperoxidase staining on remnant liver (RL) sections in control IgG-treated mice (E) and mAb-treated mice (F) are shown. A histogram of the number of accumulated neutrophils in the RL 6 h after 80%-PH shows that there are significantly fewer neutrophils in mAb-treated mice than in IgG-treated controls (G) ( $^*P < 0.05$  vs IgG group).





**Figure 9** Broad-spectrum matrix metalloproteinase-9 inhibitor GM6001 ameliorate initial injury of the liver 6 h after 80%-partial hepatectomy. We inhibited matrix metalloproteinase-9 by GM6001 in 80%-partial hepatectomy (PH) mice, and determined liver necrosis 6 h after 80%-PH. Liver damage was significantly reduced in the GM6001-treated mice compared with that in the vehicle-treated group (A, B). Serum levels of aspartate aminotransferase (AST) (C), alanine aminotransferase (ALT) (D) and total bilirubin (T-Bil) (E) were significantly lower in the GM6001-treated mice than those in the vehicle-treated mice. Representative images of *in situ* gelatin zymography analysis with DQ gelatin are shown. Gelatinolytic activity was suppressed in the GM6001-treated mice (G) compared with that in the vehicle-treated mice (F). Representative images of myeloperoxidase staining on remnant liver (RL) sections in vehicle-treated mice (H) and GM6001-treated mice (I) are shown. A histogram of the number of accumulated neutrophils in the RL after extended hepatectomy shows that there are significantly fewer neutrophils in the GM6001-treated mice than in the vehicle-treated mice (J) (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs vehicle-treated mice).

fewer accumulated neutrophils in the mAb group than in the control IgG group (mAb:  $4.2 \pm 1.2$  vs control IgG:  $9.6 \pm 1.9$ , each per 40 high-power field,  $P < 0.05$ ) (Figure 8E-G).

Similarly, the inhibition study by GM6001 showed a significant suppression in the area of liver necrosis ( $1.04\% \pm 0.36\%$  vs  $0.19\% \pm 0.13\%$ ,  $P < 0.01$ , Figure 9A) and the number of foci compared with the vehicle group ( $1.12 \pm 0.31/\text{mm}^2$  vs  $0.35 \pm 0.19/\text{mm}^2$ ,  $P < 0.05$ , Figure 9B). The levels of AST ( $660 \pm 66$  IU/L vs  $243 \pm 28$  IU/L,  $P < 0.01$ , Figure 9C), ALT ( $702 \pm 88$  IU/L vs  $212 \pm 33$  IU/L,  $P < 0.01$ , Figure 9D) and T-Bil ( $1.49 \pm 0.25$  mg/dL vs  $2.39 \pm 0.30$  mg/dL,  $P < 0.05$ , Figure 9E) were significantly lower in the GM6001 group than those in the vehicle group. *In situ* gelatin zymography analysis indicated inhibition of gelatinolytic activity in the GM6001 group (Figure 9F-I). Additionally, the accumulation of neutrophils was significantly suppressed in the GM6001 group compared with the vehicle group ( $9.9 \pm 1.5$  per 40 high-power field vs  $5.7 \pm 1.1$  per 40 high-power field,  $P < 0.05$ ) (Figure 9J). These results suggested that therapy with exogenous MMP-9 inhibition has the potential to reduce the initial injury of RL after EH.

## DISCUSSION

The mechanism of PLF is not fully understood, because of the involvement of various clinical perioperative factors, such as pre-existing liver disease, infection, transfusion, and insufficient volume of the RL. Murine and rat models have been used to determine the mechanisms for many years. Jin *et al*<sup>[15]</sup> showed necrosis of hepatocytes by oxidative injury after 87%-PH in mice. Catardegirmen *et al*<sup>[22]</sup> reported that blockage of the receptor for advanced glycation end products (RAGE) improves survival after 85%-PH and preserves liver parenchyma

with the absence of necrosis in 85%-PH. Yoshida *et al*<sup>[23]</sup> reported the efficiency of a caspase inhibitor for survival after 95%-PH in rats by attenuating apoptosis of hepatocytes. Both necrosis and apoptosis pathways appear to be involved in PLF process after EH. We previously performed marginal hepatectomy to evaluate the difference between predictive liver failure and normal recovery at the early phase. Use of hemoclips and a surgical microscope enabled us to complete the surgical procedure within 10 min and to stabilize the outcomes after surgery in our model<sup>[12]</sup>. In this previous study, a quick procedure appeared to reduce the opportunity for an infection or any complications<sup>[12]</sup>. In our preliminary study, histological changes from 80%-PH<sup>[12]</sup> were slightly improved compared with a conventional model by Higgins *et al*<sup>[24]</sup>. Our marginal PH model could be helpful for evaluation of the direct effects of insufficient volume of the RL after EH, from the viewpoint of the deletions of irrelevant factors.

With regard to the mechanism of liver failure, only a few studies have focused on the initial changes in the RL after EH. Panis *et al*<sup>[16]</sup> reported the presence of centrilobular necrosis, which varied in the severity in different areas of sections as well as a fatty change and microvascular steatosis 24 h after 85%-PH in the rat. Jin *et al*<sup>[15]</sup> reported severe sinusoidal narrowing as early as 6 h, and swelling, fatty degeneration and nuclear condensation of hepatocytes by 48 h after 87%-PH in asymptomatic mice<sup>[15]</sup>. In small-for-size grafts used in transplantation, a vacuolar cytoplasm or considerable mitochondrial swelling in hepatocytes, irregular large gaps between sinusoidal lining cells and collapse of the space of Disse have been observed<sup>[25]</sup>. Our study clearly showed multiple necroses with intrahepatic-hemorrhage in the RL, and biochemical results also revealed PLF at the early phase after 80%-PH in symptomatic mice. In-

terestingly, these histological changes were similar to the findings of a lipopolysaccharide/galactosamine-induced acute liver failure model<sup>[26]</sup>. Initial extravasation of red blood cells, sinusoidal congestion, destruction of parenchymal architecture and subsequent total destruction of micro-architecture with hemorrhage were reported 4 to 8 h after a challenge of galactosamine and lipopolysaccharide<sup>[26]</sup>. We also observed dehiscence of sinusoids 6 h after 80%-PH. The mechanism of sinusoidal cell injury is not fully understood. Many efforts after PH, such as hyperperfusion and shear stress due to portal hypertension, appear to have the potential to physically damage sinusoidal endothelial cells (SECs), and intensive decompression of the portal vein has recently been proposed as an intra-operative strategy for liver transplantation with small-for-size grafts<sup>[27]</sup>. We observed distention of the portal vein during 80%-PH. An increase in portal flow indicates an increased exposure of bacterial products or toxic agents from the gut for an insufficient RL after EH, and this condition may affect SECs. The development of SEC injury followed by parenchymal cell injury is considered as an important mechanism in monocrotaline-induced veno-occlusive disease or acetaminophen hepatotoxicity<sup>[28-30]</sup>, because SEC injury leads to hepatic microcirculatory dysfunction and hemorrhage<sup>[28,29]</sup>. SEC gap formation after treatment with galactosamine/endotoxin in mice has been reported to affect neutrophil extravasation and hemorrhage of liver parenchyma, and activated MMP is involved in SEC gap formation<sup>[31]</sup>. MMPs have been shown to be involved in several liver injury mechanisms including the fulminant liver failure model<sup>[32]</sup>, sinusoidal obstruction syndrome model<sup>[33]</sup> and hepatic ischemia/reperfusion injury model<sup>[20]</sup>. Olle *et al.*<sup>[34]</sup> have reported the importance of MMP-9 associated with tumor necrosis factor, hepatocyte growth factor, vascular endothelial growth factor and the apoptosis pathway for liver regeneration. Mohammed *et al.*<sup>[35]</sup> showed that TIMP-1 affects the hepatocyte cell cycle in the liver regeneration process. These findings indicate MMPs' negative and positive potential in liver disease. A RL with considerable damage requires emergent regeneration for survival after EH. The role of MMPs appears to be more complex.

We demonstrated that MMP-9 expression was enhanced in the damaged liver associated with necrosis, and the main source of MMP-9 was CD11b-positive nonnative leukocytes and hepatic stellate cells 6 h after 80%-PH. Our results appear to be consistent with the ischemia/reperfusion injury model and acute liver failure model<sup>[26]</sup>. Kim *et al.*<sup>[36]</sup> showed initial MMP-9 expression at 3 h in only periportal hepatocytes after 70%-PH in the rat, and positive staining gradually spread for up to 48 h. In our 80%-PH model, there was little expression of MMP-9 in hepatocytes 6 h after surgery, and our results suggested that a severe situation after 80%-PH impairs the regular process for normal regeneration observed after 70%-PH<sup>[13,36]</sup>. Yan *et al.*<sup>[26]</sup> showed bimodal alteration of MMP-9 mRNA in the acute liver failure model. We observed a change in bimodal activity (the second peak

was after 12 h) by zymography after 80%-PH (data not shown). Taken together, these results suggest that there are two different modalities for MMP-9 production. Although we previously reported that MMP-9 plays an important role in the development of parenchymal hemorrhage and necrosis in the small remnant liver after massive hepatectomy and that successful MMP-9 inhibition attenuates the formation of hemorrhage and necrosis and might be a potential therapy to ameliorate liver injury<sup>[11]</sup>, the origin of MMP-9 production was unclear. We speculate that it is necessary to separately consider the roles of MMP-9 produced from leukocytes at the early phase and from hepatocytes at the late phase of EH.

In the current study, we focused on the initial MMP-9 expression and hypothesized that it plays a role in liver injury in the RL after EH. To examine the involvement of MMP-9 in initial liver injury after EH, we evaluated the effect of inhibition of MMP-9 using three different methods *in vivo* including MMP-9-deficient mice, an inhibitory monoclonal antibody and a broad spectrum inhibitor. In all of the MMP-9 inhibitory experiments, suppression of the initial injury was clearly observed with the inhibition of MMP-9. Our results suggested that MMP-9 plays a pivotal role in liver injury, especially in the formation of necrosis, likely sinusoidal injury after 80%-PH. Perioperative factors in each experiment were not different between each experimental group such as the resected volume, the surgical time and intra-operative blood loss. In the experiments with genetic deficient mice, we examined whether TIMP-1 deficiency had any effects on the theoretical enhancement of MMP-9 activity on liver injury simultaneously. We observed the reverse effects in TIMP-1 deficient mice compared with MMP-9-deficient mice with an increased gelatinolytic activity. No exacerbation of liver injury in the TIMP-1-deleted condition suggested insufficiency of TIMP-1 to regulate MMP-9 activity after EH. Mohammed *et al.*<sup>[35]</sup> showed that TIMP-1 was slightly increased 6 to 18 h after 70%-PH in the rat. We did not assess TIMP-1 expression in the current study. However, it is possible that TIMP-1 could be used as a therapeutic target to reduce MMP-9 levels related to liver injury.

In the current study, we did not observe any survival benefits of MMP-9 inhibition in MMP-9(-/-) mice, although the other two methods of inhibition of MMP-9 showed improvement of survival. MMP-9 was shown to be important in liver regeneration in PH by Olle *et al.*<sup>[34]</sup>. Additionally, the possibility of impairment of the host defense by MMP-9 deficiency was reported in a sepsis model by Renckens *et al.*<sup>[37]</sup>. Excessive MMP-9 activity induced by shear stress after PH may have the potential to induce initial liver injury. Application of MMP-9 blockade should be precisely controlled.

In this study, we evaluated the accumulation of neutrophils in the RL after 80%-PH. Areas of necrosis were correlated with the number of accumulated neutrophils. The infiltration of polymorphonuclear neutrophils (PMNs) has been reported in several liver diseases<sup>[38-41]</sup> and even after EH<sup>[42]</sup>. We could not identify infiltrated

neutrophils as a central effector on liver injury after 80%-PH, but a possible explanation may be provided by an association between neutrophils and SEC destruction. In addition, recent studies have shown that MMP-9 derived from neutrophils affects several pathophysiological conditions such as blood brain barrier breakdown after an ischemic stroke and gut barrier dysfunction in an acute pancreatitis model<sup>[43,44]</sup>. Keck *et al.*<sup>[45]</sup> showed that PMN-derived MMP-9 contributes to the accumulation of PMNs in the inflammation site and it initiates damage to the basement membrane. Although our results are consistent with the previous work described above, the mechanism of liver damage or regeneration is complicated. Periportal infiltration of inflammatory cells will increase the liver damage, but this infiltration is paradoxically necessary to trigger the liver regeneration<sup>[46-48]</sup>. Though the balance of damage and regeneration is still unclear, we speculated that the MMP-9 produced from neutrophil may be important factor for a successful liver regeneration after EH. Further study is needed to elucidate the involvement of MMP-9 associated with neutrophils in liver injury after EH with an insufficient RL.

In summary, our data showed that the pathway of liver necrosis related to initial sinusoidal endothelial cell injury is responsible for the PLF process after EH with an insufficient RL. The initial injury is associated with MMP-9 derived from neutrophils, and blockade of MMP-9 reduces initial liver injury in an *in vivo* model. Our results suggest that MMP-9 may be a potential target for preventive care or further challenging for EH.

## ACKNOWLEDGMENTS

We are grateful to Dennis W Dickson, Monica Castanedes-Casey, Virginia R Phillips, Linda G Rousseau and Melissa E Murray (Department of Neuroscience, Mayo Clinic in Florida, Jacksonville, FL 32224, United States) for their technical support with histopathological assessment.

## COMMENTS

### Background

Postoperative liver failure (PLF) after extended hepatectomy (EH) can cause an insufficient volume of the remnant liver (RL), resulting in higher morbidity and mortality.

### Research frontiers

The mechanism of liver damage or regeneration is complicated. Periportal infiltration of inflammatory cells will increase the liver damage, but this infiltration is paradoxically necessary to trigger the liver regeneration. This PLF is progressive from the early postoperative period, but the mechanism is unknown. Authors hypothesized that matrix metalloproteinase (MMP)-9 plays a role in PLF pathogenesis.

### Innovations and breakthroughs

Though the balance of damage and regeneration is still unclear in the RL after EH, the MMP-9 produced from neutrophil may be important factor for a successful liver regeneration after EH.

### Applications

Their data showed that the pathway of liver necrosis related to initial sinusoidal endothelial cell injury is responsible for the PLF process after EH. The initial injury is associated with MMP-9 derived from neutrophils, and blockade of

MMP-9 reduces initial liver injury.

### Terminology

Their results suggest that MMP-9 may be a potential target for preventive care or further challenging for EH.

### Peer review

In this manuscript, authors attempt to investigate that the effect of MMP9 in the initial injury after hepatectomy in mice. The work is of potential interest to the readership of the *World Journal of Gastroenterology*.

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P- Reviewer Lin JY S- Editor Gou SX L- Editor A  
E- Editor Zhang DN



## MGMT and MLH1 methylation in *Helicobacter pylori*-infected children and adults

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Supported by The Fundacao de Amparo a Pesquisa do Estado de Sao Paulo, No. 2009/01813-0 and 2008/02678-6; the Conselho Nacional de Desenvolvimento Científico e Tecnológico, No. 471088/2007-2

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Received: November 21, 2012 Revised: March 26, 2013

Accepted: April 9, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To evaluate the association between *Helicobacter pylori* (*H. pylori*) infection and *MLH1* and *MGMT* methylation and its relationship with microsatellite instability (MSI).

**METHODS:** The methylation status of the *MLH1* and

*MGMT* promoter region was analysed by methylation specific methylation-polymerase chain reaction (MSP-PCR) in gastric biopsy samples from uninfected or *H. pylori*-infected children ( $n = 50$ ), from adults with chronic gastritis ( $n = 97$ ) and from adults with gastric cancer ( $n = 92$ ). *MLH1* and *MGMT* mRNA expression were measured by real-time PCR and normalised to a constitutive gene ( $\beta$  actin). MSI analysis was performed by screening MSI markers at 4 loci (Bat-25, Bat-26, D17S250 and D2S123) with PCR; PCR products were analysed by single strand conformation polymorphism followed by silver staining. Statistical analyses were performed with either the  $\chi^2$  test with Yates continuity correction or Fisher's exact test, and statistical significance for expression analysis was assessed using an unpaired Student's *t*-test.

**RESULTS:** Methylation was not detected in the promoter regions of *MLH1* and *MGMT* in gastric biopsy samples from children, regardless of *H. pylori* infection status. The *MGMT* promoter was methylated in 51% of chronic gastritis adult patients and was associated with *H. pylori* infection ( $P < 0.05$ ); this region was methylated in 66% of gastric cancer patients, and the difference in the percentage of methylated samples between these patients and those from *H. pylori*-infected chronic gastritis patients was statistically significant ( $P < 0.05$ ). *MLH1* methylation frequencies among *H. pylori*-infected and non-infected chronic gastritis adult patients were 13% and 7%, respectively. We observed methylation of the *MLH1* promoter (39%) and increased MSI levels (68%) in samples from gastric cancer patients in comparison to samples from *H. pylori*-infected adult chronic gastritis patients ( $P < 0.001$  and  $P < 0.01$ , respectively). The frequency of promoter methylation for both genes was higher in gastric cancer samples than in *H. pylori*-positive chronic gastritis samples ( $P < 0.05$ ). The levels of *MLH1* and *MGMT* mRNA were significantly reduced in chronic gastritis samples that were also hypermethylated ( $P < 0.01$ ).

**CONCLUSION:** In summary, *MGMT* and *MLH1* methylation did not occur in earlier-stage *H. pylori* infections and thus might depend on the duration of infection.

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**Key words:** *Helicobacter pylori*; Microsatellite instability; Promoter methylation; *MLH1*; *MGMT*; Gastric cancer

**Core tip:** Gastric carcinogenesis is a multistep process that is triggered by *Helicobacter pylori* (*H. pylori*) infection and characterised by multiple genetic and epigenetic alterations, including the DNA repair genes. To date, few advances have been made to determine the time duration required for to *H. pylori* infection to induce such epigenetics alteration and thus potentially induce gastric carcinogenesis. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might depend on the duration of infection because these methylation events were not observed in children.

Alvarez MC, Santos JC, Maniezzo N, Ladeira MS, da Silva ALC, Scaletsky ICA, Pedrazzoli Jr J, Ribeiro ML. *MGMT* and *MLH1* methylation in *Helicobacter pylori*-infected children and adults. *World J Gastroenterol* 2013; 19(20): 3043-3051 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3043.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3043>

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is an important pathogen in the human stomach. The natural acquisition of *H. pylori* infection occurs mainly during childhood. Once established within the gastric mucosa, the infection persists for life if left untreated. The epidemiological evidence and the rare occurrence of peptic ulcers or gastric atrophy in children<sup>[1,2]</sup> suggest that *H. pylori*-related gastric mucosal damage might be progressive through childhood into adulthood. *H. pylori* infection elicits a host inflammatory response, including mucosal infiltration by polymorphonuclear leukocytes, macrophages, and T and B lymphocytes. The inflammatory response has a slow onset and becomes chronic after a long period of infection. Although the symptoms of chronic infection are not as severe as those of acute inflammation, the condition is persistent<sup>[3]</sup>. The activated inflammatory cells release reactive oxygen and nitrogen species that can induce DNA injury and cellular apoptosis<sup>[4]</sup>. The chronic colonisation of the stomach with *H. pylori* causes inflammation within the gastric mucosa and activates multiple oncogenic pathways<sup>[5]</sup>.

The interaction of *H. pylori* with the surface mucosa results in the increased release of pro-inflammatory cytokines<sup>[6]</sup> that exacerbate the inflammatory response. The persistence of this immune response leads to chronic inflammation, one of the factors associated with DNA

methylation. DNA methylation is one of the most important epigenetic modifications and primarily occurs on the cytosine residues of CpG dinucleotides, which are frequently clustered into CpG islands within the promoter regions of certain genes<sup>[7]</sup>. DNA methylation of these promoter regions inhibits transcription through chromatin structural changes that are mediated by the interactions of the methyl-cytosines with the protein complexes that recruit histone-modifying enzymes<sup>[8,9]</sup>.

Globally, gastric cancer is the fourth most common type of cancer and the second leading cause of cancer death, and 930000 new cases of gastric cancer are projected per year. South Korea, Japan and Eastern Asia have the highest incidences of gastric cancer, followed by Eastern Europe and Latin America<sup>[10]</sup>. Since the discovery of *H. pylori* by Warren *et al* in 1982<sup>[11]</sup>, many studies have demonstrated a strong association between *H. pylori* infection and the development of gastric cancer<sup>[12,13]</sup>. Moreover, in 1994, the International Agency for Research on Cancer recognised *H. pylori* as a definitive carcinogenic agent based on several epidemiological reports<sup>[14]</sup>. The primary mechanism by which *H. pylori* induces gastric cancer is thought to include the upregulation of several genes, including cytokines, oncogenes and growth factors, as well as the downregulation of tumour suppressor genes. These alterations in gene expression are believed to result from mutations and microsatellite instability<sup>[15]</sup>. Additionally, several studies have demonstrated a close association between *H. pylori* infection and aberrant CpG island methylation<sup>[16-18]</sup>.

Many critical genes are silenced by DNA methylation during cancer development. Recent studies have shown that the silencing of certain DNA repair genes by DNA methylation might be related to the occurrence of tumorigenic mutations. The expression levels of *MLH1*, a mismatch repair gene, are frequently altered in gastric cancers, and changes in *MLH1* expression can promote tumour development<sup>[19]</sup>. Additionally, O<sup>6</sup>-methylguanine DNA methyltransferase (*MGMT*) is a protein required for the repair of alkylated guanines in DNA that arise from exposures to environmental alkylation mutagens or through endogenous mechanisms. It has been reported that the loss of *MGMT* expression might increase the occurrence of genetic mutations and thus lead to gastric cancer development<sup>[20]</sup>. Additionally, Kitajima *et al*<sup>[21]</sup> reported that the loss of *MGMT* along with mismatch repair (MMR) proteins in gastric cancer patients is an important event in tumour progression.

Microsatellite instability (MSI) is one hallmark of DNA MMR deficiency that is observed in gastric carcinogenesis. Microsatellites are short DNA sequence repeats that are scattered throughout the human genome. Errors in the DNA MMR mechanisms of tumour cells can result in the expansion or contraction of these repeated sequences and thus MSI<sup>[22]</sup>. MSI occurs in nearly every case of gastric cancer that is associated with germline mutations of the MMR genes.

The inactivation of the MMR genes *MSH2* and

*MLH1*, either through a mutation or an epigenetic mechanism, is responsible for the development of MSI in gastric cancer. The aberrant loss of expression of either the *MLH1* or *MSH2* proteins has been observed in patients exhibiting gastric cancer with MSI. In particular, altered *MLH1* expression is associated with self-gene inactivation through the hypermethylation of its promoter regions<sup>[23,24]</sup>.

It is well established that *H. pylori* infection leads to chronic inflammation in the gastric mucosa, which is a condition associated with DNA methylation. Because such epigenetic alterations play an important role in the regulation of gene expression and the maintenance of DNA integrity and stability, the aim of this study was to analyse the effects of *H. pylori* infection on the methylation statuses of *MLH1* and *MGMT* and the relationship between the methylation of these promoters and microsatellite instability in *H. pylori*-infected or uninfected children as well as infected or uninfected adults with chronic gastritis or gastric cancer.

## MATERIALS AND METHODS

### Patients

This study was approved by the following institutions: the Ethical Committee of the Paulista Medical School, State University (UNESP), Botucatu, SP, Brazil; the Sao Francisco University, Braganca Paulista, SP, Brazil; the School of Medicine of the São Paulo University, SP, Brazil; and by the National Committee of Ethics in Research, Brasília, DF, Brazil. Informed consent to participate in the study was obtained from all patients.

This study included 239 patients, of which 50 were children between the ages of 2 and 18 years old (average age =  $8 \pm 4$  years; 47% male, 53% female) who suffered from dyspepsia. Of the remaining patients, 97 suffered from chronic gastritis (average age =  $35 \pm 13$  years; 33% male, 67% female), and 92 suffered from gastric cancer (average age =  $60 \pm 12$  years; 82% male, 18% female). All of the patients were non-smokers, did not abuse alcohol and were not using prescription or recreational drugs.

### Biopsy collection

Biopsies from patients with gastric complaints were obtained from the lesser curvature of the antrum (the distal region of the stomach) within 2 cm of the pyloric ring during endoscopies. For patients with gastric cancer, biopsies were obtained during gastric surgeries (to remove gastric carcinoma). One biopsy was used for a rapid urease test<sup>[25]</sup>, 2 were used for histopathological evaluations, and 1 was used for bacterial genotyping by polymerase chain reaction (PCR). *H. pylori* infection was considered present when positive results were obtained from all of the following tests: rapid urease test, histological analysis and gastric biopsy PCR. The patients were considered uninfected when negative results were obtained for all tests.

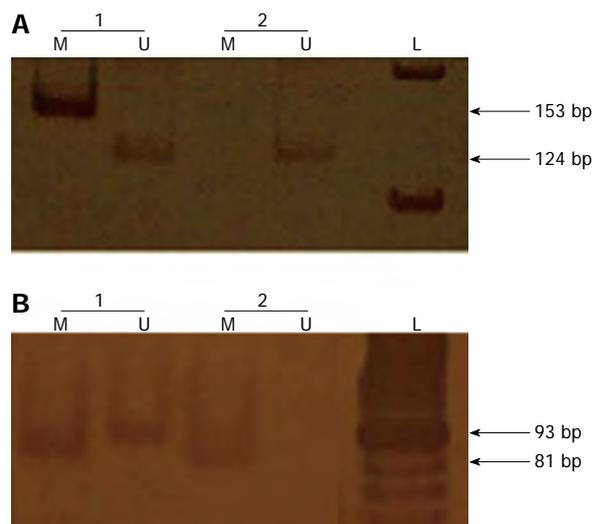


Figure 1 Example of the results from methylation-specific polymerase chain reaction analysis. A: *MLH1*; B: *MGMT*. Lane U: Amplified product with primers for unmethylated sequences; Lane M: Amplified product with primers for methylated sequences; L: Ladder.

### Histopathology

Gastric mucosal samples were fixed in 10% formalin for 24 h, dehydrated in alcohol and xylene, and embedded in paraffin. Sequential 3-5- $\mu$ m sections were cut and stained with haematoxylin-eosin for routine histology. Gastritis was classified according to Sydney's system<sup>[26]</sup>, and the presence of *H. pylori* was determined by carbolfuchsin staining of the sections.

### Bisulphite treatment and methylation-specific PCR

Bisulphite treatment was performed on 1  $\mu$ g of DNA with the EpiTect Bisulfite kit (Qiagen, Valencia, CA, United States). Methylation-specific PCR (MSP-PCR) was performed with a primer set specific to the methylated or unmethylated sequences (M or U sets, respectively)<sup>[27]</sup>. The PCR reactions were performed in a final volume of 25  $\mu$ L with approximately 200 ng of sodium bisulphite-treated DNA and 25 pmol of each primer. The PCR amplifications were performed for 40 cycles that each consisted of a denaturation step at 95  $^{\circ}$ C for 5 min, a primer-annealing step at 58  $^{\circ}$ C for 35 s and an extension step at 72  $^{\circ}$ C for 40 s, with a single final extension step at 72  $^{\circ}$ C for 7 min. The reaction products were separated by electrophoresis on 8% polyacrylamide gels and visualised by silver staining (Figure 1).

### RNA extraction and real-time PCR

The gastric biopsies were collected, snap frozen, and stored at -80  $^{\circ}$ C in RNAlater<sup>®</sup> (Qiagen, Valencia, CA, United States). Total RNA was isolated with the RNeasy tissue kit<sup>®</sup> (Qiagen). The gastric cancer biopsies were microdissected prior to RNA extraction. The PixCell<sup>®</sup> IIe Laser Capture Microdissection (LCM) System (Arc-turus Engineering, Mountain View, CA, United States)

**Table 1 Primers used for real-time polymerase chain reaction**

| Gene           | Primer    | Sequence (5'-3')        |
|----------------|-----------|-------------------------|
| <i>β-actin</i> | Sense     | ACACTGGCTCGTGTGACAAGG   |
|                | Antisense | CGGCTAATACACACTCCAAGGC  |
| <i>MGMT</i>    | Sense     | CACCACACTGGACAGCCCTTT   |
|                | Antisense | CGAACTTGCCAGGAGCTTTATTT |
| <i>MLH1</i>    | Sense     | CGGTAACTACCAATGCCTCAAC  |
|                | Antisense | TTCTCGACTAACAGCATTTC    |

was used to obtain laser captures using an amplitude of 50 mW, a duration of 800 ms and a 7.5-mm beam. RNA from the capture lids (Arcturus) that contained the microdissected tissue was extracted with the PicoPure RNA isolation kit (Arcturus). Single-stranded cDNA was synthesised from the RNA using the high capacity cDNA archive kit<sup>®</sup> (Applied Biosystems, Foster City, CA, United States) according to the manufacturer's protocol.

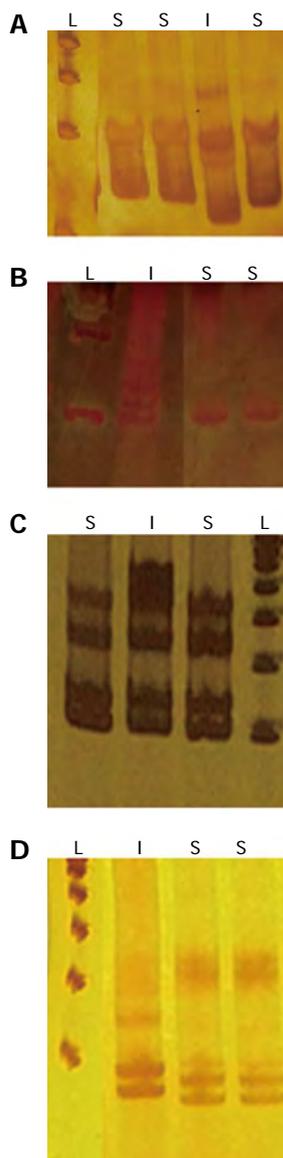
Quantitative PCR was performed on a 7300 real-time PCR system (Applied Biosystems) and the threshold cycle numbers were determined with the RQ Study software (Applied Biosystems). The reactions were run in triplicate and the threshold cycle numbers were averaged. The 50-μL reaction mixture was prepared as follows: 25 μL of Sybr Green<sup>®</sup> Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies, Alameda, CA, United States), 10 mmol/L of each primer (Table 1) and 10 μL of cDNA (100 ng). The reactions were performed with a preliminary UDG treatment for 2 min at 50 °C and denaturation for 2 min at 95 °C, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and primer extension at 72 °C for 15 s. This treatment was followed by a melting-point analysis of the double-stranded amplicons that consisted of 40 cycles of 1 °C decrement (15 s each), beginning at 95 °C. The first derivative of this plot, dF/dT, is the rate of change of fluorescence in the reaction. A significant change in fluorescence occurs at the melting point of the double-stranded PCR products. A plot of dF/dT versus temperature displays these changes as distinct peaks.

*MLH1* and *MGMT* gene expression was measured and normalised to a constitutive gene (*β-actin*). The relative expression was calculated according to the formula  $2^{-(\Delta\Delta C_t)[28]}$ , and the results are expressed as the average gene expression ± SD.

**MSI analysis**

All samples were analysed for 4 markers (BAT-25, BAT-26, D17S250 and D2S123) as recommended by the American National Cancer Institute (NCI) workshop on MSI<sup>[29]</sup>.

PCR was performed in a final volume of 25 μL with approximately 200 ng of template genomic DNA and 25 pmol of each primer. PCR amplifications were performed for 35 cycles that consisted of a denaturation step at 95 °C for 30 s, a primer-annealing step at 55-58 °C for 30 s and an extension step at 72 °C for 30 min, followed by a final single extension step at 72 °C for 10 min.



**Figure 2** Example of the results from polymerase chain reaction-single strand conformation polymorphism analysis. A: BAT25; B: BAT26; C: D2S123; D: D17S250. Lane S: Stable; Lane I: Instable; L: Ladder 10 bp.

Single strand conformation polymorphism (SSCP) analysis was performed on each sample. Briefly, 12 μL of each PCR product were mixed with 12 μL of denaturing buffer (95% Formamide, 0.05% Bromophenol blue, and 0.05% xlenocianol), denatured at 95 °C for 5 min and separated by electrophoresis on a non-denaturing 7% polyacrylamide gel for 3 h at room temperature. The single strands of the PCR products were visualised as bands by silver staining.

MSI was defined as a shift in the mobility of the DNA band from either allele or by the appearance of a new band (Figure 2). The MSI status was also confirmed by direct sequencing (data not shown). High-level instability (MSI-H) was defined by the presence of more than one instability marker. Low-level instability (MSI-L) was defined as the presence of a single instability marker. Finally, if no instability markers were present, the sample

**Table 2** Frequency of hypermethylation at the *MLH1* and *MGMT* promoter regions in chronic gastritis and gastric cancer patients *n* (%)

| Subjects                         | <i>MLH1</i>            |              | <i>MGMT</i>            |              |
|----------------------------------|------------------------|--------------|------------------------|--------------|
|                                  | Methylated             | Unmethylated | Methylated             | Unmethylated |
| Chronic gastritis                |                        |              |                        |              |
| Child <i>H. pylori</i> negative  | -                      | 28 (100%)    | -                      | 28 (100%)    |
| Child <i>H. pylori</i> positive  | -                      | 22 (100%)    | -                      | 22 (100%)    |
| Adults <i>H. pylori</i> negative | 1 (7%)                 | 13 (93%)     | 3 (21%)                | 11 (79%)     |
| Adults <i>H. pylori</i> positive | 11 (13%)               | 72 (87%)     | 42 (51%) <sup>c</sup>  | 41 (49%)     |
| Gastric cancer                   | 36 (39%) <sup>ab</sup> | 56 (61%)     | 61 (66%) <sup>de</sup> | 31 (34%)     |

<sup>a</sup>*P* < 0.05 vs *Helicobacter pylori* (*H. pylori*) negative; <sup>b</sup>*P* < 0.01 vs *H. pylori* positive chronic gastritis; <sup>c</sup>*P* < 0.05 vs *H. pylori* negative; <sup>e</sup>*P* < 0.05 vs *H. pylori* positive chronic gastritis and <sup>d</sup>*P* < 0.01 vs *H. pylori* negative chronic gastritis group.

was classified as displaying microsatellite stability (MSS)<sup>[29]</sup>.

### Statistical analysis

The association between microsatellite instability and the methylation pattern was evaluated with either the  $\chi^2$  test with Yates continuity correction or Fisher's exact test. Statistical significance for expression analysis was assessed by an unpaired Student's *t*-test. A *P* value of < 0.05 was considered statistically significant.

## RESULTS

The presence of *H. pylori* infection was analysed in biopsy specimens from 50 paediatric patients and 189 adult patients who underwent endoscopies. Infections were detected in 22 of the 50 (44%) children, 83 of the 97 (86%) adults with chronic gastritis and all (100%) of the 92 adult gastric cancer patients.

Methylation was not detected in the promoter regions of *MLH1* and *MGMT* in the paediatric samples. The samples from the chronic gastritis patients showed hypermethylation in the *MHL1* gene promoter region in 11 of the 83 (13%) samples from the *H. pylori*-infected subjects and in 1 of the 14 (7%) samples from the uninfected subjects (*P* > 0.05; Table 2). Methylation of the *MHL1* promoter region was observed in the samples from 36 of the 92 (39%) gastric cancer patients. The differences observed in the percentages of methylated samples are statistically significant when the patients with gastric cancer are compared to those with chronic gastritis, regardless of *H. pylori* infection status (*P* < 0.001; *P* < 0.05, respectively; Table 2).

In patients with chronic gastritis, *MGMT* promoter region methylation was observed in the biopsies from 42 of the 83 (51%) *H. pylori*-positive patients and 3 of the 14 (21%) *H. pylori*-negative patients (Table 2); this difference was statistically significant (*P* < 0.05). In the gastric cancer patients, *MGMT* promoter region methylation

**Table 3** Relative *MLH1* and *MGMT* mRNA expression levels

| Subjects                         | <i>MLH1</i>              |              | <i>MGMT</i>              |              |
|----------------------------------|--------------------------|--------------|--------------------------|--------------|
|                                  | Methylated               | Unmethylated | Methylated               | Unmethylated |
| Chronic gastritis                |                          |              |                          |              |
| Child <i>H. pylori</i> negative  | -                        | 0.25 ± 0.03  | -                        | 0.13 ± 0.08  |
| Child <i>H. pylori</i> positive  | -                        | 0.27 ± 0.02  | -                        | 0.12 ± 0.01  |
| Adults <i>H. pylori</i> negative | 0.35 ± 0.01 <sup>b</sup> | 0.85 ± 0.07  | 0.40 ± 0.16 <sup>b</sup> | 0.99 ± 0.21  |
| Adults <i>H. pylori</i> positive | 0.88 ± 0.10 <sup>b</sup> | 1.29 ± 0.27  | 0.79 ± 0.19 <sup>b</sup> | 1.78 ± 0.47  |
| Gastric cancer                   | 0.27 ± 0.07              | 0.25 ± 0.08  | 0.49 ± 0.10              | 0.46 ± 0.10  |

*MLH1* and *MGMT* mRNA values are expressed as the mean ± SD. <sup>b</sup>*P* < 0.01 vs unmethylated samples in the same subject category. *H. pylori*: *Helicobacter pylori*.

was observed in the biopsies from 61 of the 92 (66%) patients. The difference in the percentages of samples with *MGMT* promoter region methylation between the gastric cancer patients and the non-infected chronic gastritis patients was statistically significant (*P* < 0.01). Additionally, there was a statistically significant difference between the percentages of methylated samples from the gastric cancer patients and the *H. pylori*-infected chronic gastritis patients (*P* < 0.05). Furthermore, the frequency of promoter methylation for both genes was higher in the gastric cancer samples than in the *H. pylori*-positive chronic gastritis samples; this difference was statistically significant (*P* < 0.05).

The *MLH1* and *MGMT* gene expression levels were measured and evaluated in the context of promoter methylation. The levels of both *MLH1* and *MGMT* mRNA were significantly reduced in the methylated samples compared to the unmethylated samples in both the *H. pylori*-positive and uninfected adult chronic gastritis samples (Table 3). Overall, gastric cancer patients and paediatric patients had low levels of *MLH1* and *MGMT* mRNA expression.

Biopsy samples from children with chronic gastritis were relatively stable for the tested microsatellite markers. Only 2 samples (both *H. pylori*-negative) were scored as MSI-H, and the remaining paediatric samples were scored as MSS (stable; Table 4). MSI was present in 51 of the 83 (61%) *H. pylori*-positive chronic gastritis samples from adults. Of these, 37 (73%) samples were scored as MSI-L and 14 (27%) samples as MSI-H. MSI was observed in 9 of the 14 (64%) uninfected patients with gastritis; 6 (67%) samples were scored as MSI-L and 3 (33%) as MSI-H. MSI was observed in 63 of the 92 (68%) gastric cancer patients; 38 (60%) were scored as MSI-L and 25 (40%) as MSI-H. No significant association was found between *H. pylori* infection and MSI in the samples from chronic gastritis adult patients. However, there was a significant difference (*P* = 0.03) in the percentages of samples with MSI for between the gastric cancer patients and the *H. pylori*-infected chronic gastritis patients.

Various authors have postulated that the methyla-

**Table 4** Microsatellite instability status *n* (%)

| Subjects                         | Microsatellite instability status |         |                      |          |
|----------------------------------|-----------------------------------|---------|----------------------|----------|
|                                  | MSI-L                             | MSI-H   | MSI                  | MSS      |
| Chronic gastritis                |                                   |         |                      |          |
| Child <i>H. pylori</i> negative  | -                                 | 2 (7)   | 2 (7)                | 26 (93)  |
| Child <i>H. pylori</i> positive  | -                                 | -       | -                    | 22 (100) |
| Adults <i>H. pylori</i> negative | 6 (67)                            | 3 (33)  | 9 (64)               | 5 (36)   |
| Adults <i>H. pylori</i> positive | 37 (73)                           | 14 (27) | 51 (61)              | 32 (39)  |
| Gastric cancer                   | 38 (60)                           | 25 (40) | 63 (68) <sup>a</sup> | 29 (32)  |

MSI-H, the presence of more than one instability marker. MSI-L, the presence of a single instability marker. MSS, no instability markers were present. <sup>a</sup>*P* < 0.05 vs *Helicobacter pylori* (*H. pylori*)-positive chronic gastritis samples.

tion of the *MHL1* promoter region leads to the down-regulated expression of the *MHL1* gene, an effect that is strongly associated with MSI<sup>[23,24,30]</sup>. Therefore, we evaluated whether there was any association between the *MLH1* methylation status and MSI in our study population. The data presented in this study show a strong association between the *MLH1* methylation status and MSI in patients with gastric cancer (*P* < 0.01). However, we did not find any significant association between the *MLH1* methylation status and MSI in the samples from chronic gastritis adult patients, regardless of *H. pylori* infection status. Additionally, a multivariate analysis did not show that the *H. pylori* infection was associated with gene promoter methylation and MSI.

## DISCUSSION

Genomic DNA methylation is one of the most important epigenetic modifications in eukaryotes. DNA methylation is essential for life, and alterations of the methylation process are often associated with carcinogenesis related to chronic inflammation or persistent infections of viruses or other pathogenic microorganisms. In this setting, we evaluated the effects of *H. pylori* infection on the methylation patterns of the *MLH1* and *MGMT* promoter regions as well as the MSI statuses of paediatric and adult patients.

Children are an interesting natural model for *H. pylori* infection studies not only because they are not usually exposed to gastric mucosal irritants such as alcohol, tobacco, and anti-inflammatory medications but also because the gastric mucosal changes in children might represent an earlier stage of the inflammatory response when compared to those in adult hosts, due to the shorter duration of *H. pylori* infections in children. To our knowledge, this is the first study to evaluate the methylation patterns of DNA repair genes in paediatric samples. Our data showed an absence of the methylation in the *MLH1* and *MGMT* promoter regions and that the mRNA levels of both genes were similar in infected and uninfected children. Although our data did not indicate an effect of *H. pylori* infection on the methylation patterns of two DNA repair genes, a recent study reported an association

between *H. pylori* infection and the methylation patterns of 7 genes in paediatric samples; however, none of these genes were involved with DNA repair<sup>[31]</sup>. The methylation levels in these susceptible loci were increased in the adult samples when compared to the paediatric samples, suggesting that the altered methylation patterns might be related to the duration exposure to of *H. pylori*<sup>[31]</sup>. Accordingly, our data showed that the methylation rates were significantly higher in the samples from adults with chronic gastritis than in the samples from children with chronic gastritis.

Recently, it has been shown that prolonged bacterial infections lead to saturation of the repair capabilities of the host cells and thus to an ineffective and mutagenic DNA repair system<sup>[32]</sup>. Accordingly, it is believed that *H. pylori*-associated gastric mucosal damage might be progressive through childhood and into adulthood<sup>[1,2]</sup>. Taking this into account, our data suggest that *H. pylori* infection-mediated DNA methylation in adults may depend not only on the level of the inflammatory response but also on the persistence and duration of the infection.

Our data showed high levels of *MGMT* promoter region methylation in patients with *H. pylori*-positive chronic gastritis when compared to those with *H. pylori*-negative chronic gastritis, indicating that *MGMT* promoter methylation is significantly associated with *H. pylori* infection in chronic gastritis cases. Previous studies reported that *MGMT* CpG methylation is more frequent and extensive in *H. pylori*-infected versus uninfected patients<sup>[33]</sup>. Additionally, it has been suggested that the *MGMT* promoter methylation in *H. pylori*-infected patients is related to tumour progression<sup>[34,35]</sup>.

Our data showed no differences in *MLH1* promoter region methylation between patients with *H. pylori*-positive chronic gastritis and those with *H. pylori*-negative chronic gastritis. However, the methylation levels were higher in patients with gastric cancer than in patients with chronic gastritis. Similarly, it has been reported that *MLH1* promoter methylation occurs late in intestinal metaplasia development<sup>[36]</sup>. Taken together, our data suggest that *MLH1* methylation occurs late in the progression of gastric carcinoma and that methylation depends partly on the persistence of the *H. pylori* infection.

The data presented herein also showed that the methylation of the promoter regions significantly reduced the mRNA levels of both *MGMT* and *MLH1* in patients with chronic gastritis, regardless of *H. pylori* infection status. Conversely, no differences in mRNA levels were observed in gastric cancer samples, regardless of methylation status; this is likely consequent to other epigenetic and genetic mechanisms. Recently, it was reported that *H. pylori* infections in patients with gastritis were associated with *MGMT* hypermethylation and reduced levels of *MGMT* mRNA in the gastric epithelium<sup>[33]</sup>. Similar results were reported for *MLH1* in gastric carcinoma samples<sup>[19]</sup>. Therefore, it is possible that the hypermethylation of the *MLH1* promoter region leads to the reduced expression of its protein product. This phenomenon could permit

the accumulation of mutations due to the lack of surveillance and repair that are consequent to this deficiency in the DNA repair system. Ultimately, the deficient DNA repair process can be detected by the appearance of microsatellite instability<sup>[37]</sup>.

In this study, we screened a group of *H. pylori*-infected and uninfected paediatric patients as well as a group of adult patients who were divided as follows: *H. pylori*-infected chronic gastritis patients, uninfected chronic gastritis patients and gastric cancer patients (all of whom were infected with *H. pylori*). MSI instability was found to be a very rare event in the paediatric population. Our data showed that *H. pylori* infection was not associated with MSI among patients with chronic gastritis. The incidence of MSI in gastric cancer patients was 64%, which was consistent with results reported previously by others (incidences ranging from 58% to 76%)<sup>[38,39]</sup>. Additionally, several studies have reported the presence of MSI in patients with intestinal metaplasia and gastric cancer, suggesting that the development of MSI may be an early event in the multi-step progression of gastric carcinogenesis<sup>[40,41]</sup>.

The presence of MSI-H in sporadic carcinomas has been significantly associated with the loss of *MLH1* expression<sup>[42,43]</sup>. This phenomenon was associated with the hypermethylation of the *MLH1* promoter, which is the underlying mechanism that causes MSI in gastric adenomas and early gastric cancers<sup>[24,44]</sup>. It is well known that *H. pylori* infection causes an increased rate of cell turnover in the gastric mucosa and thus overwhelms the DNA repair system. This process might allow for the accumulation of mutations that are consequent to *H. pylori* infection and other environmental risk factors<sup>[45]</sup>.

Previously, we reported that *H. pylori* infection leads to decreased *MLH1* expression in patients with gastric cancer<sup>[46]</sup>. This result correlated with the high levels of MSI that were detected in these samples because the downregulation of *MLH1* can lead to DNA repair system failures. Moreover, when the methylation patterns were compared with the *MLH1* expression levels and the MSI levels in gastric cancer samples, we found higher methylation levels and a consequent downregulation of *MLH1* in samples that were characterised as MSI-H versus those characterised as MSI-L samples ( $P < 0.03$ ). Similarly, Mizoshita *et al.*<sup>[47]</sup> identified a strong association between the MSI phenotype and the loss of *MLH1* expression in advanced gastric cancers.

We did not find an association between *MGMT* promoter region methylation and MSI status. Furthermore, it was observed that the methylation of both the *MLH1* and *MGMT* promoter regions is a frequent event in gastric cancers. Similarly, Zou *et al.*<sup>[44]</sup> reported an increase in *MGMT* methylation during the progression from intestinal metaplasia to early gastric carcinoma.

*H. pylori* infections are generally acquired during childhood and, if left untreated, will persist indefinitely. These infections lead to chronic inflammation, one of the factors associated with epigenetic alterations and

possibly with the development of gastric cancer. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might be considered to be dependent on the duration of infection because similar methylation patterns had not been observed in children. Moreover, in gastric cancer patients it was shown that the *MLH1* expression levels, the hypermethylation pattern of the *MLH1* promoter region and the consequent increase in MSI frequency are all related events. The results of this study are in accordance with the results presented in previous studies; taken together, these findings provide a better understanding of gastric carcinogenesis in the Brazilian population. Although other authors had previously studied these genes in gastric cancers or in chronic gastritis samples from adult patients, this is the first study that included samples from children with chronic gastritis to represent the earlier stages of the inflammatory response, which supported the idea that the methylation of these genes might depend on the duration of the *H. pylori* infection, among other factors.

Another interesting point of this study is that it addresses a new concept described by Ogino *et al.*<sup>[48]</sup>, termed “Molecular Pathological Epidemiology”, a new field of epidemiology based on the molecular classification of cancer, in which a known or suspected etiologic factor is examined in relation to a specific molecular change to gain insights into the carcinogenic mechanisms. It is well known that gastric carcinogenesis is a multifactorial process that results from the interactions of factors related to diet, environment, individual genetic susceptibility and *H. pylori* infection. In this manner, this study provides insights into the carcinogenic mechanisms that are induced by *H. pylori* infection, one of the etiologic factors involved in gastric carcinogenesis, and the induction of epigenetic alterations in the early stages of this process. The chronic gastric mucosal inflammation induced by *H. pylori* infection, which is characterised by mucosal infiltration by polymorphonuclear leukocytes, macrophages and T and B lymphocytes, leads to the release of reactive oxygen species (ROS) from activated inflammatory cells. ROS can induce DNA damage and the lack of a proper MMR repair system, which is partly due to the persistence of the organisms and the associated inflammation, and thus can lead to the accumulation of DNA mutations in gastric epithelial cells that contribute to gastric carcinogenesis. Additionally, recent studies have shown that specific types of inflammation that are characterised by the expression of specific inflammation-related genes, as well as increased cell proliferation, are necessary for methylation induction. In a previous study, the authors showed that *H. pylori*-induced inflammation was able to induce methylation, unlike alcohol or saturated NaCl-induced inflammation<sup>[49]</sup>. Despite all of the evidence for *H. pylori* infection-induced methylation, we cannot exclude the presence of potential confounding factors such as differences in individual host genetics, different bacterial strains and the local microenvironment, all of which might

affect an association study. Further studies of *H. pylori*-induced molecular pathogenesis will be useful to our understanding of gastric carcinogenesis.

## COMMENTS

### Background

Gastric carcinogenesis is a multistep process that is triggered by *Helicobacter pylori* (*H. pylori*) infection and characterised by multiple genetic and epigenetic alterations, including the DNA repair genes. The action of *H. pylori* through inflammatory mediators might play a key role in the epigenetic silencing of these genes.

### Research frontiers

Impairment of the DNA MMR system is a known mechanism of carcinogenesis and tumour progression. *H. pylori* infection leads to chronic inflammation in the gastric mucosa, which is associated with DNA methylation; this epigenetic alteration plays an important role in the regulation of gene expression and the maintenance of DNA integrity and stability. To date, few advances have been made to determine the time duration required for to *H. pylori* infection to induce such epigenetics alteration and thus potentially induce gastric carcinogenesis.

### Innovations and breakthroughs

To the knowledge, this is the first study to evaluate the methylation patterns of DNA repair genes in paediatric samples. Children can be considered interesting natural models for the study of *H. pylori* infection not only because they are not usually submitted to gastric mucosal irritants such as alcohol, tobacco, and anti-inflammatory medications but also because the gastric mucosal changes in children might represent an earlier stage of the inflammatory response compared to those in adult hosts, due to the shorter duration of *H. pylori* infections in children. The results presented in this study indicate that the methylation of the *MGMT* and *MLH1* promoter regions might depend on the duration of infection because these methylation events were not observed in children.

### Applications

This study indicates that the impairment of the DNA MMR system through DNA promoter methylation is an infrequent event in the early stages of *H. pylori*-induced inflammation.

### Peer review

This is overall an interesting study that analyses the relationship between *H. pylori* infection and molecular changes in the gastric mucosa, diseases, and cancers. In this respect, this is a very unique study in the field of MPE research in non-neoplastic diseases (gastritis).

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**P- Reviewers** Ogino S, Wang K **S- Editor** Song XX  
**L- Editor** A **E- Editor** Zhang DN



## Neoadjuvant-intensified treatment for rectal cancer: Time to change?

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Received: September 10, 2012 Revised: February 19, 2013

Accepted: March 8, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To investigate whether neoadjuvant-intensified radiochemotherapy improved overall and disease-free survival in patients with locally advanced rectal cancer.

**METHODS:** Between January 2007 and December 2011, 80 patients with histologically confirmed rectal adenocarcinoma were enrolled. Tumors were clinically classified as either T3 or T4 and by the N stage based on the presence or absence of positive regional lymph nodes. Patients received intensified combined modality treatment, consisting of neoadjuvant radiation therapy (50.4-54.0 Gy) and infusional chemotherapy (oxaliplatin 50 mg/m<sup>2</sup>) on the first day of each week, plus five daily continuous infusions of fluorouracil (200 mg/m<sup>2</sup> per die) from the first day of radiation therapy until radiotherapy completion. Patients received five or six cycles of oxaliplatin based on performance status, clinical lymph node involvement, and potential risk of a non-sphincter-

conserving surgical procedure. Surgery was planned 7 to 9 wk after the end of radiochemotherapy treatment; adjuvant chemotherapy treatment was left to the oncologist's discretion and was recommended in patients with positive lymph nodes. After treatment, all patients were monitored every three months for the first year and every six months for the subsequent years.

**RESULTS:** Of the 80 patients enrolled, 75 patients completed the programmed neoadjuvant radiochemotherapy treatment. All patients received the radiotherapy prescribed total dose; five patients suspended chemotherapy indefinitely because of chemotherapy-related toxicity. At least five cycles of oxaliplatin were administered to 73 patients. Treatment was well tolerated with high compliance and a good level of toxicity. Most of the acute toxic effects observed were classified as grades 1-2. Proctitis grade 2 was the most common symptom (63.75%) and the earliest manifestation of acute toxicity. Acute toxicity grades 3-4 was reported in 30% of patients and grade 3 or 4 diarrhoea reported in just three patients (3.75%). Seventy-seven patients underwent surgery; low anterior resection was performed in 52 patients, Miles' surgery in 11 patients and total mesorectal excision in nine patients. Fifty patients showed tumor downsizing  $\geq$  50% pathological downstaging in 88.00% of tumors. Out of 75 patients surviving surgery, 67 patients (89.33%) had some form of downstaging after preoperative treatment. A pathological complete response was achieved in 23.75% of patients and a nearly pathologic complete response (stage ypT1ypN0) in six patients. An involvement of the radial margin was never present. During surgery, intra-abdominal metastases were found in only one patient (1.25%). Initially, 45 patients required an abdominoperineal resection due to a tumor distal margin  $\leq$  5 cm from the anal verge. Of these patients, only seven of them underwent Miles' surgery and sphincter preservation was guaranteed in 84.50% of patients in this subgroup. Fourteen patients received postoperative

chemotherapy. In the full analysis of enrolled cohort, eight of the 80 patients died, with seven deaths related to rectal cancer and one to unrelated causes. Local recurrences were observed in seven patients (8.75%) and distant metastases in 17 cases (21.25%). The five-year rate of overall survival rate was 90.91%. Using a median follow-up time of 28.5 mo, the cumulative incidence of local recurrences was 8.75%, and the overall survival and disease-free survival rates were 90.00% and 70.00%, respectively.

**CONCLUSION:** The results of this study suggest oxaliplatin chemotherapy has a beneficial effect on overall survival, likely due to an increase in local tumor control.

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**Key words:** Rectal cancer; Neoadjuvant treatment; Intensified radiochemotherapy; Oxaliplatin; Fluorouracil

**Core tip:** Management of rectal cancer requires a multimodality treatment approach. The objective of this study was to determine whether neoadjuvant-intensified radiochemotherapy, using traditional radiation therapy in combination with oxaliplatin and 5-fluorouracil (5-FU), could improve the overall and disease-free survival rates in patients with locally advanced rectal cancer. Conventional chemotherapeutic strategies typically only use 5-FU infusion. The results from this study indicate that the addition of oxaliplatin to the chemotherapeutic regime enhances the 5-year overall survival rate, facilitates a high rate of sphincter preservation, and reduces the local recurrence rate relative to the traditional strategies previously reported in the literature. Furthermore, oxaliplatin addition was well tolerated by patients, demonstrating an acceptable level of toxicity.

Musio D, De Felice F, Bulzonetti N, Guarnaccia R, Cazzazzo R, Bangrazi C, Raffetto N, Tombolini V. Neoadjuvant-intensified treatment for rectal cancer: Time to change? *World J Gastroenterol* 2013; 19(20): 3052-3061 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3052.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3052>

## INTRODUCTION

Management of rectal cancer requires a multimodality treatment approach. Significant progress has been made in the conventional modalities of surgery, radiotherapy and chemotherapy, typically used to treat this type of cancer. The frequent spread of neoplastic cells to mesorectal nodes and the consequent increase in local recurrence has led to surgical standardization using total mesorectal excision (TME). With standard TME surgery, the incidence of local recurrence in lymph node metastasis-negative, pN0, tumors is reduced. However, the local recurrence rate is still higher than 20% in patients with lymph node

positive, pN+, disease and chemotherapy has remained the standard adjuvant care. Technical progress in radiation techniques, identified in major accuracy planning, and further analysis of accurate timing in sequential multimodality therapy has created improvements in local control, toxicity (acute and chronic), and sphincter preservation<sup>[1-3]</sup>.

Previous work by Sauer *et al*<sup>[4]</sup> from the German Rectal Cancer Study Group identified preoperative radiochemotherapy as the standard treatment for patients with stage cT3-4 and/or N+ tumors. Based on empiric data and on the efficacy demonstrated in stage III colon cancer patients<sup>[5,6]</sup>, the addition of a second chemotherapeutic agent in a neoadjuvant setting confirmed oxaliplatin (OXP) radiosensitizing properties both *in vitro* and *in vivo*<sup>[7]</sup>.

The goal of this work was to determine whether neoadjuvant-intensified radiochemotherapy improved the overall and disease-free survival, which is typically only achieved with 5-fluorouracil (5-FU) treatment, in patients with locally advanced rectal cancer. In this study, tumor downstaging, pathological complete response (pCR), and negative radial (circumferential) margins of tumors were assessed as well as the overall and disease-free survival rates in a cohort of 80 patients, 51 of whom had already been evaluated as previously described<sup>[8]</sup>. The results indicate that oxaliplatin therapy, in addition to traditional radiation and 5-FU therapies, enhances the overall survival rate and reduces local recurrence in patients with rectal cancer.

## MATERIALS AND METHODS

### Eligibility criteria

Patients enrolled were positively diagnosed with rectal adenocarcinoma as shown by histological analysis. Tumors were within 12 cm from the anal margin and clinically classified as described below. The performance status (PS), age, normal blood parameters and normal renal function were also assessed. Patient exclusion criteria consisted of the presence of synchronous tumors, cardiovascular disease, history of neurological or psychiatric disorders, and previous pelvic radiotherapy. All patients were enrolled after providing informed consent.

### Staging

The pre-treatment staging included obtaining the complete history and careful physical examination of the patient, digital rectal examination, rectocolonoscopy, trans-rectal ultrasound and total body computerized tomography. Patient tumors were evaluated by ultrasound exam and tumor stage (T) was classified according to the American Joint Committee on Cancer tumor, nodes, metastasis (TNM) Staging System<sup>[9]</sup>. With the exception of one sample, all tumors were classified as either T3 or T4 and N+ if positive regional lymph nodes were detected without any distant metastases. In the case of an uncertain diagnosis, patients underwent abdominal-pelvic

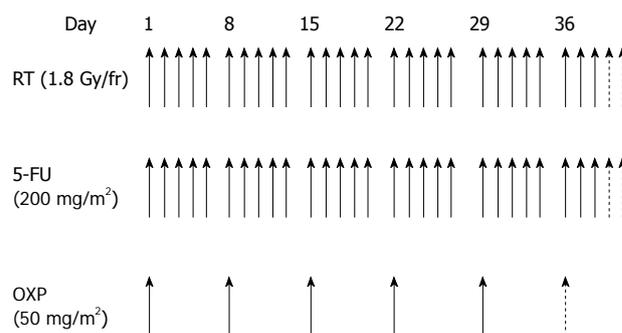
magnetic resonance imaging. The evaluation of clinically positive lymph nodes (N) was performed by trans-rectal ultrasound and TC; lymph nodes  $\geq 1$  cm were considered pathological.

### Treatment

**Radiotherapy:** As the physical positioning of a patient must remain identical for both the initial localization of the tumor by computerised tomography (CT) scanning and during subsequent treatment, a planning CT scan was performed in the treatment position. Patients were treated in the prone position using a belly-board device to displace the small bowels out of the treatment field. A radio-opaque marker was placed on the anal verge. CT images were acquired from the level of L1 to 3 cm below the anal marker with 5 mm slice spacing. CT data were analyzed using Treatment Planning Software (Pinnacle<sup>®</sup>) for target volume definition and dose solutions. The planning target volume 1 (PTV1) encompassed the primary tumor, the mesorectal and posterior pelvic sub-regions, and the regional node. The presacral, obturator and internal iliac lymph nodes were monitored in all patients. The external iliac lymph nodes were monitored if clinically positive or in the case of T4 tumor. The inguinal lymph nodes were irradiated if there was major tumor extension to the internal and external anal sphincter. The superior field border was located at the bifurcation of the common iliac vessels (L5/S1 interspace); the inferior margin was 5 cm below the inferior edge of the tumor. The lateral extension was 2 cm outside of the pelvic bones. The posterior border was placed 1 cm behind the sacrum to include sacral hollows. The anterior limit was placed at the posterior margin (cT3) or anterior margin (cT4) of the symphysis.

The PTV2 included the tumor mass with a 2 cm 3-D margin. The organs at risk were bowel (Dmax < 55 Gy), bladder (V50 60%; V60 50%), femoral heads (V50 60%), and anal canal (Dmax < 55 Gy). Patients were set up daily, using sagittal and lateral tattoos and a laser to prevent lateral rotation. Electronic portal imaging was used to check treatment organization once a week from the start to the end of treatment; portal images were compared with digitally-reconstructed radiographs from the planning CT scan. Radiation therapy was delivered with a 3-D-conformational multiple field technique at a dose of 45 Gy (in 25 daily fractions of 1.8 Gy given in 5 wk) to the whole pelvis for PTV1 and 5.4-9.0 Gy (in 3-5 daily fractions of 1.8 Gy) to the tumor volume for PTV2 with 6-15 MV energy photons.

**Chemotherapy:** All patients received a central venous access (port-a-cath) for delivering chemotherapy. Chemotherapy consisted of a 2-h oxaliplatin infusion (50 mg/m<sup>2</sup>) on the first day of each week of radiotherapy, and five daily continuous infusions of 5-FU (200 mg/m<sup>2</sup> per die). Patients received five or six cycles of oxaliplatin, dependent on PS, clinical lymph node involvement, and potential risk of a non-sphincter-conserving surgical pro-



**Figure 1 Neoadjuvant-intensified treatment protocol.** Patients received 50.4 Gy (solid arrows) or 54.0 Gy (dashed arrows) of radiation therapy (RT), 28 d (solid arrows) or 30 d [dashed arrows of fluorouracil (5-FU) and five (solid arrows) or six (dashed arrow)] weekly cycles of oxaliplatin dependent on performance status, clinical lymph node involvement, and potential risk of a non-sphincter-conserving surgical procedure. Radiation therapy was delivered with a 3-D-conformational multiple field technique at a daily dose of 1.8 Gy/fraction. All patients received a central venous access (port-a-cath) for delivering chemotherapy. Chemotherapy consisted of a 2-h oxaliplatin infusion (50 mg/m<sup>2</sup>) on the first day of each week of radiotherapy, and five daily continuous infusions of 5-FU (200 mg/m<sup>2</sup>).

cedure. Desamethasone (8 mg) and ondansetron (8 mg) were administered before the oxaliplatin infusion. Figure 1 shows the neoadjuvant treatment protocol.

Toxicity was evaluated using National Cancer Institute's Common Terminology Criteria for Adverse Events version 3.0<sup>[10]</sup>. Oxaliplatin and 5-FU dose reductions were not planned. For occurrence of hematological toxicity grade 3 or neurological toxicity grade 2, the oxaliplatin administration was interrupted; both chemotherapeutic agents were stopped if grade 3 toxicity was reached. If severe toxicity persisted, did not return to grade 1, or was classified as grade 4, chemotherapy was cancelled but radiotherapy was completed.

**Surgery:** Five weeks from the end of neoadjuvant treatment, each patient underwent digital rectal examination, total body TC, colonoscopy and trans-rectal ecography to evaluate clinical response. Surgery was planned seven to nine weeks after the end of radiochemotherapy treatment. The surgeon chose the type of surgery to perform.

**Adjuvant chemotherapy:** Adjuvant chemotherapy treatment was left to the oncologist's discretion and was recommended in patients with lymph node metastases.

### Pathologic examination of the operative specimen

Pathological staging was designated as pTNM and depended on the data acquired clinically in addition to surgical and pathologic findings. Radial margins were considered positive if there was evidence of microscopic invasion. Downstaging was defined as a reduction of at least one level in T or N staging between the baseline ultrasound exam and histopathological staging. Downsizing was defined as a reduction of lesion diameter between pre-treatment ultrasound evaluation and histopathological results. pCR was defined as the absence of any residual

**Table 1** Characteristics of patients *n* (%)

| Characteristics               | Patients   |
|-------------------------------|------------|
| Performance status            |            |
| 0                             | 52 (65.00) |
| 1                             | 27 (33.75) |
| 2                             | 1 (1.25)   |
| Localization                  |            |
| ≤ 5 cm from anal verge        | 45 (56.25) |
| > 5 to ≤ 8 cm from anal verge | 19 (23.75) |
| > 8 cm from anal verge        | 16 (20.00) |
| Stage                         |            |
| II A                          | 19 (23.75) |
| III A                         | 1 (1.25)   |
| III B                         | 30 (37.50) |
| III C                         | 30 (37.50) |

tumor cells detected in the operative specimen.

### Patient recruitment

This study was an extension of a previous study of 51 patients<sup>[8]</sup>. Between January 2007 and December 2011, 29 new patients were enrolled for a total of 80 participants: 25 females (31.25%) and 55 males (68.75%). The patients' ages ranged between 36-76 years (average = 63.55 years). Patients presented clinically with rectal bleeding (39/80, 48.75%) that may have been accompanied by a change in bowel habits, such as unexplained constipation and diarrhea (10/80, 12.50%). At a pre-treatment evaluation, 75.00% of patients showed pathological tumor-positive lymph nodes; 37.5% of patients were clinically staged as III B and 37.5% as III C. The distance of the inferior margin of the tumor lesion was located in the lower rectum at ≤ 5 cm from the anal verge in 56.25% of patients. The characteristics of patients are listed in Table 1.

### Follow-up

After surgery, all patients were monitored at three-month intervals for the first year and at six-month intervals for the subsequent years. All patients were stratified into five cohorts according to the year of the last treatment. We defined "absolute permanence" as the greatest number of months of permanence in each cohort. Absolute permanence, overall survival (OS) and disease-free survival (DFS) were measured in months from the end of the neoadjuvant treatment.

### Statistical analysis

Statistical analysis was performed using the following factors: 1 - sex, 2 - age, 3 - PS, 4 - TNM clinical staging, 5 - cranio-caudal extension of tumor lesion, 6 - tumor location, 7 - total radiotherapy dose, 8 - cycles of associated chemotherapy, 9 - interval between neoadjuvant treatment and surgery, 10 - type of surgery, 11 - toxicity, 12 - OS, 13 - DFS, 14 - pathologic downstaging, and 15 - pCR. Factors from 1 to 11 were considered "causal" or "predictive"; factors from 12 to 15 characterized the "considerable" results of therapy. Standard descriptive statistics were used to evaluate the distribution of each

factor. OS and DFS were evaluated in each cohort. To determine the association between downstaging or pCR and predictive factors, the univariate analysis was performed using the non-parametric Bernard test. Statistical tests were one-sided. Changes of OS and DFS according to predictive factors were assessed using a logistic model in multivariate analysis. Statistical analysis was performed using MATLAB software, version 7.5.0.342 (R2007b), and SAS software, version 9.1.

## RESULTS

### Compliance

Seventy-five patients completed the programmed radio-chemotherapy treatment. All patients received the radiotherapy prescribed total dose of 50.4 Gy in 75 patients (93.75%) and 54 Gy in five patients (6.25%). Five patients suspended chemotherapy indefinitely because of chemo-related toxicity after the second cycle (one patient), third cycle (two patients), or fourth cycle (two patients). Twelve patients stopped the planned neoadjuvant treatment because of acute toxicity: five patients interrupted radiation therapy only and seven patients interrupted both treatments. In these patients, radiotherapy was stopped for an average period of 10.58 d (range 2-22 d).

### Surgery

Seventy-seven out of 80 patients underwent surgery. A "wait and see" approach was recommended to only one patient; he was unfit for surgery because of type II diabetes mellitus and pericarditis co-morbidities. After neoadjuvant treatment, he underwent pelvic RM that indicated a complete clinical regression of the tumor. One patient did not undergo surgery because of liver metastases. One patient had a myocardial infarction two weeks after the end of radiochemotherapy and died.

For those patients who were eligible, surgery was planned an average of 9.30 wk (range 5-24 wk) after the end of neoadjuvant treatment. Low anterior resection was performed in 52 patients, Miles' surgery in 11 patients, and transanal endoscopic microsurgery in nine patients. Three patients had a different surgical approach. Two patients died of intra-operative complications. None had positive radial margins. At the beginning, 45 patients required an abdominoperineal resection due to a distal tumor margin distance of ≤ 5 cm from the anal verge. Only seven patients underwent Miles' surgery, and sphincter preservation was guaranteed in 84.50% of this subgroup of patients. Post-operative complications were recorded in nine patients; the most common type was perianastomotic fistula (six patients) and other post-operative complications included fever (one patient), intestinal obstruction (one patient), and adhesion (one patient).

### Pathologic complete response and downstaging

Downsizing and downstaging was evaluated by comparing clinical staging to pathological staging. Fifty patients showed tumor downsizing of ≥ 50% and associated

**Table 2** Evaluation of overall survival and disease-free survival

| Cohort | $\alpha$ | $\Omega$ | AP, mo | OS, mo | DFS, mo | OS/AP % | DFS/OS % |
|--------|----------|----------|--------|--------|---------|---------|----------|
| 2007   | 16       | 3        | 54.00  | 50.90  | 46.90   | 94.28   | 92.14    |
| 2008   | 22       | 3        | 42.90  | 39.30  | 34.90   | 91.40   | 89.01    |
| 2009   | 18       | 2        | 30.80  | 29.40  | 22.60   | 95.53   | 76.97    |
| 2010   | 13       | 0        | 17.40  | 17.40  | 15.30   | 100.00  | 87.93    |
| 2011   | 11       | 0        | 6.30   | 6.30   | 5.60    | 100.00  | 88.79    |
| Total  | 80       | 8        | 29.80  | 27.90  | 23.90   | 93.45   | 85.88    |

$\alpha$ : Survived;  $\Omega$ : Died; AP: Absolute permanence; OS: Overall survival; DFS: Disease-free survival.

downstaging in 88.00% of tumors. Out of 75 patients surviving surgery, 67 patients (89.33%) had some form of downstaging from preoperative treatment. After surgery, 25 patients (33.33%) harbored tumors that were classified as Stage I. Of these, 18 patients (72.00%) had clinical positive nodes at diagnosis. Pathologic complete response, defined as the absence of tumor cells in the operative specimen, was observed in 18 patients (22.50%); only six of them had clinical negative lymph nodes at diagnosis. Six patients had a nearly pathologic complete response (stage ypT1ypN0). During surgery, intra-abdominal metastases were found in one patient only (1.25%).

### Adjuvant chemotherapy

Fourteen patients received postoperative chemotherapy. Twelve patients had lymph node-positive tumors previously identified by trans-anal ultrasound (eight patients with cN2 and four patients with cN1). One patient had positive lymph nodes identified by histopathological examination and distant metastases were detected intraoperatively in one patient. Of 16 patients with ypT3ypN0 disease, only four were assigned to adjuvant chemotherapy.

### Overall survival and disease-free survival

Until January 2012, surviving patients were monitored with a follow-up program; the average follow-up time was 27.28 mo and the median follow-up time was 28.50 mo (range 2-58 mo). Twenty-four patients were followed for 25-36 mo, 18 patients followed for 37-48 mo, and 11 patients were followed for at least 49 mo.

In the full analysis of study cohort, eight of the 80 patients died. Seven deaths were related to rectal cancer and one death was caused by unrelated causes. Of these, one patient died before surgery, two died during surgery, and four deaths occurred during the follow-up program. Of the latter, two patients had local recurrence 6 and 9 mo, respectively, after the end of neoadjuvant treatment and two patients had distant metastases 13 and 15 mo, respectively, after the end of preoperative radiochemotherapy. Three out of the four patients had stage III B disease at diagnosis.

The absolute permanence (AP), OS, and DFS times were evaluated for each cohort (Table 2 and Figure 2), as well as the permanence/OS and permanence/DFS ratios and the total number of patients surviving ( $\alpha$ ) or

deceased ( $\Omega$ ) at the time of analysis (Table 2). It is important to note that OS times were nearly equivalent to absolute permanence times; the permanence/OS ratios were greater than 90% in all years evaluated. Remarkably, comparisons between OS and DFS demonstrated that the DFS/OS ratio was always higher than 88%, except in the 2009 cohort.

Of 19 patients with a complete response (18 pathological and one clinical), 18 patients are still disease-free survivors. One patient had local recurrence 19 mo after the end of neoadjuvant treatment. In the full analysis of the study participants, locoregional recurrence was observed in seven patients (8.75%) an average of 21.29 mo (range 6-39 mo) after the end of preoperative radiochemotherapy, six patients showed downstaging, and one patient had no benefit from neoadjuvant treatment. Local recurrence was located in the perianastomotic region (six cases) or pre-sacral region (one case). Distant metastases were recorded in 17 cases (21.25%) where eight patients presented pulmonary metastases after an average of 22.75 mo (range 7-38 mo) after the end of neoadjuvant treatment and six patients had liver metastases after an average of 6.83 mo (range 1-16 mo) after the end of radiochemotherapy. Individual cases of brain and ovarian metastases were recorded 6 and 12 mo, respectively, after the end of preoperative treatment. One patient presented distant metastases in multiple locations. Of these 17 patients, only five showed negative lymph nodes at diagnosis.

### Toxicity

**Acute toxicity:** Table 3 summarizes the incidence of acute toxicity. Proctitis, grade G2, was the most common symptom (63.75%) and the earliest manifestation of acute toxicity. On average, proctitis symptoms appeared during the ninth day of radiation therapy (range 2-25 d). Grade G1-2 diarrhea was noted in 31.00% of patients. Grades 3-4 toxicity was seen only in nine patients, with symptoms of diarrhea (three patients), proctitis (four patients), and abdominal pain (two patients). Genitourinary toxicity was observed in 32.50% of patients. Symptoms included an increase in frequency and dysuria, usually during the end of third week of radiation therapy (range 3-26 d). Of the 23 patients with dysuria, 47.83% (11 patients) were classified as G2. Radiation dermatitis was reported in 30 patients; ten patients' symptoms were graded as G1, 17 patients as G2, and three patients as G3. There was a significant correlation between radiation dermatitis and tumors located within 5 cm of the anal verge ( $P$  value, 0.0076). No hematological toxicity was observed. Hypersensitivity reactions were recorded in six patients. One patient stopped chemotherapy after the second cycle. One patient completed all five prescribed cycles of intensified chemotherapy, but during the last two cycles was administered 5-FU only. Four patients completed the prescribed treatment after taking a one-week break.

Sixteen patients presented neurologic toxicity due to oxaliplatin. Neuropathy was classified as G3 in only one patient, who had sensory loss and paresthesia during the

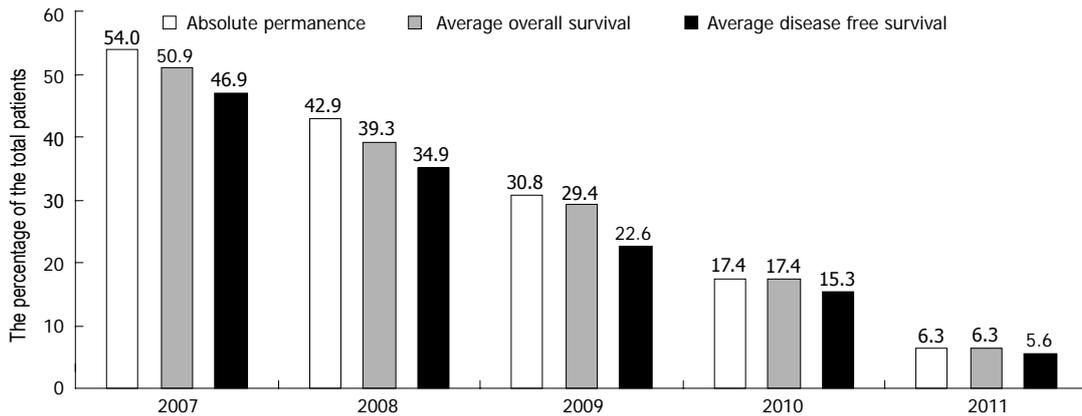


Figure 2 Absolute permanence (white column), overall survival (grey column), and disease-free survival (black column) comparisons. Patient data was stratified by year and is represented as a percentage of the total cohort of patients.

Table 3 Incidence of acute toxicity *n* (%)

|                                    | G1        | G2         | G3       |
|------------------------------------|-----------|------------|----------|
| Blood-bone marrow                  |           |            |          |
| Neutrophils-granulocytes           | 1 (1.25)  |            |          |
| Allergy-immunology                 |           |            |          |
| Allergic reaction-hypersensitivity | 6 (7.5)   |            |          |
| Cardiac arrhythmia                 |           |            |          |
| Palpitation                        | 1 (1.25)  |            |          |
| Constitutional symptoms            |           |            |          |
| Fatigue                            | 10 (12.5) | 5 (6.25)   |          |
| Fever                              | 7 (8.75)  | 1 (1.25)   |          |
| Dermatology-skin                   |           |            |          |
| Pruritus-itching                   | 2 (2.5)   |            |          |
| Rash-desquamation                  | 2 (2.5)   | 6 (7.5)    |          |
| Radiation-dermatitis               | 10 (12.5) | 17 (21.25) | 3 (3.75) |
| Gastrointestinal                   |           |            |          |
| Constipation                       | 2 (2.5)   | 15 (18.75) |          |
| Diarrhea                           | 14 (17.5) | 11 (13.75) | 3 (3.75) |
| Nausea                             | 12 (15)   |            |          |
| Proctitis                          | 6 (7.5)   | 51 (63.75) | 4 (5)    |
| Vomiting                           | 5 (6.25)  | 1 (1.25)   |          |
| Neurology                          |           |            |          |
| Neuropathy: sensory                | 14 (17.5) | 1 (1.25)   | 1 (1.25) |
| Pain                               |           |            |          |
| Pelvic pain                        | 1 (1.25)  | 3 (3.75)   |          |
| Abdominal pain or cramping         | 6 (7.5)   | 2 (2.5)    | 2 (2.5)  |
| Renal-genitourinary                |           |            |          |
| Dysuria-painful urination          | 12 (15)   | 11 (13.75) |          |
| Urinary frequency                  | 3 (3.75)  |            |          |
| Cardiovascular                     |           |            |          |
| Vascular thromboembolism           | 7 (8.75)  | 2 (2.5)    | 2 (2.5)  |

end of the fifth cycle of chemotherapeutic treatment. In total, 11 patients (13.75%) had adverse cardiovascular events. Two patients experienced acute myocardial infarction (IMA), two patients experienced pulmonary embolism, and seven patients experienced deep vein thrombosis requiring anticoagulant therapy. Embolic events G3-4 arose after the end of neoadjuvant-intensified radiochemotherapy by an average of 4.42 mo (range 1-8 mo). One patient died 2 wk after the end of treatment; it was considered an IMA-related death. One patient died dur-

ing surgery of cardiac infarction.

**Late toxicity:** Late toxicity was defined as long-term toxic effects occurring at least 6 mo after the end of radiochemotherapy treatment. Considering all enrolled patients, gastrointestinal toxicity was the most evident late side effect and was recorded in 37 patients (46.25%). Fecal incontinence was reported in 32.43% of patients and proctitis in 32.43% of patients. Four patients had diarrhea and five patients had an increase in stool frequency. Seven patients reported sexual dysfunction. Urinary incontinence was observed in three patients with colostomy. In 11 out of 60 patients with acute proctitis, the symptoms continually persisted as G1-2 grade. No correlation was observed between acute toxicity and late toxicity. Neuropathy with loss of deep tendon reflexes and paresthesia that did not interfere with activities of daily living was documented in five patients and acute neuropathic toxicity was reported in 16 patients.

### Correlation analysis

We evaluated 11 “predictive” factors: (1): sex; (2): age; (3): PS; (4): TNM clinical staging; (5): cranio-caudal extension of the tumor lesion; (6): tumor location; (7): total radiotherapy dose; (8): cycles of associated chemotherapy; (9): interval between neoadjuvant treatment and surgery; (10): type of surgery; and (11): toxicity. Univariate analysis did not show any significant correlation between “predictive” factors and downstaging or pCR. There was no causal significant correlation between “predictive” factors and OS or DFS in the multivariate analysis.

Two additional statistical analyses were of interest and enhanced the evaluation of the data. First, the linear relationship between the infusion of six cycles of chemotherapy and tumor sizes smaller than 5 cm was statistically significant (*P* value, 0.0334). Second, a causal correlation was found between surgery performed seven weeks before the end of neoadjuvant treatment and OS or DFS, although the data was not stratified by the surgery characteristics.

## DISCUSSION

### *Multimodality treatment in rectal cancer*

In the past two decades, several clinical trials have been performed to determine the role of a multimodal approach in treating rectal cancer. These clinical trials faced three problems: (1) determination of temporal sequencing of treatment modalities; (2) integration of radiotherapy and chemotherapy; and (3) radiation dose fractionation. Testing and analysis of new approaches should be aimed at defining therapeutic strategies to improve local tumor control and overall survival benefit<sup>[11]</sup>.

Year after year, local recurrence has represented the prevailing method of treatment failure. Now, with improvement in surgical and radiotherapeutic techniques, local tumor control rates have improved. Neoadjuvant radiotherapy and concurrent 5-FU based chemotherapy, as compared with the same protocol delivered after surgery, has improved local control<sup>[4]</sup>. The addition of 5-FU to radiation therapy also has been shown to significantly reduce the incidence of local recurrences<sup>[4,11,12]</sup>. Likewise, survival has improved with standardization of total mesorectal excision surgery<sup>[13-16]</sup>. The decrease in local recurrence rates emphasizes the need to investigate the risk of distant metastases and requires new treatment strategies to improve distant tumor control.

Although most patients achieve tumor downstaging after preoperative radiochemotherapy, the debate over monochemotherapy *vs* polichemotherapy still remains. Specifically, whether 5-FU infusion, the standard of care in rectal cancer, is better than multichemotherapeutic agents for reducing the risk of distant metastases. The prevalence of distant metastases (24%-30%) stresses the importance of a more effective systemic treatment<sup>[11]</sup>.

### *Evaluation*

In this study, the effects of a multimodal therapy approach for rectal cancer were evaluated. Disease-free survival was considered the most efficient indicator of the absence of disease and is the most robust indicator of effective treatment. In our previously reported study<sup>[8]</sup>, we evaluated the toxicity and efficacy of preoperative-intensified radiochemotherapy, and the subsequent pathologic complete response, downstaging, and sphincter preservation rates. In the current study, the patient cohort from the previous study was expanded with 29 new cases and the overall survival and disease-free survival rates were evaluated and stratified per year.

The aim of this study was to verify efficacy of neoadjuvant-intensified treatment. Oxaliplatin (50 mg/m<sup>2</sup> per week) was added to the standard 5-FU chemotherapy, normally given in continuous infusion of 200 mg/m<sup>2</sup> per die during each day of radiation therapy. Weekly administration of oxaliplatin, with the cumulative dose of 300 mg/m<sup>2</sup>, was chosen so that its toxic effects were reduced and to optimize its radiosensitizing properties. This intensified radiochemotherapy regime was used to test the hypothesis that it could produce greater OS and

DFS within tolerable toxicity. An empirical analysis of the study results confirms our hypothesis; the treatment was well tolerated, with high compliance and a relatively good level of toxicity. All patients had received the total prescribed radiotherapy dosage and at least five cycles of oxaliplatin were administered to 91.25% of patients.

The compliance rate (93.75%) was slightly higher than rates registered in studies in which intensified radiochemotherapy regimes were adopted (range 64%-85%)<sup>[17-21]</sup>. Most of the acute toxic side effects observed in this study were classified as grade 1-2. Grade 3-4 acute toxicity was reported in 30% of patients and was only slightly higher than data from the STAR-01 trial (24%) and the ACCORD study (25%)<sup>[17,18]</sup>. Of note, cardiovascular toxicity represented 45.83% of grade 3-4 acute toxic effects.

### *Thromboembolic risk*

Thromboembolic risk could be ascribed to the type of chemotherapy administered or to surgery. In the literature, there is not enough available data describing this risk, although chemotherapy is recognized as an independent risk factor for a thromboembolic event<sup>[22]</sup>. Ng *et al*<sup>[23]</sup> examined the frequency and pattern of cardiotoxicity in 153 patients treated with capecitabine used in addition with oxaliplatin for advanced colorectal cancer and found that 6.7% of patients developed thromboembolic events. Randomized studies, aimed to assess the efficacy of neoadjuvant radiochemotherapy treatment in locally advanced rectal cancer, with or without oxaliplatin, did not analyze cardiovascular toxicity. Chua *et al*<sup>[24,25]</sup> reported that during induction chemotherapy using capecitabine and oxaliplatin, 10 patients (8.5%) had cardiac or thromboembolic events and four patients died in a phase II trial. The incidence rate of that study was slightly lower than the rate reported here (13.75%). The high rate of thromboembolic risk may be associated with properties of oxaliplatin that boost the 5-FU thrombogenic effect<sup>[26]</sup>. Certainly, the rise in cardiovascular, chemo-related toxicity must be monitored carefully and kept under control. Because of the risk, neoadjuvant-intensified radiochemotherapy must be interrupted if cardiac symptoms appear.

### *Toxicity*

Excluding cardiovascular toxicity, grade 3 or 4 acute toxic effects occurred only in 13 patients (16.25%) with diarrhea occurring just in three patients (3.75%). In both STAR-01 and ACCORD trials, addition of oxaliplatin to 5-FU based radiochemotherapy increased toxicity rates. In those studies, grade 3-4 diarrhea was recorded in 15% and 13% of patients given oxaliplatin *vs* 4% and 3% of those in the control group, respectively<sup>[17,18]</sup>. The lowest rates of acute gastrointestinal toxicity in this study were observed in the oxaliplatin regimen, given as 2-h infusion at a dose of 50 mg/m<sup>2</sup> per week. Grade 3-4 long-term toxic effects were not recorded.

**Neoadjuvant-intensified radiochemotherapy efficacy**

It was difficult to determine if the cause of fecal incontinence and sexual function was due to radiation therapy, surgery, or both treatments. Sixty-four patients, or 80% of the enrolled patients, underwent conservative surgery. Of these patients, 10.45% reported sexual dysfunction and 17.91% fecal incontinence.

The sphincter preservation rate, evaluated in the total patient cohort and in a subgroup of 45 patients with a tumor localization  $\leq 15$  cm from the anal verge, was 80% and 84.50%, respectively. These results are slightly better than the ACCORD trial, in which conservative surgery was performed in 77.20% of patients, and similar to the STAR-01 study, in which 82% of patients had sphincter preservation. In the literature, the incidence of pCR and negative radial margins, in patients who received preoperative radiochemotherapy with 5-FU and oxaliplatin, ranged from 10% to 24% and from 80.95% to 100%, respectively<sup>[18-20,24,25,27,28]</sup>. Randomized trials confirmed an improvement in the local tumor control rate. Neoadjuvant treatment has been associated with a reduction of local recurrence of 6%-15%, although the incidence of distant metastases was still 30%<sup>[29-32]</sup>. A retrospective study showed that pathologic downstaging and pCR were essential for an accurate prediction of disease-free survival and overall survival<sup>[33]</sup>.

In 22.5% of patients, no residual cancer cells were identified and the tumor was staged as pT0N0. Pathological complete response rates reached 23.75% and were evaluated by post-treatment RM. One patient is still a disease-free survivor 21 mo after the end of radiochemotherapy. The majority of patients, 83.75%, had some form of tumor downstaging after preoperative treatment; involvement of the radial margin was never present.

The 5-year overall survival rate (90.91%) in this study was better than results observed in randomized trials of locally advanced rectal cancer, which only observed survival rates of 65%-68%<sup>[12,13,26]</sup>. The results of this study indicate that intensification of radiochemotherapy using oxaliplatin is effective in preoperative treatment and showed that a strong response to neoadjuvant treatment increased the overall patient survival.

Considering a median follow-up of 28.5 mo (range 2-58 mo) in the total cohort, the cumulative incidence of local recurrences was 8.75% and the incidence of OS and DFS was 90% and 70%, respectively. The most common type of first recurrence was pulmonary metastases, which arose in eight patients. This observation confirmed the need to include CT thorax controls during the follow-up program. Although some questions remain, such as the need for adjuvant chemotherapy and whether oxaliplatin boosts the 5-FU trombogenic effect, the radiochemotherapy regime used (OXP 50 mg/m<sup>2</sup> and 5-FU 200 mg/m<sup>2</sup>) seems to be appropriate to produce good clinical results and to maintain toxicity at tolerable levels.

In conclusion, the results of this study indicate that neoadjuvant-intensified radiochemotherapy in patients with rectal cancer carcinoma improved pathological

complete response, negative radial margins, and sphincter preservation. The chemotherapy regime of OXP 50 mg/m<sup>2</sup> and 5-FU 200 mg/m<sup>2</sup> was well tolerated, with few severe toxic effects. Despite the small number of patients enrolled, this study suggests that the addition of oxaliplatin has a beneficial effect on overall survival, likely due to an increase in local control, although there was not a clear increase in distant metastases control. On the basis of these results, the importance and validity of intensified radiochemotherapy to reduce local recurrence should be emphasized. Currently, we are waiting for longer follow-up data from the randomized phase III trials. Future studies will address the treatment and management of recurrent distant metastases in rectal cancer.

**COMMENTS****Background**

Management of rectal cancer needs a multimodality treatment approach. Significant progress has been made in the conventional modalities of surgery, radiotherapy and chemotherapy used to treat this type of cancer. The decrease in local recurrence rates emphasizes the need to investigate the risk of distant metastases and indicates that new treatment strategies are necessary to improve distant control.

**Research frontiers**

Although most patients achieve a tumor downstaging after preoperative radiochemotherapy, the question of monochemotherapy versus polichemotherapy still remains. Infusion of 5-fluorouracil (5-FU), the standard of care in rectal cancer, is better than multi-chemotherapeutic agents for reducing the risk of distant metastases. The incidence of distant metastases (24%-30%) stresses the importance of a more effective systemic treatment.

**Applications**

The objective of this study was to determine whether neoadjuvant-intensified radiochemotherapy improved the overall and disease-free survival rates in patients with locally advanced rectal cancer. Typically, improved survival rates have only been achieved with 5-FU treatment.

**Terminology**

Oxaliplatin is a chemotherapeutic agent. Studies *in vitro* and *in vivo* confirm its radiosensitizing properties.

**Peer review**

This is a retrospective review in a significant number of patients with favourable results and worth publication.

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**P- Reviewer** Cheung HYS **S- Editor** Song XX  
**L- Editor** A **E- Editor** Zhang DN



## Glucmannan for abdominal pain-related functional gastrointestinal disorders in children: A randomized trial

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Received: November 17, 2012 Revised: April 4, 2013

Accepted: April 9, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To assess the efficacy of glucmannan (GNN) as the sole treatment for abdominal pain-related functional gastrointestinal disorders (FGIDs).

**METHODS:** We conducted a double-blind, placebo-controlled, randomized trial. Patients were recruited among children referred to the Department of Paediatrics, Medical University of Warsaw. Included in the study were children aged 7-17 years with abdominal pain-related FGIDs classified according to the Rome III diagnostic criteria. The children were randomly assigned to receive GNN, a polysaccharide of 1,4-D-glucose and D-mannose, a soluble fiber from the Japanese Konjac plant, at a dosage of 2.52 g/d (1 sachet of 1.26 g 2 times a day), or a comparable placebo (maltodextrin) at the same dosage. The content of each sachet was dissolved in approximately 125 mL of fluid and was consumed twice daily for 4 wk.

**RESULTS:** Of the 89 eligible children, 84 (94%) completed the study. "No pain" and "treatment success" (defined as no pain or a decrease  $\geq$  2/6 points on the

FACES Pain Scale Revised) were similar in the GNN ( $n = 41$ ) and placebo ( $n = 43$ ) groups [no pain (12/41 vs 6/43, respectively; RR = 2.1, 95%CI: 0.87-5.07) as well as treatment success (23/41 vs 20/43; RR = 1.2, 95%CI: 0.79-1.83)]. No significant differences between the groups were observed in the secondary outcomes, such as abdominal cramps, abdominal bloating/gasiness, episodes of nausea or vomiting, or a changed in stool consistency. GNN demonstrated no significant influence on the number of children requiring rescue therapy, school absenteeism, or daily activities.

**CONCLUSION:** In our setting, GNN, as dosed in this study, was no more effective than the placebo in achieving therapeutic success in the management of FGIDs in children.

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**Key words:** Functional abdominal pain; Abdominal pain-related functional gastrointestinal disorders; Children

**Core tip:** This study focused on abdominal pain-related functional gastrointestinal disorders (FGIDs) which are a common problem in children. The aim of the study was to assess the effectiveness of glucmannan (GNN), a soluble fiber of the Japanese Konjac plant, in alleviating the frequency and the severity of pain in children with FGIDs. We have demonstrated through our prospective, double-blind, placebo-controlled, randomized study that GNN in this setting for 4 wk was not effective in treatment of the FGIDs. The obtained results led us to the conclusion that further studies are needed to explore the role of GNN in the pathophysiology of functional disorders.

Horvath A, Dziechciarz P, Szajewska H. Glucmannan for abdominal pain-related functional gastrointestinal disorders in children: A randomized trial. *World J Gastroenterol* 2013; 19(20): 3062-3068 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Functional abdominal pain (FAP) is one of the most common reasons for a referral to a physician<sup>[1]</sup>. According to various estimates of the prevalence of FAP, approximately 13% to 38% of children and adolescents report functional abdominal problems<sup>[2]</sup>.

The diagnosis of FAP is symptom-based. The Rome III criteria for abdominal pain-related functional gastrointestinal disorders (FGIDs) have been validated and assist clinicians make diagnoses<sup>[2]</sup>. The vast majority of children with FGIDs are subsequently diagnosed with functional dyspepsia (FD), irritable bowel syndrome (IBS), and childhood abdominal pain (AP)<sup>[3,4]</sup>. The symptoms associated with these diagnoses have a great impact on the patients' quality of life, daily activities, and school absenteeism, which in turn can result in long-term psychological implications<sup>[2]</sup>. Therefore, patients as well as caregivers and physicians are interested in safe and reliable treatments to relieve troublesome symptoms. Unfortunately, the medical cause for these symptoms remains unclear, thus limiting currently available therapies. FGIDs continue to represent a significant challenge in approaches to patient management.

For most children with FGIDs, no organic causes for their pain are found on physical examination or during investigations<sup>[5]</sup>. One of the recent hypotheses regarding the pathophysiology of FGIDs proposes that the biological and environmental factors that alter the enteric flora and visceral perception cause the development of functional disturbances<sup>[2,6]</sup>. The associations between the enteric microbiota, immune activation, and the role of lumen-mucosa interaction have been explored recently<sup>[2,7,8]</sup>. However, a precise explanation of the etiology of FGIDs remains to be expounded.

Regardless of the lack of a biophysiological model for FGIDs, a variety of therapeutic options have been tried. Unfortunately, studies have proven that most options do not exert any significant influence on the natural history of FGID development<sup>[2]</sup>. Two recent Cochrane systematic reviews have revealed weak evidence for the benefits of medication or dietary manipulation in children with functional abdominal pain<sup>[9,10]</sup>. Only 2 small, randomized clinical trials (Christensen 1982, Feldman 1985) have been conducted in children with FGIDs, and they compared the effects of added dietary fiber with placebo<sup>[9,11,12]</sup>. The results and conclusions of these trials were not consistent. Nevertheless, many clinicians routinely recommend the use of bulking agents or dietary fiber to stimulate regular bowel movements and to improve the symptoms associated with FGIDs<sup>[2]</sup>.

The goal of our study was to assess the efficacy of glucomannan (GNN) as the sole treatment for FGIDs in children. GNN, a polysaccharide of 1,4-D-glucose and

D-mannose, is a soluble fiber from the Japanese Konjac plant. GNN exhibits properties that are typical of dietary fiber. Thus, it may provide a positive effect on many aspects of gut physiology and the appropriate functioning of enteric microbiota in patients with FGIDs<sup>[8,13]</sup>.

## MATERIALS AND METHODS

### *Trial design*

We conducted a double-blind, placebo-controlled, randomized trial (RCT) from January 2009 to October 2011. The recommendations of the CONSORT 2010 Statement for reporting parallel group randomized trials were followed<sup>[14]</sup>.

### *Participants*

Patients were recruited among children referred to the Department of Paediatrics, the Medical University of Warsaw, from January 2009 to October 2011. The RCT included children aged 7-17 years with abdominal pain-related FGIDs classified according to the Rome III diagnostic criteria (Table 1)<sup>[5]</sup>. Patients with organic gastrointestinal diseases (as established by the medical history, complete blood count, urinalysis, stool examination for occult blood, ova and parasites, blood chemistries, abdominal ultrasound, breath hydrogen testing, and endoscopy, if needed), other chronic disease, or growth failure were excluded from the study. Additionally, during the time of the study, subjects were instructed not to take any medications that could influence the enteric flora, including antibiotics and commercially available probiotic or prebiotic preparations.

### *Interventions*

At the randomization visit, the inclusion criteria were checked. Potentially eligible patients were evaluated using a full review of their clinical histories and the results of a physical examination. The included subjects were randomized into a group receiving either GNN (Dicoman Junior, Vitis Pharma, Poland) at a dosage of 2.52 g/d, *i.e.*, 1 sachet of 1.26 g 2 times a day, or a group receiving a comparable placebo (maltodextrin) at the same dosage. The content of each sachet was dissolved in approximately 125 mL of fluid and was consumed twice daily for 4 wk. The manufacturer had no role in the conception, design, or conduct of the study or in the data analysis.

Subjects were instructed to ingest the preparation twice a day, in the morning and in the evening, for 4 wk and to record any symptoms in a questionnaire at the end of the study. At randomization, parents received 28 sachets for the first 2 wk of the study. After this period, the parents were contacted to examine the children's compliance with the treatment, provide them with the next 28 sachets, and schedule the exact timing of a final visit. The assessment of all outcome measures was based on the patients' questionnaires collected at the final visit. Additionally, the subjects and/or caregivers were asked to report the following information in their diaries: compli-

**Table 1 Study population baseline characteristics *n* (%)**

|  | Placebo    | Glucomannan |
|--|------------|-------------|
| Patients recruited                             | 45         | 44          |
| Male/female                                    | 21/22      | 21/20       |
| Age mean, yr                                   | 11.3 ± 2.5 | 11.6 ± 3.0  |
| Self-reported frequency of pain <sup>1</sup>   |            |             |
| Pain 1-3 times per month                       |            |             |
| Pain 1-2 times per week                        | 12 (27.9)  | 9 (21.9)    |
| Pain > 2 times per week                        | 13 (30.2)  | 9 (21.9)    |
| Pain every day                                 | 14 (32.6)  | 14 (34.1)   |
|  | 4 (9.3)    | 9 (21.9)    |
| Severity of pain <sup>1</sup>                  |            |             |
| “face 0”                                       | 0 (0)      | 0 (0)       |
| “face 1”                                       | 5 (11.6)   | 2 (4.8)     |
| “face 2”                                       | 18 (40.8)  | 13 (31.7)   |
| “face 3”                                       | 15 (34.8)  | 16 (39.0)   |
| “face 4”                                       | 5 (11.6)   | 8 (19.5)    |
| “face 5”                                       | 0 (0)      | 2 (4.8)     |
| Self-reported absenteeism from school          | 21         | 21          |
| Self-reported alterations in daily activities  | 29         | 27          |
| Number of subjects who required rescue therapy | 20         | 25          |

<sup>1</sup>Wilcoxon's test.

ance with consumption of the product and information on any other treatment given to the child during the study. The diary was constructed in a simple, understandable mode. The FACES Pain Scale Revised was used to assess the severity of pain<sup>[15]</sup>. The scale consists of 6 faces that reflect different levels of pain, ranging from a relaxed face on the left (no pain, indicating a score of 1 points - “face 0”) to a face showing intense pain on the right (highest pain possible, indicating a score of 6 points - “face 5”).

### Outcomes

The primary outcome measures included the proportion of patients with self-reported no pain and that of patients with treatment success, which was defined as no pain or a decrease of  $\geq 2/6$  points on the FACES Pain Scale Revised<sup>[15]</sup>. The subjective assessment of pain frequency, abdominal cramps, abdominal bloating/gasiness, the number of episodes of nausea or vomiting; changes in stool consistency (loose or constipated stools) during the study were the secondary outcome measures. Furthermore, the frequencies of school absenteeism and changes in daily activities were assessed, as was the percentage of children requiring rescue therapy. Finally, all adverse effects were recorded, and their possible relation to the study product consumption was evaluated. Compliance was assessed by direct questioning of the subjects or their caregivers during clinic visits.

### Randomization

Block randomization, with a block size of 6, was performed using a computer-generated random number list prepared by an investigator with no clinical involvement in the trial. The sequence was concealed until all data were analyzed.

### Blinding

Both the participants and the researchers conducting the study were blinded. The study intervention product was prepared centrally by the hospital pharmacy at the Medical University of Warsaw with the assistance of independent personnel who were not involved in the conduct of the trial. The active product and the placebo were packaged in identical sachets and labeled with one of two codes, each allocated to the experimental product or placebo. This procedure was performed by an independent pharmacist, who was the only person aware of the codes' meanings. The randomization numbers had been previously generated, and every patient eligible for inclusion received a consecutive number from the list. The appearance and texture of the dry placebo product were identical to those of the active product. When mixed with water, GNN turned into a substance of jelly-like consistency; however, this only occurred if the solution was not consumed within a few minutes, which was the recommended time limit for consumption.

### Statistical analysis

The sample size was based on the treatment success outcome (*i.e.*, the proportion of participants with no pain or a decrease of  $\geq 2/6$  points on the FACES Pain Scale Revised). It was estimated that an initial sample size of 80 patients would be sufficient to reveal a difference in the treatment effect of 30% (70% of the participants receiving GNN compared with 40% of the participants receiving placebo) considering that  $\alpha = 0.05$  and a power (beta) of 80%. The number of 80 for the children accounted for approximately 10% withdrawals or losses.

The computer software “R” [version 2.13.1 (2011-07-08)] was used for the analysis. Analyses of continuous data were performed with a parametric analysis (Student's *t*-test) in the case of a normal distribution of variables. The Mann-Whitney test was implemented for non-normally distributed variables. The  $\chi^2$  or Fisher's exact test were used, as appropriate, for the analysis of dichotomous outcomes. The RR or the mean difference (MD) with a 95%CI was calculated using the computer software StatsDirect [version 2.7.8b (2011-11-09)]. The differences between the study groups were considered to be statistically significant when the *P* value was  $< 0.05$  or when the 95%CI: for the RR did not exceed 1.0 or, for the MD, did not exceed 0. The results were analyzed using an Available Case Analysis (ACA).

### Ethical considerations

Parents and children were fully informed about the schedule and the aims of the study. Informed consent was obtained from at least one caregiver of each child included in the study and from all children older than 12 years of age.

The Ethics Committee of The Warsaw Medical University approved the study. The trial was registered at ClinicalTrials.gov (<http://clinicaltrials.gov>), number NCT 01495806.

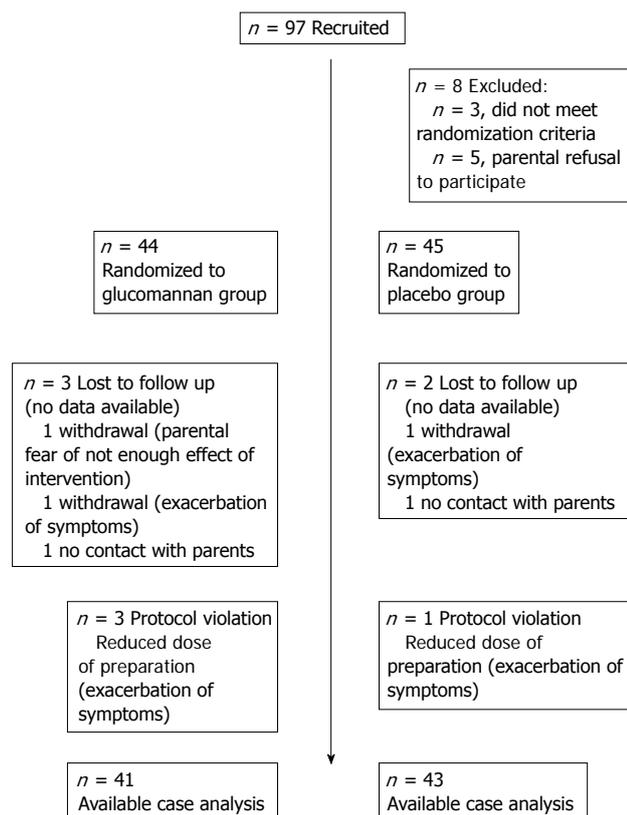


Figure 1 Flowchart of patients participating in the study. Declaration of funding interests: This study was funded in full by the Medical University of Warsaw.

## RESULTS

### Patients' characteristics and compliance

Figure 1 shows a flowchart of the subjects' progression through the study. Of the 97 eligible children, 89 underwent randomization. Among them, 44 children were assigned to the experimental GNN group, and 45 children were assigned to the placebo group. Of the 89 randomized children, 84 (94%) completed the study. There was no significant difference in the drop-out rate between the 2 groups. The baseline demographic and clinical characteristics were similar between the experimental and placebo groups (Table 1).

### Primary outcomes

Overall, 18 of the 84 (21%) subjects reported the primary outcome measure of 'no pain'. The subjects in the GNN group were more likely to experience "no pain" (29%) than the subjects in the placebo group (14%); however, the difference was not statistically significant (RR = 2.1, 95%CI: 0.87-5.07).

"Treatment success" (defined as no pain or a decrease  $\geq 2/6$  points on the FACES Pain Scale Revised) was similar between the study groups. Of the 41 children in the GNN group, 23 (56%) experienced treatment success, compared with 20 of the 43 (47%) in the placebo group (RR = 1.21, 95%CI: 0.79-1.83).

Originally, we had also planned to conduct a subgroup analysis (*e.g.*, FD, FAP, IBS). However, due to the small number of patients included in the subgroups and the overlap of symptoms between the patients with various types of functional disorders, we decided against conducting this analysis.

### Secondary outcomes

No significant differences between the GNN and placebo groups were observed in the secondary outcome measures, such as the subjective assessment of gastrointestinal symptoms: (1) Abdominal cramps (32% *vs* 49%, respectively;  $P = 0.12$ ); (2) Abdominal bloating/gassiness (44% *vs* 51%, respectively;  $P = 0.39$ ); (3) Number of episodes of nausea or vomiting (7% *vs* 2%, respectively;  $P = 0.34/24\%$  *vs* 33%, respectively;  $P = 0.31$ ); or (4) Change in stool consistency (loose stools: 29% *vs* 39%, respectively;  $P = 0.33$ ; constipated stools: 27% *vs* 21%, respectively;  $P = 0.53$ ).

### Rescue therapy

The percentage of patients requiring rescue therapy was similar in both groups (GNN 19% *vs* placebo 14%, respectively;  $P = 0.53$ ).

### Subjects' activities

The GNN supplementation showed no significant influence on the frequency of school absenteeism (10% *vs* 14%, respectively;  $P = 0.56$ ) or on changes in daily activities (27% *vs* 19%, respectively;  $P = 0.37$ ) during the study.

### Adverse effects

The GNN was well tolerated, and no adverse effects were recorded in any of the patients. However, 4 patients in the GNN group complained of an exacerbation of symptoms (1 discontinued the therapy; 3 required a dose reduction) compared with 2 patients in the placebo group (1 discontinued therapy; 1 required a dose reduction). Nonetheless, it was difficult to establish causality in these cases because the course of FGIDs was defined by exacerbations and periods of recovery.

## DISCUSSION

This prospective, double-blind, placebo-controlled, randomized study showed that GNN, a soluble fiber of the Japanese Konjac plant, as used in this study and setting for 4 wk, was not effective in reducing the frequency or severity of pain in children with abdominal pain-related FGIDs. Although "no pain" was more likely to occur in the GNN group, the difference was of borderline statistical significance.

The study groups did not differ with regard to any of the secondary outcomes, such as the proportion of patients with gastrointestinal symptoms during the study period, the need for rescue therapy, and the occurrence of adverse effects.

### Fiber as a treatment option for FGIDs

The optimal management strategy for abdominal pain-related FGIDs in children is a matter of ongoing debate. Because of their obscure pathophysiology, the management of FGIDs remains challenging. This difficulty has prompted interest in new and safe treatment options, among them dietary interventions.

Given their safety profiles, prebiotics (especially soluble fiber) appear to be attractive therapeutic options for FGIDs<sup>[16]</sup>. Dietary fiber is not digested by human enzymes but is instead fermented by the flora of the large intestine<sup>[17,18]</sup>. There are several reasons why these agents might, in theory, prove to be beneficial in the management of functional disorders. First, some studies have demonstrated the positive effects of prebiotics on changes in the intestinal microbiota through the selective stimulation of the growth of potentially protective bacteria (bifidobacteria and, in part, lactobacilli) and the simultaneous inhibition of potentially pathogenic microorganisms<sup>[8,19-23]</sup>. Second, fiber increases biomass, feces weight and defecation frequency, which can alter the volume and composition of the stool and gas<sup>[8,13,18,24]</sup>. These changes in intestinal contents can affect the gastrointestinal symptoms associated with functional disturbances<sup>[2,8,18]</sup>. Additionally, a reduction in the pH and the release of short-chain organic acids stabilize the intestinal environment<sup>[8,16,19,25]</sup>. Finally, prebiotic-induced changes could indirectly modulate various parameters of the immune system, such as the NK-cell activity, the secretion of Il-10 and interferon, and the lymphocyte proliferation that may establish intestinal regularity<sup>[8,18,26]</sup>.

Dietary fiber consumption has remained low in many populations, especially in children. The American Academy of Pediatrics recommends a daily dietary fiber intake for children of 0.5 g/kg body weight, up to 35 g/d<sup>[27]</sup>. Williams *et al.*<sup>[28]</sup> proposed a minimum daily fiber intake equivalent to the age in years plus 5 g/d for children older than 2 years of age.

Few studies have evaluated the response of functional abdominal disorders to prebiotics. Furthermore, most data on the possible use of prebiotics in the management of functional disorders and the rationale for their use are derived from the studies of adults with IBS<sup>[16,19,26]</sup>. Until now, there have been only 3 published papers (Christensen 1982, Christensen 1986, Feldman 1985), which described 2 trials and assessed the effect of fiber supplementation on FGIDs in children<sup>[9]</sup>. These 2 trials, which met the inclusion criteria for the Cochrane systematic review, concerned the dietary interventions for recurrent abdominal pain (RAP) and irritable bowel syndrome (IBS) in children<sup>[9]</sup>. Both studies involved a total of 92 children with recurrent abdominal pain, and they evaluated the effect of fiber supplementation on the improvement in gastrointestinal complaints<sup>[11,12]</sup>. According to this meta-analysis, the pooled odds ratio for improvement in the frequency of abdominal pain was 1.26 (95%CI: 0.25-6.29)<sup>[9]</sup>. Feldman *et al.*<sup>[12]</sup> (52 children recruited) described changes in the intensity of pain; however, the differences between

the fiber and placebo groups were not statistically significant. Christensen *et al.*<sup>[11]</sup> (40 children recruited) reported the mean number of episodes of pain during the study period, but the differences in the results between groups were also not statistically significant. The lack of recent findings concerning the potential application of fiber in the management of children with FGIDs instigated our decision to examine the effectiveness of GNN, which is composed of a dietary fiber, *i.e.*, water-soluble polysaccharide.

### Dose and duration of treatment

The optimal dose and treatment duration of GNN therapy have not yet been clearly established. Recently, we used GNN to treat functional constipation in children, where it proved to be relatively safe<sup>[13]</sup>. In the present study, regardless of the dietary recommendations for daily fiber intake, we used only one standard dose of fiber for all participants to provide a relatively easy administration regimen for the clinicians and parents. We applied a daily intake of 2.52 g/d, which exceeded the minimum dose of fiber suggested in the literature for therapeutic purposes<sup>[13]</sup>.

Considering the chronic nature of functional disorders, the chosen duration of treatment (4 wk) appears to be optimal for evaluating GNN's potential therapeutic influence on gastrointestinal complaints. The administration of GNN, a soluble fiber preparation, was the sole intervention implemented, allowing one to draw conclusions regarding its effectiveness without any confounding influence from other treatments.

### Participants

Evaluating children as study participants is a complicated issue because they complain of nonspecific chronic abdominal pain and constitute a heterogeneous group of patients. To minimize the heterogeneity of our study's population, the diagnosis was based on the well-recognized Rome III criteria<sup>[5]</sup>.

The trial was conducted in a pediatric department oriented towards the diagnosis and treatment of children with functional abdominal pain. Nevertheless, the possibility that only less affected patients were included in the study could not be entirely eliminated. The pattern of the response to treatment may differ from that in patients with more severe courses of functional disturbances.

### Strengths and limitations of the study

We used the appropriate methods to generate the allocation sequence and allocation concealment. We then strived to maintain the blinding of the selection, treatment, monitoring, data management, and data analyses throughout the study. In addition, the follow-up was appropriate. Data were obtained from more than the 94% of the participants. All of these features minimize the risk of systematic bias.

The lack of an apparent effect of GNN compared with the placebo may be explained by an inadequate dose

and an overly brief treatment period. Although the optimal dose and duration of treatment for GNN have not yet been established, a tentative conclusion can be drawn: subjects with abdominal pain-related FGIDs require a modification of the GNN dose and an extension of the period of supplementation to improve their gastrointestinal symptoms. Additionally, nutritional habits can modify the natural course of abdominal complaints. We realized that precise data on the daily fiber intake would be useful. However, collecting that type of information would have required the use of a food diary. We abstained from this process for 2 major reasons. First, our study population of children aged 7-17 years included children who attended school. Collecting reliable information from children in this age group represents a particularly challenging task. Second, the use of a food diary, especially in this group of patients, provides only an approximate evaluation of dietary intake. Moreover, the validity of paper diary records is sometimes questionable. Well-known problems with paper diaries include poor adherence and retrospective recording<sup>[29]</sup>. As there were no differences between the groups in terms of the baseline characteristics, we may expect that the randomization was implemented properly and that the daily fiber intake was similar in both groups.

It is known that success in treating patients with abdominal pain-related FGIDs depends on the relationship established between a patient and a physician. An “active listening approach” and an enthusiastic, positive, and encouraging attitude towards treatment help improve subjects’ responses to both the therapeutic attempts and the placebo<sup>[30]</sup>. The placebo effect in adults ranges from 10%-70% for FD and from 0%-84% for IBS<sup>[6]</sup>. We used a placebo control group, which is considered an essential requirement for interventional studies<sup>[31]</sup>. According to our previous experiences based on earlier studies conducted in children with functional abdominal pain and functional constipation, we were able to predict the high proportion of children who were responsive to the placebo<sup>[3,13]</sup>. Considering our positive patient-physician relationship, we expected a drop-out rate of approximately 10% (6% at the end of the trial), in contrast to the 20% drop-out rate estimated for most trials. Thus, paradoxically, these positive relationships can be a potential weakness of our investigation.

Finally, another potential limitation of this trial is that although the overall number of patients was adequate, the study did not allow the analysis of the data for a specific diagnosis of abdominal pain-related FGIDs, *e.g.*, FD, IBS or FAP.

Consequently, an obvious next step in future research should be to identify the characteristics of children with specific abdominal pain-related FGIDs who respond to GNN treatment. Identifying this group of patients will allow for the selection of the optimal GNN dose to improve long-term gastrointestinal symptoms. Further studies are also needed to explore the role of GNN in the pathophysiology of functional disorders.

In our setting, GNN as dosed in this study was no more effective than the placebo in achieving therapeutic success in the management of abdominal pain-related FGIDs in children.

## COMMENTS

### Background

Abdominal pain-related functional gastrointestinal disorders (FGIDs) are widespread complaints in the pediatric population, affecting from 13% to 38% of children and adolescents. The symptoms associated with FGIDs have a great impact on patients’ quality of life and their daily activities, which in turn can result in long-term psychological implications. Therefore a variety of therapeutic options have been considered to date.

### Research frontiers

Many clinicians routinely recommend the use of bulking agents or dietary fiber to stimulate regular bowel movements and to improve symptoms associated with FGIDs. However, only two small, randomized clinical trials (Christensen 1982, Feldman 1985) conducted in children with FGIDs, which compared the effects of added dietary fiber with placebo, were identified. The results and conclusions of these trials were not consistent.

### Innovations and breakthroughs

Development of the optimal management strategy for abdominal pain-related FGIDs in children is difficult. Soluble fiber seems to be an attractive therapeutic option for FGIDs. Only a small number of studies that have evaluated the response of functional abdominal disorders to fiber are available. Additionally, the optimal dose and treatment duration have not been clearly established yet. In the present study, the standard daily dose (2.52 g/d) of glucomannan was used for all participants in order to provide a relatively easy administration regimen for clinicians and parents. The dose was equal to the minimum dose of fiber suggested in the literature for therapeutic purposes. Because of the chronic nature of functional disorders, the 4 wk duration of treatment was chosen to evaluate glucomannan’s potential therapeutic influence on gastrointestinal complaints. The most important contribution of this study and its strength is its methodology. The adequate methods for generation of the allocation sequence, allocation concealment and blinding of selection, treatment, monitoring, data management, and data analyses were used throughout the study. Data were obtained from more than the 94% of the participants. All of these features minimize the risk of systematic bias.

### Applications

The study results suggest that, glucomannan was no more effective than placebo in achieving therapeutic success in the management of abdominal pain-related FGIDs in children.

### Terminology

Glucomannan, a polysaccharide of 1,4-D-glucose and D-mannose, is a soluble fiber from the Japanese Konjac plant. Glucomannan exhibits properties that are typical of dietary fiber. There are several reasons why these agents might, in theory, prove to be beneficial in the management of functional disorders. Some studies have demonstrated positive effects of fiber on changes in the intestinal microbiota by selective stimulation of the growth of potentially protective bacteria with simultaneous inhibition of potentially pathogenic microorganisms. Fiber increases biomass, feces weight and defecation frequency, which can in turn alter the volume and composition of stool and gas. These changes in intestinal contents can affect the gastrointestinal symptoms associated with functional disturbances.

### Peer review

This study employs a simple, methodologically sound model. The main conclusion of the study is the lack of influence of glucomannan supplementation on the level and duration of pain in children with FGIDs.

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P- Reviewers Martellucci J, Sarin YK S- Editor Wen LL  
L- Editor A E- Editor Zhang DN



## Efficacy and safety of 0.4 percent sodium hyaluronate for endoscopic submucosal dissection of gastric neoplasms

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Supported by BMI Korea, Co., Ltd

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Received: December 29, 2012 Revised: March 19, 2013

Accepted: April 13, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To evaluate the efficacy and safety of sodium hyaluronate solution (SH) in endoscopic submucosal dissection (ESD) of gastric neoplasms.

**METHODS:** A prospective multicenter randomized, double blind, controlled trial was designed and utilized in this study. A total of 76 patients with 5-20 mm sized gastric neoplasms were enrolled at three academic hospitals in South Korea from June 2011 to October 2011. Patients were randomly assigned to the 0.4% sodium

hyaluronate or control groups. All lesions underwent endoscopic ESD. ESD was performed with 0.4%SH and normal saline (NS) solution for submucosal injection. Efficacy was assessed using *en bloc* resection and the number of additional injections. Secondary evaluation variables were the volume of injection material, steepness of mucosal elevation, bleeding rate, procedural time and operator satisfaction. Finally, the safety was assessed by analyzing adverse events during the study.

**RESULTS:** The usefulness rate in the 0.4%SH group and the controlled group had statistically significant difference under intention to treat (ITT) analysis (90.91% vs 61.11%  $P = 0.0041$ ). Under per protocol (PP), the usefulness rate is statistically significant different (93.10% vs 61.76%,  $P = 0.0036$ ). The difference in volume of the solution injected between 0.4%SH group and the controlled group and NS group was also statistically significant under intention to treat and per protocol analysis (ITT:  $0.03 \pm 0.02$  mL vs  $0.06 \pm 0.03$  mL,  $P = 0.0003$ , PP:  $0.03 \pm 0.02$  mL vs  $0.06 \pm 0.03$  mL,  $P = 0.0004$ ). Satisfaction above the grade good was significantly higher in the SH group under intention to treat and per protocol analysis (ITT: 90.91% vs 61.11%,  $P = 0.0041$ , PP = 93.11% vs 61.77%,  $P = 0.0022$ ). Adverse events above grade 3 were not noticed in either group. All adverse events were treated and were judged as not associated with the submucosal injection solutions.

**CONCLUSION:** 0.4%SH solution is a safe and effective agent that doesn't cause any significant adverse events and is useful for submucosal injection during ESD.

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**Key words:** Sodium hyaluronate; Endoscopic submucosal dissection; Gastric neoplasm; Endoscopic mucosal resection

**Core tip:** Saline-assisted endoscopic mucosal resection is an established method for excision of nonpolypoid early neoplastic lesions of the gastrointestinal tract. However, it is sometimes difficult to maintain a desired level of tissue elevation after injection of saline, especially when using a one-channeled endoscope. Adequate elevation of the mucosa and sufficient elevation time is achieved more effectively when a material more viscous than normal saline (NS) is used. The 0.4% sodium hyaluronate solution (SH) used in this study provides a more effective and prolonged cushion effect for large lesions without serious adverse events compared to NS. Therefore, endoscopic submucosal dissection (ESD) with SH is more useful than ESD with NS.

Kim YD, Lee J, Cho JY, Kim SW, Kim SH, Cho YK, Jang JS, Han JS, Cho JY. Efficacy and safety of 0.4 percent sodium hyaluronate for endoscopic submucosal dissection of gastric neoplasms. *World J Gastroenterol* 2013; 19(20): 3069-3076 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3069.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3069>

## INTRODUCTION

Endoscopic mucosal resection (EMR) using submucosal saline injection is an established method for excision of nonpolypoid early neoplastic lesions of the gastrointestinal tract<sup>[1,2]</sup>. Numerous resection methods have been developed using the submucosal injection technique. In 1998, Hosokawa developed an IT knife (insulated tipped electro-surgical knife) useful for endoscopic submucosal dissection (ESD)<sup>[3]</sup>, which has made *en bloc* resection of not only elevated lesions but depressive lesions without ulcers and flat lesions possible, compared to EMR. The procedure has minimal limitations regarding location and size of the lesions. The technical advancement of ESD combined with development of many endoscopic accessories has made ESD a standard treatment of early gastric cancer in selected cases.

The submucosa is a thin connective tissue layer with a lax structure compared to the mucosa or the muscularis propria. The injection of solutions to this layer forms a bulla and lifts the lesion above. Submucosal injection during ESD facilitates the removal of the lesions, provides a safety cushion during resection, and prevents perforations during the procedure. The most commonly used material is normal saline (NS). However, it is sometimes difficult to maintain a desired level of tissue elevation after an injection of saline, especially when using a one-channeled endoscope<sup>[4]</sup>.

An ideal submucosal injection solution would have a prolonged cushion effect, and be easily available and inexpensive, nontoxic, and easy to inject<sup>[5]</sup>. Sodium hyaluronate solution (SH) is a macromolecular polysaccharide composed of *D*-glucuronate and *N*-acetyl-*D*-

glucosamine. The high viscosity, elasticity, and lack of antigenicity or toxicity<sup>[6-8]</sup> have led to its extensive use in ophthalmologic surgical procedures and intra-articular injections. Since Yamamoto *et al*<sup>[9]</sup> reported the use of SH in porcine stomach yielding a more distinct and prolonged submucosal elevation compared to NS, numerous studies have used SH for difficult cases.

The authors aimed to compare the usefulness rate and safety of 0.4%SH in ESD as compared to NS in a multicenter prospective randomized double-blind control study.

## MATERIALS AND METHODS

### Patient selection

From June 2011 to October 2011, 76 patients with less than 20 mm sized early gastric cancer or gastric adenoma were enrolled in 3 independent academic hospitals in South Korea. Experienced endoscopists with more than 300 cases of ESD experience performed the ESD. Criteria for inclusion in the study were: (1) patients between the ages of 20 and 80; (2) patients with 5- to 20-mm early gastric cancer or gastric adenoma; and (3) patients who gave written informed consent. The limitation of the lesion size to less than 20 mm was based on the absolute indication criteria of ESD. Exclusion criteria of the study were: (1) residual or recurrent lesion; (2) lesions accompanied by ulcers; (3) undifferentiated cancer; (4) advanced malignant neoplasm; (5) patient with pacemakers; (6) history of hypersensitivity to hyaluronic acid; (7) serum creatinine  $\geq$  1.5 mg/dL or creatine clearance  $\leq$  50 mL/min; (8) patients with severe liver disease; (9) severe cardiovascular disease; (10) patient taking immunosuppressants including prednisolone or anti-cancer agents; (11) alcohol- or drug-addicts; (12) pregnant or lactating patients; and (13) patients judged by a physician as inappropriate for inclusion.

### Study design

The clinical trial was approved by the Korean Food and Drug Administration (KFDA) and the study protocol was approved by the Institutional Review Board of each center. Written informed consent was obtained from all the patients.

The study was double-blinded by having an independent investigational device manager that supplied the solutions used for the study. The endoscopist was blind to the material used. During the procedure, an experienced assistant did the injection of fluids to the submucosa. Although the endoscopist could visualize the elevation effect, the difference in pressure during injection of the material was known only to the injecting assistant. The injecting assistant did not take any part in evaluating the satisfaction grade of the materials. All lesions were resected through ESD. The margin of the lesion was marked through electrocautery and submucosal injection was done at the normal mucosa adjacent to the lesion. ESD was initiated after an adequate amount of solution was injected to lift the lesion. The maximum amount of

0.4%SH solution (Endo-Mucoup, BMI Korea, Co., Ltd) for the procedure was limited to 40 mL, which is 1/10 the nontoxic level administered into the peritoneum. The amount of SH solution supplied for the procedure was limited to 40 mL and any additional amount of injection needed used NS. Necessary medication or procedure for the treatment of coexisting disorders of the patients was allowed. Except for the use of epinephrine and indigo carmine, any other material for submucosal injection solution was prohibited.

The study was designed as a multicenter, randomized, double blind, and placebo-controlled trial. The primary outcome of the study was *en bloc* complete resection and additional injections of the solution used. *En bloc* complete resection was defined as *en bloc* resection with negative resection margins confirmed histopathologically. Additional resection was defined as the number of additional injects required to maintain the mucosal lifting for the procedure. The usefulness rate was defined as the percentage of *en bloc* complete resection with 1 or less additional injection during the procedure (Table 1). The 40 mL of SH was decided for the efficacy evaluation as it is 1/10 the nontoxic level administered into the peritoneum.

Secondary outcomes of the study included: (1) volume of the solution injected; (2) the steepness of the lift; (3) presence or absence of bleeding; (4) procedure time; and (5) satisfaction of the solution. The volume of the solution used for evaluation was the total amount of the solution divided by the area of the lesion (long diameter  $\times$  short diameter). The steepness of the lift was graded as steep, mild, or non lifted. The percentage of each grade was used for evaluation. Bleeding was defined as the need for electrocauterization before the incision and after injecting the submucosal solution. The procedure time was defined as the period from the marking of the margins to the completion of the excision. Satisfaction rate of the solution was comprehensively assessed by evaluating the *en bloc* complete resection rate and the number of additional injections (Table 1).

The safety of the solution was assessed through 5 grades of symptoms and signs unwarranted during the study. Grade 1 adverse event was defined as symptoms or signs that do not require treatment and do not inhibit daily activities. Grade 2 adverse events were defined as symptoms or signs that required treatment but did not inhibit daily activities. Grade 3 adverse events were when the patient experienced substantial discomfort leading to limitation of daily activities and requiring admission. Life-threatening adverse events were defined as grade 4. Death was defined as grade 5. For each adverse event, the clinician assessed the association of the response to the submucosal injection solution as definitely related, probably related, possibly related, probably not related, definitely not related, or unknown.

### Sample size

This multi center randomized, double-blind, placebo-controlled study was designed with a level of significance

$\alpha = 0.05$  and detection power  $1 - \beta = 0.8$  to test the superiority of the 0.4%SH solution compared to NS for the lift and maintenance of the submucosa during ESD. The calculated sample size was 76 patients. Thirty-four patients were analyzed in each group assuming a 10% rate of non-comparability in the two groups.

### Statistical analysis

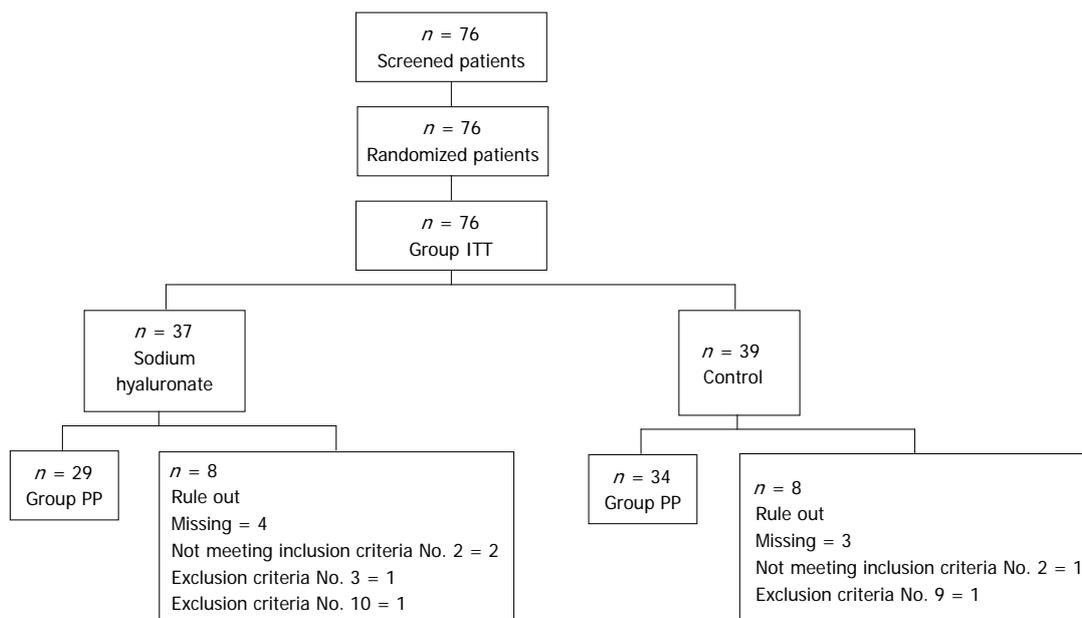
Statistical analysis was done using SAS software 9.1 version (SAS Institute, Cary, NC). Intention to treat analysis and per protocol analysis was used in the study. The clinical usefulness was assessed using both methods, with intention to treat as the primary method. Per protocol analysis was used for demographic data and adverse effects. Continuous variables for the two groups were compared using *t*-test and  $\chi^2$  test or the Fisher's exact test, which was used for categorical variables. The primary outcome, the usefulness rate, was analyzed using the  $\chi^2$  test and 95% CIs are given. The secondary outcomes, volume of the solution used and procedure time were analyzed using *t*-test. Presence or absence of bleeding and satisfaction of the solution was assessed using  $\chi^2$  test. Safety evaluation using the total number of the adverse effects and the rate of patients with more than 1 adverse effect was done using the  $\chi^2$  test. Null hypotheses of no difference were rejected if *P*-values were less than 0.05.

## RESULTS

Seventy-six patients who underwent screening examination and who gave informed written consent were included in this study. Using randomization, 37 patients were assigned to the 0.4%SH group and 39 patients were assigned to the control group that used NS for submucosal injection. All these patients were included in the intention to treat analysis. Eight patients from the 0.4%SH group and 5 patients from the control group were either lost to follow up or had a clinical trial protocol violation; the remaining 63 patients were included in the per protocol analysis (Figure 1). There were no statistically significant differences in the patients' age, sex, percentage of gastric cancer, or location of the lesion between the two groups (Table 2).

### Clinical usefulness

Usefulness, the primary outcome of the study, was assessed using both intention to treat analysis and per protocol analysis, with intention to treat as the primary method. Under intention to treat analysis, the usefulness rate of the 0.4%SH group (90.91%, 30/33) was found to be significantly greater than the control group (61.11%, 22/36) ( $P = 0.0041$ ). Using the per protocol analysis, the usefulness rate of the 0.4%SH group and the control were 93.10% (27/29) and 61.76% (21/34), respectively. The difference of the usefulness rate using per protocol analysis was also statistically significant ( $P = 0.0036$ ). The usefulness according to the site of the gastric neoplasm,



**Figure 1 Trial profile.** Inclusion criteria No. 2, 5 mm ≤ adenoma or adenocarcinoma ≤ 20 mm; exclusion criteria No. 3, undifferentiated adenocarcinoma; exclusion criteria No. 9, severe functional abnormalities of cardiovascular system; exclusion criteria No. 10, concomitant medication with systemic prednisolone, anticancer agents or immunosuppressive agents. ITT: Intention to treat; PP: Per protocol.

**Table 1 Measurement of usefulness and satisfaction**

| Complete <i>en bloc</i> resection | Additional injection | Usefulness | Satisfaction |
|-----------------------------------|----------------------|------------|--------------|
| Complete                          | 0                    | Usefulness | Excellent    |
| Complete                          | 1                    | Usefulness | Good         |
| Complete                          | ≥ 2                  | Useless    | Moderate     |
| Incomplete or not evaluated       | -                    | Useless    | Poor         |

using the intention to treat (ITT) analysis revealed that 0.4%SH was statistically significantly useful when used during procedures for gastric neoplasms at the body and angle. However, there was no statistically significant difference in procedures done at the antrum. Using a per-protocol analysis, 0.4%SH showed statistically significant usefulness in all the sites of the stomach.

Secondary outcomes of the study were analyzed including volume of the solution injected, the steepness of the lift, presence or absence of bleeding, procedure time, and satisfaction of the solution (Table 3).

The volume of the solution used for evaluation was the total amount of the solution divided by the area of the lesion (long diameter × short diameter). The volume of the solution injected for the 0.4%SH group (0.03 ± 0.02 mL) was significantly less compared to the control group (0.06 ± 0.03 mL) (P = 0.0003). The difference in volume of the solution injected between the 0.4%SH group and NS group using per protocol analysis was also statistically significant (P = 0.0004).

The procedure time was defined as the period from the marking of the margins to the completion of the excision. The procedure time analyzed by intention to treat showed 23.42 ± 16.76 min in the 0.4%SH group

and 21.64 ± 16.52 min in the control group. Using the per protocol analysis the procedure times were 23.79 ± 17.51 min in the 0.4%SH group and 19.71 ± 12.65 min in the control group. Although the procedure time for the 0.4%SH group was shorter compared to the control group, the difference was not statistically significant.

The steepness of the lift was graded as steep, mild, or non lifted in the two study groups and showed no statistically significant differences.

The absence of bleeding during injection of the submucosal solution was higher in the 0.4%SH group (96.97%, 32/33) compared to the control group (88.89%, 32/36) using the intention to treat analysis. Using the per protocol analysis, the absence of bleeding was 100% (29/29) in the 0.4%SH group and 88.24% (30/34) in the control group. However, the difference was not statistically significant in both analyses.

Satisfaction with the solution was comprehensively assessed by evaluating the *en bloc* complete resection rate and the number of additional injections. ESD with *en bloc* complete resection and no additional submucosal injection was defined as excellent. *En bloc* complete resection with 1 additional submucosal injection was defined as good. Satisfaction above the grade good was significantly higher in the 0.4%SH group (90.91%, 30/33) compared to the control group (61.11%, 22/36) using intention to treat analysis (P = 0.0175). Using per protocol analysis showed statistically significant difference in the satisfaction above the grade good in 93.11% (27/29) and 61.77% (21/34) in the 0.4%SH group and control group, respectively (P = 0.0022).

The ESD that had additional submucosal injections was analyzed by the site of the lesion using the usefulness rate, volume of solution injection and procedure time (Table 4).

**Table 2** Demographic characteristics of the patients at base line

|                         | Sodium hyaluronate | Control      | P-value |
|-------------------------|--------------------|--------------|---------|
| Age                     | 62.59 ± 9.23       | 62.44 ± 9.93 | 0.943   |
| Sex (male/female)       | 25/12              | 26/13        | 0.933   |
| Diagnosis, <i>n</i> (%) |                    |              |         |
| Adenocarcinoma          | 5 (13.51)          | 7 (17.95)    | 0.869   |
| Adenoma                 | 31 (83.78)         | 31 (79.49)   |         |
| Atypia                  | 1 (2.7)            | 1 (2.56)     |         |
| Size (mm)               | 14.2 ± 5.47        | 13.5 ± 4.35  | 0.293   |
| Location, <i>n</i> (%)  |                    |              |         |
| Antrum                  | 23 (62.16)         | 29 (74.35)   | 0.465   |
| Angle                   | 3 (8.10)           | 3 (7.69)     |         |
| Body                    | 11 (29.72)         | 7 (17.94)    |         |

**Table 3** Summary of results *n* (%)

|                     | Sodium hyaluronate | Control       | P-value |
|---------------------|--------------------|---------------|---------|
| ITT                 |                    |               |         |
| Usefulness rate     | 30/33 (90.91)      | 22/36 (61.11) | 0.004   |
| Volume of injection | 0.03 ± 0.02        | 0.06 ± 0.03   | 0.000   |
| Procedural time     | 23.42 ± 16.76      | 21.64 ± 16.52 | 0.658   |
| Steepness           |                    |               | 0.228   |
| Steep               | 26 (78.79)         | 27 (75.00)    |         |
| Mild                | 6 (18.18)          | 4 (11.11)     |         |
| Non lifted          | 1 (3.03)           | 5 (13.89)     |         |
| Absence of bleeding | 32/33 (96.97)      | 32/36 (88.89) | 0.359   |
| Satisfaction        | 30/33 (90.91)      | 22/36 (61.11) | 0.002   |
| PP                  |                    |               |         |
| Usefulness rate     | 27/29 (93.10)      | 21/34 (61.76) | 0.004   |
| Volume of injection | 0.03 ± 0.02        | 0.06 ± 0.03   | 0.000   |
| Procedural time     | 23.79 ± 17.51      | 19.71 ± 12.65 | 0.288   |
| Steepness           |                    |               | 0.474   |
| Steep               | 24 (82.76)         | 26 (76.47)    |         |
| Mild                | 4 (13.79)          | 4 (11.76)     |         |
| Non lifted          | 1 (3.45)           | 4 (11.76)     |         |
| Absence of bleeding | 29/29 (100.00)     | 30/34 (88.24) | 0.118   |
| <i>n</i> (%)        | 27/29 (93.11)      | 21/34 (61.77) | 0.002   |

ITT: Intention to treat; PP: Per protocol.

The sites of the lesion were divided into the antrum group and body/angle group. Under intention to treat analysis on antrum, there is no significant difference of the usefulness rate in the 0.4%SH group (90.00%, 18/20) compared to the control group (66.67%, 18/27) ( $P = 0.086$ ). But using per protocol analysis, the antrum had a significantly higher usefulness rate in the 0.4%SH group (93.75%, 15/16) compared to the control group (65.38%, 3/8) ( $P = 0.036$ ). The usefulness rate analyzed in the lesions located at the body/angle using intention to treat analysis was 92.30% (12/13) in the 0.4%SH group compared to the 66.67% (6/9) in the control group. The difference was statistically significant ( $P = 0.007$ ). Per protocol analysis also revealed a statistically significant higher usefulness rate of 92.30% (12/13) in the 0.4%SH group compared to 37.50% (3/8) in the control group ( $P = 0.007$ ).

The amount of volume injected at the antrum using intention to treat analysis was  $0.03 \pm 0.02$  mL in the 0.4%SH group and  $0.06 \pm 0.04$  mL in the control group.

**Table 4** Summary of results on location

|                     | Sodium hyaluronate | Control        | P-value |
|---------------------|--------------------|----------------|---------|
| ITT                 |                    |                |         |
| Usefulness rate     |                    |                |         |
| Antrum              | 18/20 (90.00%)     | 18/27 (66.67%) | 0.086   |
| Body/angle          | 12/13 (92.30%)     | 6/9 (66.67%)   | 0.007   |
| Volume of injection |                    |                |         |
| Antrum              | 0.03 ± 0.02        | 0.06 ± 0.04    | 0.003   |
| Body/angle          | 0.03 ± 0.02        | 0.05 ± 0.02    | 0.047   |
| Procedural time     |                    |                |         |
| Antrum              | 19.40 ± 7.46       | 18.41 ± 11.67  | 0.741   |
| Body/angle          | 28.08 ± 25.47      | 31.33 ± 24.68  | 0.768   |
| PP                  |                    |                |         |
| Usefulness rate (%) |                    |                |         |
| Antrum              | 15/16 (93.75%)     | 17/26 (65.38%) | 0.036   |
| Body/angle          | 12/13 (92.30%)     | 3/8 (37.50%)   | 0.007   |
| Volume of injection |                    |                |         |
| Antrum              | 0.03 ± 0.02        | 0.06 ± 0.04    | 0.011   |
| Body/angle          | 0.03 ± 0.02        | 0.05 ± 0.03    | 0.039   |
| Procedural time     |                    |                |         |
| Antrum              | 19.06 ± 6.78       | 18.23 ± 11.88  | 0.800   |
| Body/angle          | 28.08 ± 25.47      | 24.50 ± 14.70  | 0.723   |

ITT: Intention to treat; PP: Per protocol.

The smaller amount used in the 0.4%SH group was statistically significant ( $P = 0.003$ ). Using per protocol analysis, the difference in the amount of volume injected 0.4%SH group ( $0.03 \pm 0.02$  mL) and control group ( $0.06 \pm 0.04$  mL) was statistically significant ( $P = 0.011$ ).

The amount of volume injected at the body/angle using intention to treat analysis was  $0.03 \pm 0.02$  mL in the 0.4%SH group and  $0.05 \pm 0.02$  mL in the control group. The smaller amount used in the 0.4%SH group was statistically significant ( $P = 0.047$ ). Using per protocol analysis, the difference in the amount of volume injected 0.4%SH group ( $0.03 \pm 0.02$  mL) and control group ( $0.05 \pm 0.03$  mL) was statistically significant ( $P = 0.039$ ).

The procedure time using both intention to treat analysis and per protocol analysis based on the site of the lesion showed no statistically significant differences.

### Adverse events

Adverse events were seen in 8 out of the 76 patients, with 13 incidents in the intention to treat analysis. Grade 1 adverse events were noticed in 12.50% (5/37) of the SH group and 2.44% (1/39) of the control group. Grade 2 adverse events were seen in 7.50% (3/37) and 9.76% (4/39) of the 0.4%SH group and control group, respectively. Adverse events above grade 3 were not noticed in both groups. Gastrointestinal symptoms were the most common adverse events with 10 out of the 13 incidents. Five cases of nausea, 3 cases of vomiting, and each 1 case of dyspepsia and hematemesis were noticed. All adverse events were treated and were judged as not associated with the submucosal injection solutions.

## DISCUSSION

ESD is now a standard treatment for early gastric neo-

plasms in the gastrointestinal tract. The EMR used currently are strip-biopsy method or lift and cut EMR<sup>[10]</sup>, endoscopic resection after local injection of a solution of hypertonic saline and epinephrine<sup>[11]</sup>, endoscopic double-snare polypectomy<sup>[12]</sup>, EMR using an over-tube<sup>[13]</sup>, strip-biopsy using two small diameter endoscopes<sup>[14]</sup>, EMR with a cap-fitted panendoscope<sup>[15]</sup>, an EMR using a ligation device<sup>[16,17]</sup>. The submucosal injection of solutions during the resection of gastrointestinal neoplasm is an essential part of the resection procedure<sup>[18-21]</sup>. Tanabe *et al.*<sup>[10]</sup> introduced strip biopsy with submucosal injection using NS as a safety cushion. Since Ikeda *et al.*<sup>[22]</sup> injected a mixture of hypertonic saline and epinephrine to lift the lesion in 1986, lifting the lesion with a safety cushion underneath the lesion has become an essential procedure in EMR. ESD can resect a larger lesion without regard to the location of the lesion compared to EMR. To achieve an *en bloc* resection of a large lesion, adequate lift of the lesion is needed for a prolonged period. Numerous solutions are under research and have been used for mucosal resection. These agents include NS, mixture of NS and epinephrine, hypertonic saline (3.8%), hypertonic glucose solution (20%, 50%), 10% glycerin + 5% fructose + 0.9% NS, SH, hydroxypropyl methylcellulose, and a fibrinogen mixture. NS is easily available, cheap, and causes minimal tissue injury due to its isotonic property. However, NS is easily absorbed to the tissue and multiple injections are needed. The mixture of NS and epinephrine (1:10000) is most widely used. The steepness and maintenance of the cushion is not significantly different from NS, but the mixture of epinephrine causes vasoconstriction of the vessels leading to hemostasis. The addition of indigo carmine to the mixture assists in identifying the submucosa and muscularis propria during resection of the deep margins. However, during ESD, the cushion is easily dissipated and multiple injections are needed. This leads to a prolonged procedure time and the risk of perforation is high when resecting the lesion without adequate cushion. Therefore, an ideal agent would have a higher viscosity to maintain the cushion longer and require fewer additional injections during the procedure. Although hypertonic saline and hypertonic glucose solution had a steeper elevation compared to NS, this was not statistically significant. The hypertonic solution has a propensity to cause tissue injury and may delay healing of the artificial ulcer after the procedure.

SH has a prolonged cushion-effect and causes minimal tissue injury. However, the solution is expensive and has special requirements for storage. Hydroxypropyl methylcellulose has a similar viscosity and tensile strength compared to SH and has an adequate cushion-effect, is inexpensive, and is easy to store. However, the synthetic material can cause cross-antigen reaction in the body. Fibrinogen mixture is a 340 kDa high molecular glycoprotein separated from blood. The viscosity is high and has a microvascular hemostatic effect. However, the high viscosity requires a large diameter injection tip. Lee *et al.*<sup>[23]</sup> report that the fibrinogen mixture is superior to NS in

the procedure time, total volume of solution injection, and additional injection rates in ESD.

SH is considered the best solution for submucosal dissection. However, its use is limited due to the high cost of the solution. The molecular weight and dilution rate of SH is continuously researched to decrease the cost and find the dilution rate for effective lifting. Fujishiro *et al.*<sup>[24,25]</sup> report that the optimal viscosity and tensile strength was 1% 1900-kDa solution. However, 1% 800-kDa, 0.5% 1900-kDa, 0.5% z800-kDa, and 0.25% 1900-kDa solutions all showed similar results. Concentration of the solution below 1% still maintained adequate viscoelasticity while not increasing osmolarity or causing tissue injury. Despite the fact that it is an ideal substance for submucosal injection, the high cost and special requirements for storage led to a mixture of it with other hypertonic solutions.

This study evaluated the clinical usefulness and safety of 0.4%SH compared to NS. All procedures for the resection of the lesions were ESD. The size of the lesion was limited to 2cm due to the absolute indication of ESD for early gastric cancers without lymph node metastasis<sup>[14]</sup>. This led to the limitation of gastric adenoma size to less than 2cm. The primary outcome, usefulness rate, analyzed using intention to treat and per protocol analysis revealed higher and statistically significant rates in the 0.4%SH group. Secondary outcome of the study including volume of the solution injected, the steepness of the lift, presence or absence of bleeding, procedure time, and satisfaction with the solution were analyzed. The volume of the solution injected was statistically significantly decreased and satisfaction of the solution was significantly superior in the 0.4%SH group. The limitation of the size in the inclusion criteria may have limited the analysis of the resection rate in the two groups. The usefulness rate may have showed a more significant difference in the two groups as the size of the lesion is increased. The procedure time of the two groups in this study showed no statistically significant difference. This may have been due to the fact that the size of the lesion was limited to less than 2 cm. The experienced clinicians participating in this study may have decreased the difference in time required for the 2 groups. However, if larger lesions with scars were included in the study, even experienced clinicians may show a difference in the procedure time for the 2 groups due to the multiple injections that may be required to maintain the cushion-effect during the procedure. Therefore, a prospective study including gastric adenoma that has no limitation of size in *en bloc* resection using ESD may show a difference in the usefulness rate, volume of solution injected and satisfaction with the solution. The procedure time may show a significant difference because 0.4%SH had a prolonged cushion-effect and requires less additional injections compared to NS. There were grade 1 and grade 2 adverse events observed in this study. However, there was no statistically significant difference in the two groups and clinicians judged that the adverse events were not related to the solution

injected. Therefore, the use of 0.4%SH for submucosal injections during ESD is considered safe.

This study has a size limitation of 2 cm. Gotoda *et al.*<sup>26]</sup> reported that due to the lack of lymph node metastasis, mucosal gastric cancer less than 3 cm accompanied by an ulcer and submucosal gastric cancer invading the SM1 layer less than 3 cm are also indications of endoscopic therapy. This has formed the basis of the expanded criteria for endoscopic therapy in early gastric cancer. However, the current study was approved by the KFDA for absolute indications of ESD for early gastric cancer. The size of the gastric adenomas was also limited for evaluation of the two materials without bias.

The recent development of endoscopic accessories with more skilled clinicians has led to *en bloc* resection of lesions without regard to the location and size. This has led to a more extended indication of ESD and a solution with a long lasting cushion-effect is needed. Using NS for lesions that are easily approachable and small is cost-effective. However, for lesions that are large and difficult to approach, the procedure time could be prolonged with more frequent complications. In these cases, using 0.4%SH for the submucosal injection would be more cost-effective.

In conclusion, the ideal agent would have a prolonged cushion effect, be easily available, nontoxic, easy to inject, and inexpensive. SH has a long-lasting cushion effect compared to the widely used NS and leads to a safe and effective procedure. SH has no significant adverse events compared to NS and is safe. With the recent advances in the indication of ESD in early gastric cancer, development of endoscopic accessories, and more trained clinicians, the resection of large lesions without concern regarding the location of the lesion is possible. Therefore, with appropriate measures taken regarding price and storage issues, 0.4%SH may further enhance the ease and safety of ESD.

## COMMENTS

### Background

Endoscopic submucosal dissection (ESD) has made *en bloc* resection of early gastric neoplasms possible. A sufficient amount of submucosal fluid cushion is one of the important requisites for ESD. The ideal injection agent should provide a long-lasting submucosal cushion.

### Research frontiers

The submucosal injection during ESD is a requisite for safety during and after ESD. An ideal submucosal injection solution would have a prolonged cushion effect, be easily available, nontoxic, easy to inject, and inexpensive. Numerous solutions are being studied to determine if they are safe and effective in the procedure.

### Innovations and breakthroughs

There are no studies evaluating the efficacy and safety of sodium hyaluronate solution (SH) solution in ESD for gastric neoplasm in a Korean population.

### Applications

0.4%SH solution gives gastric mucosa long lasting lifting compared to normal saline (NS), thereby ESD using it is more effective compared to the control group. The solution is safe when comparing adverse events to the NS group. Therefore, 0.4%SH is a useful agent for submucosal injection during ESD.

### Peer review

This is a randomized controlled trial on efficacy and safety of 0.4% sodium hyaluronate for gastric ESD. It is worthy publishing.

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P- Reviewer Mishra PK

S- Editor Huang XZ L- Editor A E- Editor Lu YJ



## Robotic cholecystectomy with new port sites

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Received: December 26, 2012 Revised: March 23, 2013

Accepted: March 28, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To introduce robotic cholecystectomy (RC) using new port sites on the low abdominal area.

**METHODS:** From June 2010 to June 2011, a total of 178 RCs were performed at Ajou University Medical Center. We prospectively collected the set-up time (working time and docking time) and console time in all robotic procedures.

**RESULTS:** Eighty-three patients were male and 95 female; the age ranged from 18 to 72 years of age (mean  $54.6 \pm 15.0$  years). All robotic procedures were successfully completed. The mean operation time was  $52.4 \pm 17.1$  min. The set-up time and console time were  $11.9 \pm 5.4$  min (5-43 min) and  $15.1 \pm 8.0$  min (4-50 min), respectively. The conversion rate to laparoscopic or open procedures was zero. The complication rate was 0.6% ( $n = 1$ , bleeding). There was no bile duct injury or mortality. The mean hospital stay was  $1.4 \pm 1.1$  d. There was a significant correlation between the

console time and white blood cell count ( $r = 0.033$ ,  $P = 0.015$ ). In addition, the higher the white blood cell count (more than 10000), the longer the console time.

**CONCLUSION:** Robotic cholecystectomy using new port sites on the low abdominal area can be safely and efficiently performed, with sufficient patient satisfaction.

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**Key words:** Robotic cholecystectomy; Port sites; Operation time; Abdominal area; Gallbladder disease

**Core tip:** The robotic procedure is safe; however, it is not acceptable as a standard operation for gallbladder disease because of its lack of benefits for patients as a result of the high cost and prolonged operating time. In the previous studies, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. In this study, we changed the port placements from the upper abdominal area to the lower abdominal area.

Kim JH, Baek NH, Li G, Choi SH, Jeong IH, Hwang JC, Kim JH, Yoo BM, Kim WH. Robotic cholecystectomy with new port sites. *World J Gastroenterol* 2013; 19(20): 3077-3082 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3077.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3077>

### INTRODUCTION

Laparoscopic cholecystectomy is a standard technique for treatment of gallbladder diseases<sup>[1]</sup>. However, there are some disadvantages to using laparoscopic techniques<sup>[2]</sup>, and laparoscopic surgery can have a steep learning curve<sup>[3]</sup>. To overcome these limitations of laparoscopic techniques, the robotic-assisted procedure de-

veloped, the 3-dimensional view, magnification, tremor suppression, and flexibility of the instruments<sup>[4,5]</sup> have allowed precise operating techniques in general surgery<sup>[6-8]</sup>. Since the first robotic-assisted cholecystectomy was performed in 1997, many reports have been published, including comparative<sup>[8-10]</sup> and non-comparative studies<sup>[6,11-15]</sup>. All authors agreed on the safety and feasibility of the robotic procedure. However, most of them concluded that this procedure is not acceptable as a standard operation because of the lack of benefits for patients due to the high cost and prolonged operating time. In this regard, the benefits of the robotic procedure in gallbladder diseases have not yet been established.

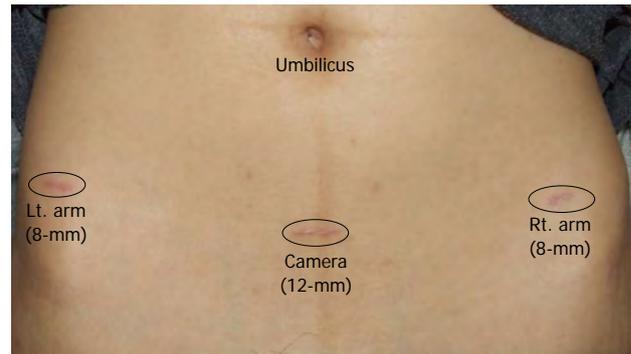
Based on the above reports, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. As a result, we performed robotic cholecystectomy, changing the port placements from the upper abdominal area to the lower abdominal area. In this study, we examined robotic cholecystectomy using port sites located on the low abdominal area and evaluated its surgical outcomes.

## MATERIALS AND METHODS

From June 2010 to June 2011, a total of 178 robotic cholecystectomies were performed at Ajou University Medical Center. We prospectively collected the set-up time (working time and docking time) and console time in all robotic procedures. The initial indications of surgery included gallbladder polyp or symptomatic gallstones. Exclusion criteria were the presence of acute cholecystitis and previous history of extensive upper abdominal surgery. Informed consent was obtained for the robotic cholecystectomy. We retrospectively reviewed the medical records of all patients and analyzed data, including demographic information, clinical presentation, results of laboratory studies, operative records, postoperative complications, and postoperative hospital stay.

In this study, the operating time was defined as the time from skin incision to wound closure. The working time extended from the first skin incision until the decision to bring the da Vinci into place was made. The docking time spanned the setup of the robot onto the surgical field. The set-up time was defined as the time from skin incision until the start the dissection. The console time was defined as the time from the start of dissection until the moment the gallbladder was completely freed from the liver.

The robotic-assisted operations were performed with the 4-arm da Vinci robot system (Intuitive Surgical, CA, United States). The operating team consisted of one operating surgeon and one assisting resident. The assisting resident replaced instruments and paced clips during cholecystectomy. Robotic cholecystectomy was performed using a three or four port technique. A total of three trocars were utilized as shown in Figure 1. First, a 12-mm trocar was inserted through a vertical incision



**Figure 1** Port sites of robotic cholecystectomy. A 12-mm trocar was inserted through a vertical incision below the umbilicus using an open method. The 8-mm ports were placed 7 to 10 cm distant from the endoscope.

below the umbilicus using an open method. CO<sub>2</sub> gas was introduced through this trocar to obtain an intraperitoneal pressure of 12 mm Hg. All other ports were placed under direct visualization. The 8-mm ports were placed 7 to 10 cm distant from the endoscope. An additional fourth trocar (3 or 5 mm) was placed in the right anterior axillary line in the upper quadrant in cases of severe inflammation of gallbladder and thickening of the gallbladder wall and used for retraction and clip/loop placement.

The patient was then placed in reverse Trendelenburg position with the right side up. The da Vinci surgical robot was then brought into position and docked. The Maryland forceps was inserted into the right robotic positioner, and a cadere grasper was placed into the left positioner. The dissection was performed according to the critical view method as described by Strasberg *et al.*<sup>[16]</sup>. After clear identification of the cystic duct and cystic artery, only the cystic duct was ligated manually with clips. In contrast, the cystic artery was coagulated just around the gallbladder and not ligated. The gallbladder was dissected from the fossa and placed in an endoscopic retrieval bag. Once fully dissected, the gallbladder was removed through the umbilical port in an endopouch. The robot was then withdrawn, and the 12-mm port site was closed with absorbable sutures.

Patients were discharged 1 d after surgery if sufficiently recovered and if pain and nausea had receded. All patients were seen for examination and reassessment at the outpatient clinics 1 wk after surgery. Laboratory tests were performed only if indicated.

### Statistical analysis

Statistical analysis was performed with independent *t*-test, and Spearman's correlation. *P*-value < 0.05 was considered statistically significant.

## RESULTS

### Clinical findings

Eighty-three patients were male and 95 female; the age

Table 1 Clinical characteristics

|   | RC (n = 178)    |
|---|-----------------|
| Age (yr)                                  | 40.1 ± 9.8      |
| Gender (male/female)                      | 83/95           |
| Laboratory findings                       |                 |
| White blood cell count (/m <sup>3</sup> ) | 7483.4 ± 2670.8 |
| AST (IU/L)                                | 62.9 ± 124.0    |
| ALT (IU/L)                                | 68.9 ± 134.4    |
| Total bilirubin (mg/dL)                   | 1.0 ± 0.9       |
| Combined diseases                         |                 |
| Diabetes mellitus                         | 5               |
| Hypertension                              | 29              |
| Ischemic heart disease                    | 0               |
| COPD                                      | 0               |

RC: Robotic cholecystectomy; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; COPD: Chronic obstructive pulmonary disease.

Table 2 Operation time of robotic cholecystectomy

|                      | RC (n = 178) |
|----------------------|--------------|
| Operation time (min) | 52.4 ± 17.1  |
| Set-up time (min)    | 11.9 ± 5.4   |
| Working time (min)   | 7.6 ± 4.2    |
| Docking time (min)   | 4.3 ± 2.5    |
| Console time (min)   | 15.1 ± 8.0   |

RC: Robotic cholecystectomy.

ranged from 18 to 72 years of age (mean  $54.6 \pm 15.0$  years). Table 1 shows the clinical characteristics of patients who underwent robotic cholecystectomy. The associated diseases were hypertension ( $n = 29$ ), diabetes mellitus ( $n = 5$ ), hypothyroidism ( $n = 2$ ), and hepatitis ( $n = 2$ ). The previous operations were appendectomy ( $n = 10$ ), C-section ( $n = 10$ ), and hysterectomy ( $n = 1$ ). Endoscopic retrograde cholangiopancreatogram was performed in 18 patients. After surgery, two patients in robotic cholecystectomy (RC) group were diagnosed with gallbladder (GB) cancer. A 53-year-old man who was diagnosed with a gallbladder mass underwent cholecystectomy with lymph node dissection. Unfortunately, tumor penetrated the serosa layer of the gallbladder (T3N0). We recommended re-operation; however, he refused the surgery in our hospital and did not follow up. The other patient was a 60-year-old man who had undergone surgery for gallbladder polyp and was diagnosed with T1a gallbladder cancer. There was no recurrence for 8 mo after surgery.

### Surgical outcomes

All robotic procedures were successfully completed. The mean operation time was  $52.4 \pm 17.1$  min. The set-up time and console time were  $11.9 \pm 5.4$  min (5-43 min) and  $15.1 \pm 8.0$  min (4-50 min), respectively (Table 2). The conversion rate to laparoscopic or open procedures was zero. The complication rates was 0.6% ( $n = 1$ , bleeding) (Table 3). The patient who had complications was a

Table 3 Surgical outcomes of robotic cholecystectomy

|                                 | RC (n = 178)  |
|---------------------------------|---------------|
| Complications                   |               |
| Bleeding                        | 1             |
| Bile duct injury                | 0             |
| Open conversion                 | 0             |
| Total hospital stay (d)         | $2.9 \pm 1.8$ |
| Postoperative hospital stay (d) | $1.4 \pm 1.1$ |

RC: Robotic cholecystectomy.

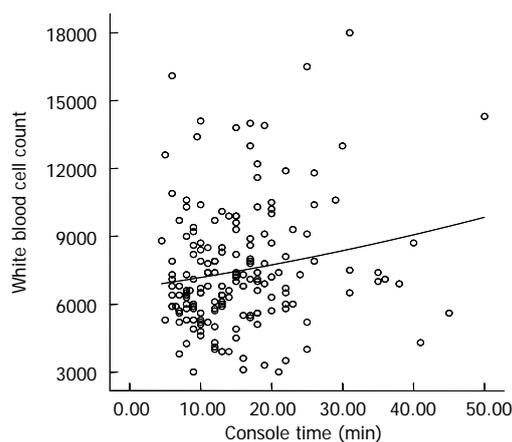


Figure 2 Correlation between console time and white blood cell count. There was a significant correlation between console time and white blood cell count ( $r = 0.182$ ,  $P = 0.015$ ).

34-year-old female who underwent re-operation on postoperative day 1; previous incisions on the low abdominal area were employed during the surgery. We identified the focus of bleeding on the gallbladder bed and coagulated the bleeder. She was finally discharged from the hospital without any symptoms. There was no bleeding associated with the cystic artery. There was no bile duct injury and mortality. The mean postoperative hospital stay was  $1.43 \pm 1.16$  d.

We analyzed the relationship between console time and other factors. The results showed that the only significant correlation was between console time and white blood cell count ( $r = 0.182$ ,  $P = 0.015$ ) (Figure 2). In addition, the higher the white blood cell count (more than 10000), the longer the console time (Table 4).

## DISCUSSION

Since 2005, when the robotic system was first introduced in South Korea, other investigators have also reported experience with surgical robotics in a variety of surgical procedures, including cholecystectomy, gastrectomy, and thyroidectomy<sup>[17-19]</sup>. However, hepato-biliary surgeons in South Korea stopped performing robotic-assisted cholecystectomy because of the lack of advantages for patients compared to its high cost<sup>[8,9,20]</sup>. In our hospital, we experienced the first fully robotic procedure in a pa-

**Table 4 Relationship between console time and inflammation of gallbladder**

|                    | WBC (< 10000)<br>(n = 151) | WBC (≥ 10000)<br>(n = 27) | P value |
|--------------------|----------------------------|---------------------------|---------|
| Console time (min) | 14.6 ± 7.5                 | 18.3 ± 9.9                | 0.025   |

WBC: White blood cell.

tient who was diagnosed with choledochal cyst; the patient underwent resection of the cyst and intracorporeal hepaticojejunostomy. After that, we decided to change the trocar placements when using the robotic system in gallbladder diseases. Before beginning robotic surgery, Professor Kim (Kim WH) also enrolled in the Intuitive Surgical da Vinci training course.

In the present study, we used only three of four arms of the da Vinci system to reduce the instrument-related costs. Most importantly, port sites were also much lower than the umbilical line. The 12-mm camera port site was located almost 10 cm from the umbilicus. The other two 8-mm port sites were located around the right and left anterior superior iliac spine. This line can be called the “Panty line” or “Bikini line”. All patients were satisfied with both the degree of postoperative pain and scarring. However, we experienced a problem in that the length of the robotic arms were too short to dissect the gallbladder, especially in big or obese patients. Indeed, Cadière *et al*<sup>[21]</sup>. Previously suggested that the robotic approach requires new operative strategies and a change in the pattern of trocar placement. Most surgeons will likely agree that, although Nio *et al*<sup>[9]</sup>. Reported on altered positioning of trocars in robot-assisted laparoscopic cholecystectomy, their locations were not different from the laparoscopic technique. In the early period of the study, we included patients who were diagnosed with gallbladder polyp or minimal symptomatic gallstones. As the number of cases increased, we attempted robotic procedures in cases of inflamed gallbladder, such as acute cholecystitis, empyematous cholecystitis, and gangrenous cholecystitis. According to previous reports, some authors did not perform robotic procedures in acute cholecystitis<sup>[11,22,23]</sup>. In study by Ruurda *et al*<sup>[14]</sup>, the rates of acute cholecystitis were 17%, and there was one conversion to an open procedure, caused by the surgeons’ inability to expose the gallbladder sufficiently because of severe cholecystitis. In the present study, there was no conversion to laparoscopic or open procedures after including cases of acute cholecystitis.

Thus far, most authors have reported that the operation time of robotic procedures was much longer than cases that used laparoscopic technique. The operation time varied, with a range of 55-152 min<sup>[13,14,21,23-28]</sup>. The reason for the various results in those studies was that the definition of the operation time was heterogeneous; the operation time was defined as the time from skin incision to skin closure, or it included anesthesia or time

in the robotic room. In the present study, the operation time was 81.3 ± 19.0, similar to other results<sup>[14,21,24-28]</sup>. Marescaux *et al*<sup>[24]</sup> reported that the median time for dissection was 25 min (range 14-109 min) and the overall operative time was 108 min, similar to times of conventional laparoscopy. In this study, the console time was 15.1 ± 8.0 min, similar to another report<sup>[24]</sup>. The console time in cases of inflamed gallbladder was longer than that of cases of non-inflamed gallbladder.

The conversion rates of robotic procedures in other studies have been almost zero<sup>[9,13,21,22,25,27]</sup>; our data showed similar results. However, Miller *et al*<sup>[28]</sup> reported that conversion to conventional laparoscopic techniques was necessary due to malfunction of graspers in three consecutive procedures. The other reasons for conversion were in cases of acute cholecystitis<sup>[24]</sup>, presence of severe adhesions, and poor visualization<sup>[26]</sup>. In this study, we found that the fourth 5-mm instrument was effective in grasping and retracting the edematous gallbladder. Until now, we have experienced no conversion after the indications were expanded to include severe acute cholecystitis. There are two published reports of 8-mm port-site hernias, so it should be recommended that any port greater than 5 mm in diameter should be routinely sutured closed<sup>[22,29]</sup>. We did not experience an 8-mm port-site hernia, likely due to the short follow-up period after surgery.

To our knowledge, this study is the first large series of robotic cholecystectomy in South Korea. Some advantages of robotic cholecystectomy from this study are as follows. First, patient satisfaction regarding a lower lying wound was very high because of the absence of a scar on the upper abdominal area. Second, the subjective perception of the surgeons is that the robotic system makes dissection easier at Callot’s triangle. Furthermore, the use of the robotics allowed the surgeon to remain in an ergonomic position throughout the procedure. This could reduce the fatigue experienced during prolonged or difficult operations, especially in cases of severe acute cholecystitis. Third, the robotic procedure is safely performed in patients who underwent upper abdominal surgery, because an adhesiolysis can be easily performed throughout the lower lying port sites.

In summary, robotic cholecystectomy using the new port sites on the low abdominal area can be safely and efficiently performed, with sufficient patient satisfaction. However, we recommend that the more difficult cases (due to acute inflammation) were likely excluded from participation in an early period.

## COMMENTS

### Background

To overcome the limitations of laparoscopic techniques, the robotic-assisted procedure developed, the 3-dimensional view, magnification, tremor suppression, and flexibility of the instruments have allowed precise operating techniques in general surgery. The robotic procedure is safe; however, it is not acceptable as a standard operation for gallbladder disease because of the lack

of advantages for patients compared to its high cost.

### Research frontiers

Since the first robotic-assisted cholecystectomy was performed in 1997, the benefits of the robotic procedure in gallbladder diseases have not yet been established. In the previous studies, port sites of robotic cholecystectomy were located on the supraumbilical area, similar to laparoscopic surgery. As a result, we performed robotic cholecystectomy, changing the port placements.

### Innovations and breakthroughs

Recent reports have highlighted the importance of changing the port placements from the upper abdominal area to the lower abdominal area. This is the first study to report that robotic cholecystectomy with lower lying ports can be safely and efficiently performed.

### Applications

This study may represent a new strategy for surgical intervention in the treatment of patients with gallbladder diseases.

### Terminology

Da Vinci robot system (Intuitive Surgical, CA, United States) is composed of the surgeon's viewing and control console and a movable cart with four articulated robot arms.

### Peer review

This study is a feasibility study that demonstrates that robotically assisted cholecystectomy may be performed. The technical details of port placement by the authors will make it helpful to the general surgeon who wishes to use the robot to assist with cholecystectomy.

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**P- Reviewers** Kumar A, Leitman IM **S- Editor** Huang XZ  
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## High-intensity focused ultrasound ablation: An effective bridging therapy for hepatocellular carcinoma patients

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Received: September 29, 2012 Revised: October 29, 2012

Accepted: January 11, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To analyze whether high-intensity focused ultrasound (HIFU) ablation is an effective bridging therapy for patients with hepatocellular carcinoma (HCC).

**METHODS:** From January 2007 to December 2010, 49 consecutive HCC patients were listed for liver transplantation (UCSF criteria). The median waiting time

for transplantation was 9.5 mo. Twenty-nine patients received transarterial chemoembolization (TACE) as a bridging therapy and 16 patients received no treatment before transplantation. Five patients received HIFU ablation as a bridging therapy. Another five patients with the same tumor staging (within the UCSF criteria) who received HIFU ablation but not on the transplant list were included for comparison. Patients were comparable in terms of Child-Pugh and model for end-stage liver disease scores, tumor size and number, and cause of cirrhosis.

**RESULTS:** The HIFU group and TACE group showed no difference in terms of tumor size and tumor number. One patient in the HIFU group and no patient in the TACE group had gross ascites. The median hospital stay was 1 d (range, 1-21 d) in the TACE group and two days (range, 1-9 d) in the HIFU group ( $P < 0.000$ ). No HIFU-related complication occurred. In the HIFU group, nine patients (90%) had complete response and one patient (10%) had partial response to the treatment. In the TACE group, only one patient (3%) had response to the treatment while 14 patients (48%) had stable disease and 14 patients (48%) had progressive disease ( $P = 0.00$ ). Seven patients in the TACE group and no patient in the HIFU group dropped out from the transplant waiting list ( $P = 0.559$ ).

**CONCLUSION:** HIFU ablation is safe and effective in the treatment of HCC for patients with advanced cirrhosis. It may reduce the drop-out rate of liver transplant candidate.

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**Key words:** Ablation; Bridging therapy; Cirrhosis; Hepatocellular carcinoma; High-intensity focused ultrasound; Liver transplant; New technology

Cheung TT, Fan ST, Chan SC, Chok KSH, Chu FSK, Jenkins CR, Lo RCL, Fung JYY, Chan ACY, Sharr WW, Tsang SHY, Dai WC, Poon RTP, Lo CM. High-intensity focused ultrasound ablation: An effective bridging therapy for hepatocellular carcinoma patients. *World J Gastroenterol* 2013; 19(20): 3083-3089 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3083.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3083>

## INTRODUCTION

Deceased donor liver transplantation provides one of the best treatments to patients with hepatocellular carcinoma (HCC) and cirrhosis. The numbers of donations and cases performed are on a rising trend. However, the scarcity of liver grafts in many parts of the world, especially Asia, leads to a significant dropout rate of patients from liver transplant waiting lists, particularly patients with HCC and a low model for end-stage liver disease (MELD) score<sup>[1]</sup>. In order to reduce the dropout rate, different bridging therapies have been proposed. Among them, transarterial chemoembolization (TACE) and radiofrequency ablation (RFA) are most popular. Despite of treatment applied before liver transplantation, the dropout rate for TACE ranged from 15% to 35% in different studies<sup>[1,2]</sup>. RFA seems to have produced better results but the dropout rate also ranged from 5.8% to 14% in various studies<sup>[3,4]</sup>.

High-intensity focused ultrasound (HIFU) ablation is one of the latest treatments. It provides a totally non-invasive therapy to HCC and is viable even in patients with severe cirrhosis. In one of our previous studies, it achieved a complete ablation rate of 82.4% for HCCs smaller than 3 cm in with just one treatment session. It is well tolerated even in patients with advanced cirrhosis and age<sup>[5]</sup>. The current study is the first study that investigates whether HIFU therapy can be safely performed in HCC patients with cirrhosis and whether it can reduce the dropout rate of liver transplant candidates.

## MATERIALS AND METHODS

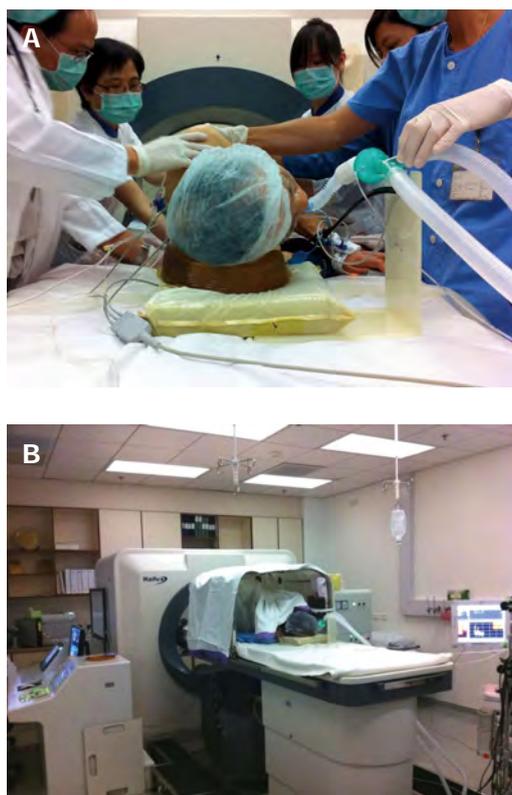
From January 2007 to December 2010, 49 consecutive HCC patients were listed for deceased donor liver transplantation (UCSF criteria). The diagnosis of HCC was confirmed by histology, elevated level of serum alpha-fetoprotein (> 400 ng/mL), or typical radiological appearance of lesion shown by contrast computed tomography or contrast magnetic resonance imaging. The median waiting time for transplantation was 9.5 mo. Patients who were listed for transplantation received TACE as a bridging therapy before transplantation. HIFU ablation has been used as a standard local ablative therapy since 2006 for HCC patients who have poor liver function and cannot tolerate hepatectomy<sup>[5]</sup>. This is a retrospective study performed with prospectively collected data. Informed consent to treatment and to the use of data for research was obtained beforehand.

Five patients received HIFU ablation and 29 patients received TACE as a bridging therapy. Fifteen patients received no treatment before transplantation. Another five patients with the same tumor staging (within the UCSF criteria) who were not on the transplant waiting list but received HIFU ablation were included for comparison. All patients were comparable in terms of Child-Pugh and MELD scores, tumor size and number, and cause of cirrhosis.

Contraindications to TACE included main portal vein thrombosis, arteriovenous shunting, Child-Pugh C cirrhosis, and extrahepatic metastasis. Cisplatin was used as the chemotherapeutic agent and was delivered with Lipiodol, followed by Gelfoam particle embolization. Selective cannulation and embolization of the feeding arteries of the tumors were performed whenever possible. During the procedure, 10 mL of Lipiodol was mixed with 10 mg of cisplatin into a 20 mL emulsion. Depending on the tumor size and number, 4-60 mL of the Lipiodol emulsion was injected into the catheter placed in the artery supplying the tumor, or into the hepatic artery proper beyond the gastroduodenal artery for bilobar disease. Light embolization of the feeding artery was then performed with pellets sized 1 mm × 2 mm mixed with 40 mg of gentamycin. Gelfoam injection was stopped when the blood flow in the artery supplying the tumor slowed down but before occlusion occurred. TACE was repeated every 2 to 3 mo. Patients were monitored every month for hepatic and renal functions and alpha-fetoprotein level. TACE was terminated if there was evidence of further derangement of liver function (bilirubin > 50 μmol/L, ascites not controlled by diuretics, or hepatic encephalopathy), progression of disease, extrahepatic metastasis, or any other major complication.

HIFU ablation was offered to patients with poor liver function or decompensated cirrhosis as documented by (1) presence of gross ascites; (2) disease at Child-Pugh B or above; and (3) tumor located at site considered difficult for percutaneous RFA. The treatment probe can target lesions as deep as 10 cm beneath the skin; any lesion within this range can be ablated. Contraindications to HIFU ablation included serum bilirubin level above 100 μmol/L and subcutaneous tissue thicker than 3.5 cm as adipose tissue would absorb a substantial amount of energy from the energy pathway.

All HIFU treatments were carried out by experienced hepatobiliary surgeons and radiologists. The JC HIFU system (Chongqing Haifu Technology, Chongqing, China) was used. The system comprises a real-time diagnostic imaging unit, a therapeutic unit, a degassed water circulation unit, and a computer system. The real-time diagnostic imaging unit provides direct visualization of the tumor. The therapeutic unit consists of an ultrasound energy transducer which focuses the ultrasound energy at a 12-cm focal point. The degassed water circulation unit provides a medium for ultrasound transmission outside the body. The computer system controls these three units.



**Figure 1** Locations of patient and control panel to the treatment console. A: The patient was placed in a right lateral position on the treatment console; B: The control panel is located next to the treatment console.

Before operation, the skin of the patient was cleaned by 70% alcohol followed by degassed water to remove all the grease from the skin. A dose of antibiotics (1.2 g of amoxicillin/clavulanic acid) was given on induction. A dose of proton pump inhibitor was also given on induction. Every patient was subjected to general anesthesia to aid comfort as the whole procedure could last for 3 h and the patient had to lie still and endure long periods of breath-holding. In addition, general anesthesia allowed manipulation of tumor location by the Valsalva maneuver during the procedure. If the tumor was at the dome of the liver, artificial right pleural effusion was induced before treatment. If the tumor was located at the right lobe of the liver, the patient was put in a right lateral position (Figure 1A). For better ultrasound conduction, the patient was put in a water bath. The surgeon and the radiologist controlled the operation from the control panel next to the treatment unit (Figure 1B). The treatment was performed under real-time ultrasound image guidance. The lesion was localized by a 3.6-MHz diagnostic ultrasound probe (Philips) incorporated at the center of the transducer. Parallel slices of the target tumor with 5-mm separations were planned and then ablated slice by slice with focused ultrasound energy produced by the transducer operating at 0.8 MHz. Grey-scale changes of the ablated sites were observed during the ablation procedure, indicating the temperature change inside the target lesion.

After the procedure, the patient was usually sent to the general ward and was closely monitored for vital signs, particularly body temperature. Transient hypothermia could occur as the patient had been immersed in a water bath. For patients who had received artificial pleural effusion, radiograph of the chest was taken to check for pneumothorax.

Tumor response was categorized according to the RECIST criteria: (1) complete response was denoted by disappearance of all target lesions; (2) partial response was denoted by at least a 30% decrease in the sum of the largest diameters of the target lesions; (3) progressive disease was denoted by at least a 20% increase in the sum of the largest diameters of the target lesions or appearance of one or more new lesions; and (4) stable disease was denoted by the absence of sufficient shrinkage of tumor qualified as partial response and the absence of sufficient increase of tumor qualified as progressive disease<sup>[6]</sup>. Contrast computed tomography or contrast magnetic resonance imaging was performed one month after the HIFU treatment and then every three month to evaluate tumor response before transplantation.

### Statistical analysis

The baseline characteristics of patients were expressed as medians with range. The Mann-Whitney *U* test was used to compare continuous variables and a  $\chi^2$  test was used to compare discrete variables. Statistical significance was denoted by  $P < 0.05$ . All statistical calculations were made with the SPSS/PC + computer software (SPSS, Chicago, IL, United States).

## RESULTS

The TACE group and the HIFU group had no difference in age, hepatitis B virus infection, or hepatitis C virus infection. The two groups of patients had similar liver function in terms of serum levels of albumin, aspartate aminotransferase and alanine aminotransferase, prothrombin time, indocyanine green retention rate, and international normalized ratio. One patient in the HIFU group had gross ascites before treatment. No patient in the TACE group had gross ascites. The two groups showed no difference in terms of tumor size and tumor number (Table 1). Table 2 shows the Child-Pugh and MELD scores of the patients. The median number of sessions of TACE was 3 (range, 1-7).

The median hospital stay was 1 d (range, 1-21 d) in the TACE group and two days (range, 1-9 d) in the HIFU group ( $P < 0.000$ ). Seven patients in the TACE group dropped out from liver transplant waiting list. One of them developed extrahepatic metastasis and six of them had local progression of disease rendering them unqualified for transplantation. No patient in the HIFU group dropped out during the study period ( $P = 0.559$ ).

According to the RECIST criteria, nine patients (90%) had complete response and one patient (10%) had partial response in the HIFU group. In the TACE group, only

**Table 1 Patient demographics**

|                                     | HIFU<br>( <i>n</i> = 10) | TACE<br>( <i>n</i> = 29) | <i>P</i> value |
|-------------------------------------|--------------------------|--------------------------|----------------|
| Age (yr)                            | 59.5 (49-76)             | 57 (43-65)               | 0.107          |
| Sex (male/female)                   | 7/3                      | 24/5                     | 0.399          |
| Child-Pugh A disease                | 3 (30)                   | 17 (58.6)                | 0.267          |
| Child-Pugh B disease                | 6 (60)                   | 12 (41.4)                |                |
| Child-Pugh C disease                | 1 (10)                   | 0                        |                |
| Carrier of hepatitis B virus        | 5 (50)                   | 28 (96.5)                | 0.002          |
| Carrier of hepatitis C virus        | 4 (40)                   | 1 (3.4)                  | 0.011          |
| Serum bilirubin (μmol/L)            | 14.5 (6-36)              | 25 (4-49)                | 0.074          |
| Serum albumin (g/dL)                | 32 (27-38)               | 34 (20-43)               | 0.606          |
| Platelet count (10 <sup>9</sup> /L) | 67 (28-166)              | 59 (23-144)              | 0.688          |
| Aspartate transaminase (U/L)        | 52 (29-141)              | 47 (15-104)              | 0.440          |
| Alanine transaminase (U/L)          | 44 (26-109)              | 36 (9-132)               | 0.376          |
| Alpha-fetoprotein (ng/mL)           | 8 (2-160)                | 24 (1-1151)              | 0.101          |
| International normalized ratio      | 1.25 (0.9-1.5)           | 1.3 (1.0-1.5)            | 0.960          |
| Largest tumor size (cm)             | 2.6 (1.2-4.0)            | 2.0 (0.8-4.3)            | 0.252          |
| Tumor number                        | 1 (1-2)                  | 1 (1-3)                  | 0.172          |

Data are expressed as absolute *n* (%) or median (range). HIFU: High-intensity focused ultrasound; TACE: Transarterial chemoembolization.

one patient (3%) had response to the treatment while 14 patients (48%) had stable disease and 14 patients (48%) had progressive disease (*P* = 0.00).

Three out of the five patients in the HIFU group subsequently received transplantation. The median waiting time was nine months (range, 3-36 mo). Histopathological examination showed coagulation necrosis with no active tumor cells in two of the excised livers. One patient had 90% necrosis of the HCC. None of the patients who had received HIFU ablation as a bridging therapy developed complication due to intolerance of the procedure. The other two patients who were still waiting for transplantation had stable disease during the study period.

## DISCUSSION

The incidence of HCC is increasing throughout the world. The annual incidence of HCC in hepatitis B carriers is around 0.5%. The incidence in patients with liver cirrhosis is even higher at around 2.5% annually<sup>[7,8]</sup>. Hepatitis-B-related cirrhosis is common in Asia, where HCC is endemic. Other risk factors for the development of HCC include hepatitis C infection, alcoholic cirrhosis, genetic hemochromatosis, and primary biliary cirrhosis. These patients should be offered regular surveillance in order to identify small tumors that may be potentially treatable. However, most patients with small HCCs have no symptoms. Resection is the main hope of cure for HCC but is only possible in 25% of the patients because the disease is usually so advanced at presentation and is frequently associated with cirrhosis<sup>[9,10]</sup>. For patients with unresectable HCC, liver transplantation appears to be the only viable option. The chance of receiving a liver graft varies worldwide. Liver donation rate is highest in Spain where there are 33.7 donations per one million of the population. In contrast, the donation rates in Asia range from only 0.05 to 4.3 donations per one million of the popula-

**Table 2 Patients' model for end-stage liver disease and Child-Pugh scores**

|                                      | HIFU ( <i>n</i> = 10) | TACE ( <i>n</i> = 29) |
|--------------------------------------|-----------------------|-----------------------|
| MELD score ( <i>P</i> = 0.687)       |                       |                       |
| 14                                   | 0                     | 2                     |
| 13                                   | 1                     | 4                     |
| 12                                   | 1                     | 4                     |
| 11                                   | 4                     | 7                     |
| 10                                   | 1                     | 3                     |
| 9                                    | 1                     | 3                     |
| 8                                    | 0                     | 1                     |
| 7                                    | 1                     | 4                     |
| 6                                    | 1                     | 1                     |
| Child-Pugh score ( <i>P</i> = 0.096) |                       |                       |
| 5                                    | 0                     | 10                    |
| 6                                    | 3                     | 7                     |
| 7                                    | 5                     | 4                     |
| 8                                    | 1                     | 5                     |
| 9                                    | 0                     | 2                     |
| 10                                   | 1                     | 1                     |

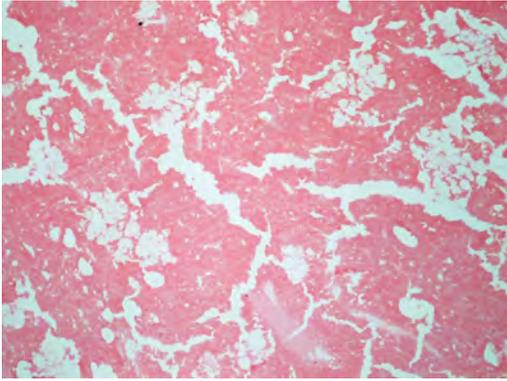
MELD: Model for end-stage liver disease; HIFU: High-intensity focused ultrasound; TACE: Transarterial chemoembolization.

tion. The general lack of suitable deceased donors makes successful liver transplantation for HCC difficult<sup>[11,12]</sup>. In order to maximize the benefit of utilizing this scarce resource, different liver graft allocation systems are adopted worldwide. The principle of allocation is to prioritize the sickest and yet maintain the highest survival rate possible. As a corollary, patients with very high MELD scores have priority. In most countries, patients with unresectable HCC and yet lower MELD scores have a low priority.

The results of liver transplantations in the early period of development were not satisfactory, with a 5-year survival rate below 40%. This urged recognition of poor prognostic factors in liver transplantation in patients with HCC<sup>[13]</sup>. Mazzaferro *et al.*<sup>[14,15]</sup> showed that a subgroup of patients with radiological evidence of a single tumor smaller than 5 cm in diameter or two to three tumors each smaller than 3 cm in diameter had better survival outcome. The Milan criteria were established in 1996 and have led to the improvement of the 5-year survival rate to 83%. At many transplant centers, patients with tumor status beyond the Milan criteria are not accepted for transplantation and those on transplant waiting lists are delisted if their tumors enlarge to beyond the criteria, ensuring that liver grafts are allocated to patients predicted to have longer survival<sup>[16]</sup>.

In order to make sure patients receive appropriate treatment before transplantation so as to remain listed, different bridging therapies have been tried. This is particularly important for patients whose treatment options are limited by poor liver reserve and portal hypertension. TACE and RFA are the most popular bridging therapies.

TACE is a standard bridging therapy at some centers, achieving a rate of down-staging of tumors of around 40%. However, about 20% of patients develop tumor progression after TACE, rendering them delisted<sup>[1,2,17,18]</sup>. RFA is an effective thermal ablative treatment modality and is widely practiced to treat small HCCs. RFA is



**Figure 2** The tumor nodule shows coagulative necrosis. The necrotic tumor tissue shows a barely preserved architectural pattern and loss of cellular details. Hematoxylin and eosin stain,  $\times 200$ .

also used as a bridging therapy. Successful tumor downstaging is observed in 70%-85% of patients. However, the dropout rate after RFA is around 14%<sup>[3,19-21]</sup>. TACE and RFA seem to be effective bridging therapies, but only for selected patients. They are not safe for patients with liver decompensation such as gross ascites and thrombocytopenia.

The concept of using ultrasound energy as a penetrating force to destroy something remote originated in last century and was summarized by Kremkau<sup>[22]</sup>. In the 1950's, researchers brought the phenomenon of piezoelectricity to the clinical setting, treating Parkinson's disease and other neurological conditions with focused ultrasound energy<sup>[23-26]</sup>.

Nowadays clinical HIFU ablation for liver tumors utilizes a unique frequency of ultrasound wave, 0.8-3.5 MHz, which can be focused at a distance from the therapeutic transducer. The accumulated energy at the focused region induces necrosis of the target lesion by elevating the tissue temperature to above 60 °C<sup>[27,28]</sup>. Temperature outside the focus point remains static as particle oscillation remains minimal. This is an advantage of HIFU over RFA in which inadvertent collateral damage is unavoidable. Patients with derangement of liver function and thrombocytopenia usually show intolerance of RFA<sup>[29]</sup>. A bridging therapy must not cause further liver decompensation. The slow process of heating by HIFU energy propagation followed by resting leads to little tissue destruction beyond the focused point.

The presence of gross ascites facilitates HIFU treatment. As ultrasound energy travels much better in water than in air, ascites encourages energy propagation to the target lesion. In addition, the presence of ascites acts as a cushion of coolant inside the peritoneal cavity and prevents the muscle wall and skin absorbing too much energy from the beam pathway where subcutaneous tissue burn could happen.

HIFU ablation is a totally extracorporeal non-invasive treatment modality using focused ultrasound energy that is capable of causing coagulative necrosis of the target lesion via intact skin without the need of surgical incision.

The unique needleless design of the HIFU system makes HIFU ablation superior to RFA, as percutaneous needle penetration may induce hemorrhage from a hypervascular tumor in a patient with coagulopathy and a low platelet count. Furthermore, without needle puncture, there is no risk of direct tumor seeding to the surrounding major vessels<sup>[30]</sup>. For tumors located at the dome of the liver, open RFA would be required if HIFU ablation is not used.

Figure 2 demonstrates the effect of HIFU ablation on the HCC in one of the excised liver in the series in the present study. Coagulative necrosis of the tumor was observed microscopically on almost the whole cut surface. Although histological examination showed the presence of viable tumor cells at a few focuses, there was no gross tumor progression. HIFU ablation was a successful bridging therapy in this case in which TACE and RFA were not considered acceptable treatment due to the poor liver reserve.

Repeated sessions of HIFU or adjuvant TACE should be performed to enhance the effect of tumor necrosis when liver function allows and the waiting time is prolonged.

Wu *et al.*<sup>[31]</sup> reported the results of HIFU treatment for 68 patients with liver tumors. Thirty patients subsequently received liver resection. In histological examination, all the lesions showed complete coagulation necrosis.

We recently published the results of HIFU treatment for 49 patients with HCC. The complete ablation rate was comparable to that of RFA, ranging from 79.5% to 82.4%. The complication rate was around 8.2%. The complications were mainly mild skin edema and injury due to energy accumulation at the ultrasound beam pathway. The treatment was well tolerated in most of the patients. The median hospital stay was 4 d<sup>[5]</sup>.

In conclusion, to the best of our knowledge, we are the first liver transplant center investigating HIFU ablation as a bridging therapy before liver transplantation. In this study, HIFU ablation was shown to be an effective thermal ablation method. The treatment stopped gross tumor progression in a patient with severe cirrhosis when TACE and RFA were contraindicated. HIFU treatment can potentially reduce the dropout rate of patients from the transplant waiting list. In a broader sense, HIFU may prolong the survival of selected patients with decompensated cirrhosis for which liver transplantation is not an option.

## COMMENTS

### Background

The lack of liver grafts in many places, particularly Asia, is the major obstacle to liver transplantation for patients with hepatocellular carcinoma (HCC). An effective bridging therapy which is well tolerated by patients with decompensated liver cirrhosis is important and much needed.

### Research frontiers

High-intensity focused ultrasound (HIFU) ablation is a relatively new technique which provides a non-invasive treatment for HCC. However, its efficacy and safety in candidates of liver transplantation have not been known. In this study, the authors demonstrated that it is an effective treatment modality for liver

transplant candidates who have decompensated cirrhosis.

### Innovations and breakthroughs

This is the first original study on the effect of HIFU ablation as a bridging therapy for liver transplantation. Unlike radiofrequency ablation, HIFU ablation does not require any needle puncture and so eliminates the risk of disease dissemination. It can even be performed in patients with gross ascites without the risk of precipitating liver decompensation. In the study, the ablation caused no adhesion. The absence of adhesion renders the subsequent liver transplantation easier.

### Applications

With its advantages, safety and efficacy as demonstrated in this study, HIFU ablation should be considered as a treatment option for HCC. This study already proved that it is a safe and effective bridging therapy before liver transplantation for HCC.

### Terminology

HIFU ablation utilizes a unique frequency of ultrasound wave of 0.8 to 3.5 MHz, causing a cavitation effect in the target lesion.

### Peer review

The authors studied the effect of HIFU ablation on HCC performed before liver transplantation. The study demonstrated that HIFU ablation is an effective ablation modality which is totally non-invasive. It can effectively reduce the drop-out rate of liver transplant candidates by giving effective control on their tumors. The histological examination of the excised livers provided evidence that necrosis is effective in an *in vivo* model, which makes this article unique of its kind.

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**P-Reviewer** Mearini L **S-Editor** Gou SX **L-Editor** A  
**E-Editor** Li JY



## Development and application of a real-time polymerase chain reaction method for *Campylobacter jejuni* detection

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Supported by The General Program of National Natural Science Foundation of China, No. 81271789; and the Major State Basic Research Development Program, No. 2013CB127204

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Received: January 15, 2013 Revised: March 7, 2013

Accepted: April 9, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To develop a real-time polymerase chain reaction (PCR) method to detect and quantify *Campylobacter jejuni* (*C. jejuni*) from stool specimens.

**METHODS:** Primers and a probe for real-time PCR were designed based on the specific DNA sequence of the *hipO* gene in *C. jejuni*. The specificity of the primers and probe were tested against a set of *Campylobacter spp.* and other enteric pathogens. The optimal PCR conditions were determined by testing a series of conditions with standard *C. jejuni* template. The detection limits were obtained using purified DNA from bacterial culture and extracted DNA from the stool specimen. Two hundred and forty-two specimens were analyzed for the presence of *C. jejuni* by direct bacterial culture and real-time PCR.

**RESULTS:** The optimal PCR system was determined using reference DNA templates, 1 × uracil-DNA glycosylase, 3.5 mmol/L MgCl<sub>2</sub>, 1.25 U platinum *Taq* polymerase, 0.4 mmol/L PCR nucleotide mix, 0.48 μmol/L of each primer, 0.2 μmol/L of probe and 2 μL of DNA template in a final volume of 25 μL. The PCR reaction was carried as follows: 95 °C for 4 min, followed by 45 cycles of 10 s at 95 °C and 30 s at 59 °C. The detection limit was 4.3 CFU/mL using purified DNA from bacterial culture and 10<sup>3</sup> CFU/g using DNA from stool specimens. Twenty (8.3%, 20/242) *C. jejuni* strains were isolated from bacterial culture, while 41 (16.9%, 41/242) samples were found to be positive by real-time PCR. DNA sequencing of the PCR product indicated the presence of *C. jejuni* in the specimen. One mixed infection of *C. jejuni* and *Salmonella* was detected in one specimen and the PCR test for this specimen was positive.

**CONCLUSION:** The sensitivity of detection of *C. jejuni* from stool specimens was much higher using this PCR assay than using the direct culture method.

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**Key words:** *Campylobacter jejuni*; Real time polymerase chain reaction; Application

**Core tip:** In the present study, we developed an effective real-time polymerase chain reaction method based on the specific DNA sequence of the *hipO* gene in *Campylobacter jejuni* (*C. jejuni*). The detection limit of this assay is 4.3 CFU/mL. A study of 242 clinical stool specimens from diarrheal patients indicated that this method is more sensitive than direct bacterial culture for the identification of *C. jejuni* from stool specimens.

Zhang MJ, Qiao B, Xu XB, Zhang JZ. Development and application of a real-time polymerase chain reaction method for *Campylobacter jejuni* detection. *World J Gastroenterol* 2013;

19(20): 3090-3095 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3090.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3090>

## INTRODUCTION

*Campylobacter* spp., *Salmonella* spp., *Yersinia* spp., *Shigella* spp., and *Escherichia coli* (*E. coli*) O157 are the leading causes of human bacterial gastroenteritis worldwide<sup>[1]</sup>. *Campylobacter jejuni* (*C. jejuni*) is the main species of *Campylobacter* that affects humans<sup>[2,3]</sup>. *Campylobacter* infections have been investigated and followed with considerable interest since the early 1980s in China, and there have been numerous reports on *C. jejuni* infection in patients with diarrhea. However, reports and the frequency of isolation of *C. jejuni* from diarrheal patients have decreased since the late 1990s. Improvement in sanitary conditions may explain this decline in numbers; however, the difficulty in detecting this fastidious pathogen might be another contributor to the decreased number of reports and frequency of isolation. Our recent pilot study revealed that the ratio of isolation of this pathogen from stool specimens of diarrheal patients was considerably different among surveillance spots in different laboratories (2%-15%, unpublished). Sensitive and accurate detection of this pathogen is important, both for the treatment of patients and for prompt epidemiological investigation.

With the development of genomic DNA sequencing, online databases, and bioinformatic analyses, nucleic acid-based methods, particularly polymerase chain reaction (PCR) methods, have become promising tools for the rapid, reliable, and sensitive detection and diagnosis of pathogenic infection<sup>[4-8]</sup>. In this study, we developed a real-time PCR assay to detect *C. jejuni*. We then combined this PCR assay with DNA sequence analysis and compared this method with the direct culture method to detect *C. jejuni* in stool specimens obtained from patients with diarrhea. The results obtained in this study provide a proof of concept for PCR-based detection of *C. jejuni* infection in patients with diarrhea, may lead to the development of a pre-screening approach for isolating *C. jejuni*.

## MATERIALS AND METHODS

### Sample collection and culture-based detection

Two hundred and forty-two stool specimens were collected from diarrheal patients (with ages ranging from 6 to 72 years) in the outpatient facilities of 11 hospitals. The specimens were transferred to a laboratory within 4 h for bacterial culture. The remainder of the samples was frozen at -80 °C for DNA extraction. A sterile cotton swab was twisted in each stool sample and streaked directly on selective Skirrow agar plates consisting of Columbia agar base (CM331, Oxoid, Basingstoke, United Kingdom), selective supplement (SR0069, Oxoid), and 5% defibrinated sheep blood. The plates were incubated in a microaerobic atmosphere (5% O<sub>2</sub>, 10% CO<sub>2</sub> and 85% N<sub>2</sub>) at 42 °C for

48 h. Suspected colonies were picked and identified by gram staining, oxidase and catalase tests, and hippurate hydrolysis analysis, according to our previous report<sup>[9]</sup>. All specimens were also examined for the presence of *Shigella* spp., *Salmonella* spp., *Yersinia* spp. and *E. coli* O157 by culture on xylose lysine deoxycholate agar, deoxycholate hydrogen sulfide lactose agar, cefsulodin-irgasan-novobiocin agar, and sorbitol MacConkey agar, respectively. Suspected colonies were identified biochemically using API 20E strips, and the cultures were confirmed up to the species level by further serotype or PCR analysis, as previously described<sup>[10,11]</sup>.

### DNA preparation from bacterial culture

Reference genomic DNA was extracted from the *Campylobacter* isolates cultured in this study by using a QIAamp DNA mini kit (Qiagen, Düsseldorf, Germany). DNA templates from other reference pathogens were gifts from Drs. Huaiqi Jing and Biao Kan. The reference DNA templates are shown in Table 1.

### Primers and probe design

Specific DNA fragments of the *hipO* gene were compared using BLASTn and Vector NTI suite 6.0 software. The primers and probe sets were designed and synthesized by Shanghai Huirui Biotechnology Co., Ltd. The sequences of the primers and probe used in this study were: *hipO*-F, 5'-CGGATAGTTATAGTATTGAAGT-TATTGG-3', *hipO*-R, 5'-GAAGCAGCATAAATAG-GATCTTTT-3', and *hipO*-P, 5'-FAM-TTCTGGAG-CACTTCCATGACCACC-BHQ1-3'.

### Optimization for real-time PCR

The optimal PCR conditions were determined by testing a series of conditions with standard *C. jejuni* template. The standard PCR curve was constructed using genomic DNA from *C. jejuni* strain NCTC11168. The detection limits of this assay from pure culture and stool specimens were determined using 10-fold dilutions of quantified reference *C. jejuni* genomic DNA templates (1 × 10<sup>0</sup> to 1 × 10<sup>6</sup> CFU/mL) and the same amount of bacteria inoculated into stool specimens that were previously confirmed to contain no *Campylobacter*. The specificity of this assay was verified using genomic DNA from other enteric pathogens. The *C. jejuni*-inoculated samples were also cultured to compare the detection limits.

### Evaluation of repeatability

To evaluate the reproducibility of the assay, five serial dilutions with three replicates per concentration were performed for two separate *C. jejuni* genomic DNA templates (NCTC11168 and ATCC33560). The serial dilutions were also performed on three different *C. jejuni* strains (81116, ATCC49349 and ICDCCJ07001) simultaneously. The curves were constructed on the basis of the log pg/mL of the genomic DNA and threshold cycle (Ct). Reproducibility was assessed using the SD of the Ct value and the SD of the slope of each dilution curve, respectively.

### PCR detection of *C. jejuni* from stool specimens and DNA sequencing of PCR products

After direct culture, the stool specimens were stored at -80 °C until DNA extraction using a QIAamp DNA stool mini kit (Qiagen), which was performed in accordance with the manufacturer's instructions. Conventional PCR with the universal primers targeting conserved bacterial 16s rRNA gene sequences was carried out to evaluate the validity of the template used in this study. Real-time PCR was performed in a 25- $\mu$ L volume with 2  $\mu$ L of purified DNA obtained directly from stool specimens. All the samples with Ct < 35 were considered positive. Each of the positive PCR products, except for the ones from the culture-positive specimens, was confirmed with conventional PCR using the same primers, and the PCR products were purified and sequenced. Online sequence BLAST was performed using the NCBI BLASTN suite ([http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST\\_PROGRAMS=megaBlast&PAGE\\_TYPE=BlastSearch&SHOW\\_DEFAULTS=on&LINK\\_LOC=blasthome](http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)).

## RESULTS

### Bacterial culture

Twenty *C. jejuni* (8.3%, 20/242) isolates, 34 (14%, 34/242) *Salmonella* isolates, and one (0.4%, 1/242) *Shigella* isolate were obtained by direct plating of the 242 stool specimens. *Salmonella* was the most frequently isolated pathogenic genus from the stool specimens and *C. jejuni* was the second most frequently isolated. No *Yersinia* or *E. coli* O157 isolates were found. In another laboratory, serotyping methods were used to further identify *Salmonella* and *Shigella* colonies.

### Optimized PCR assay

The optimal PCR system was determined using reference DNA templates, 1  $\times$  uracil-DNA glycosylase, 3.5 mmol/L MgCl<sub>2</sub>, 1.25 U platinum *Taq* polymerase, 0.4 mmol/L PCR nucleotide mix, 0.48  $\mu$ mol/L of each primer, 0.2  $\mu$ mol/L of probe, and 2  $\mu$ L of DNA template in a final volume of 25  $\mu$ L run with the following parameters: 95 °C for 4 min, followed by 45 cycles of 10 s at 95 °C and 30 s at 59 °C. Fluorescence signals were measured at the end of the annealing step of every cycle. PCR products were verified using DNA electrophoresis through 2% agarose gels. The size of the PCR product was consistent with the expected size (85 bp) in each case. The specificity of the primers and probe were tested against *Campylobacter spp.* and other enteric pathogens. Only *C. jejuni* showed positive reactions. All the other templates gave negative results, including other *Campylobacter spp.*

### Detection limit and stability of the PCR assay

Serial dilutions from 10<sup>0</sup> to 10<sup>6</sup> CFU/ml of the target template were subjected to the real-time PCR assay (Figure 1A). The standard curve based on the dilutions of genomic DNA showed a linear relationship between Log

Table 1 Reference DNA templates used in this study

| Bacteria                                      | Strain name |
|---|-------------|
| <i>Campylobacter jejuni</i>                   | NCTC11168   |
| <i>Campylobacter jejuni</i>                   | 81116       |
| <i>Campylobacter jejuni</i>                   | ATCC33560   |
| <i>Campylobacter jejuni</i> sub <i>doylei</i> | ATCC49349   |
| <i>Campylobacter jejuni</i>                   | ICDCCJ07001 |
| <i>Campylobacter coli</i>                     | ATCC33559   |
| <i>Campylobacter coli</i>                     | WHO-10.2    |
| <i>Campylobacter fetus</i>                    | ATCC27374   |
| <i>Campylobacter lari</i>                     | ATCC35221   |
| <i>Escherichia coli</i> O157:H7               | Isolate-1   |
| Enterotoxigenic <i>Escherichia coli</i>       | ETEC10407   |
| Enterotoxigenic <i>Escherichia coli</i>       | EAECO42     |
| Enteroinvasive <i>Escherichia coli</i>        | EIEC44825   |
| Enteropathogenic <i>Escherichia coli</i>      | Isolate-2   |
| <i>Salmonella</i> Serovar <i>typhi</i>        | CT18        |
| Typhoid-paratyphoid A                         | Isolate-3   |
| <i>Salmonella typhimurium</i>                 | Isolate-4   |
| Cholera O1                                    | Isolate-5   |
| <i>Shigella</i>                               | Isolate-6   |
| <i>Salmonella enteritidis</i>                 | Isolate-7   |
| <i>Yersinia enterocolitica</i> O3             | 52203       |
| <i>Vibrio parahaemolyticus</i>                | Isolate-8   |
| <i>Enterococcus</i>                           | Isolate-9   |
| <i>Listeria monocytogenes</i>                 | Isolate-10  |

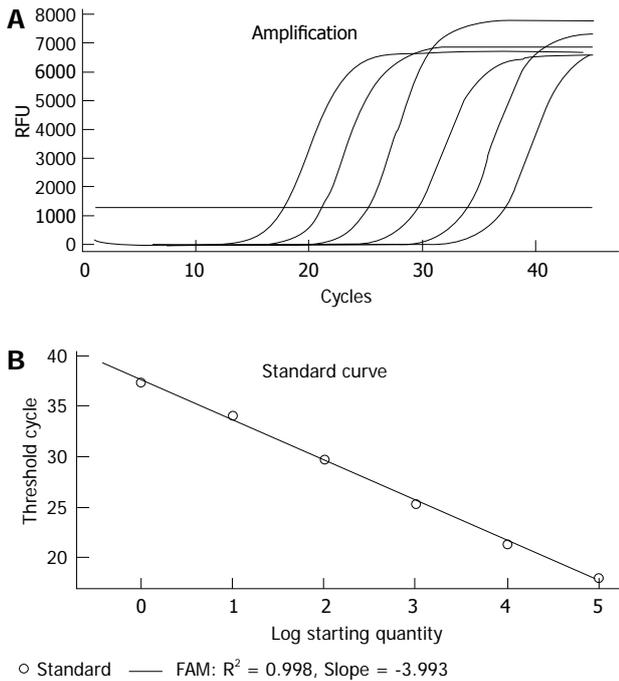
CFU/mL and threshold cycles (Ct; Figure 1B). Copy numbers could be obtained by the following equation using the standard curve:  $Y(Ct) = -3.993X (\log \text{CFU/mL}) + 37.51$  ( $R^2 = 0.998$ ). When Ct = 35, the detection limit of the PCR assay for pure culture was approximately 4.3 CFU/mL. However, when Ct = 35, the detection limit for *C. jejuni* in the stool specimens was 10<sup>3</sup> CFU/g, and we could not obtain a linear relationship between log CFU/g and Ct using serial bacterial inoculation in different stool specimens. The curves constructed from the serial dilution replicates of the individual *C. jejuni* templates and those generated from the three different *C. jejuni* templates are shown in Figure 2. The variation of the Ct value and the slopes of the curves indicated that this assay could be stably reproduced.

### PCR detection from stool specimens

The detection limit from both bacterial culture and real-time PCR (with Ct < 35) for the inoculated stool samples was 10<sup>3</sup> CFU/g; therefore, a Ct value of  $\leq 35$  was considered positive for the real-time PCR assay in this study. Forty-one (16.9%, 41/242) samples were found to be positive by the real-time PCR method. All culture-positive specimens showed positive PCR results (Ct < 34). DNA sequencing of the positive PCR products for the culture-negative samples indicated the presence of *C. jejuni*. The samples containing *Salmonella* and *Shigella* isolates tested negative by real-time PCR (Ct > 40), except for one sample that contained both *C. jejuni* and *Salmonella* (Ct = 27).

## DISCUSSION

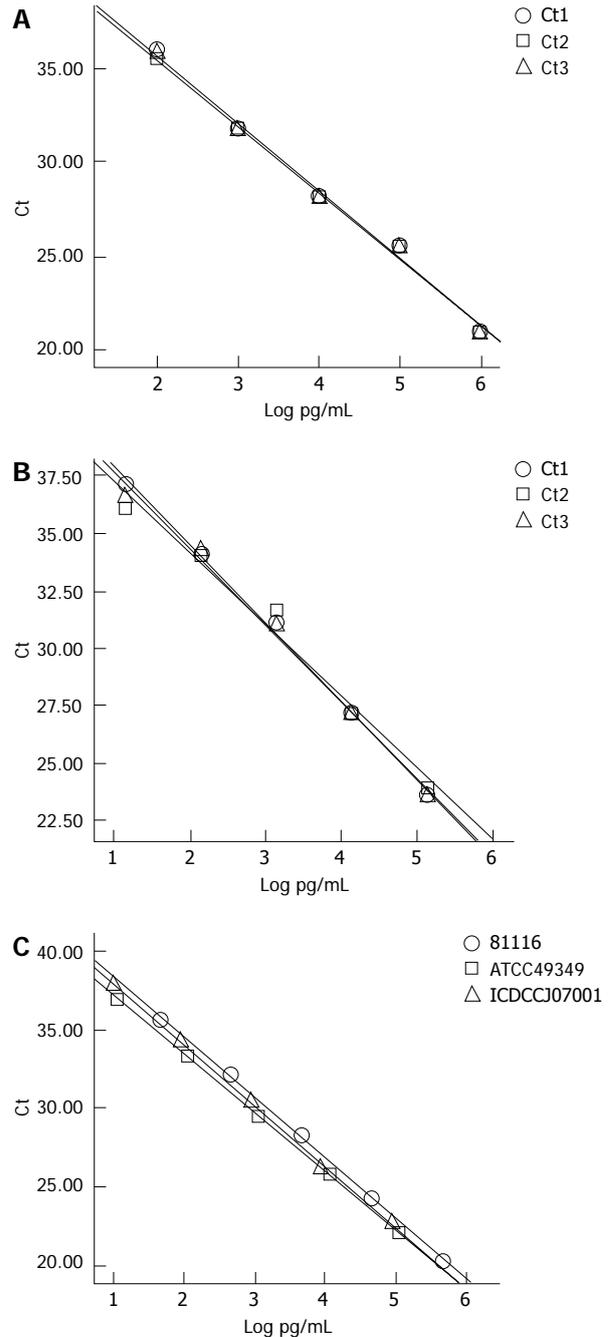
*C. jejuni* is one of the major causes of food borne disease



**Figure 1** Real time polymerase chain reaction standard curve and the linear relationship between *Campylobacter jejuni* quantity and polymerase chain reaction cycle threshold. A: The standard curve was constructed using genomic DNA from *Campylobacter jejuni* isolates. Serial dilutions ranging from 100 to 10<sup>6</sup> CFU/mL of target template were subjected to real time polymerase chain reaction assay; B: The linear relationship between Log CFU/mL and threshold cycles. RFU: Relative fluorescence units.

worldwide<sup>[12]</sup>. It is an important pathogen of acute bacterial diarrhea in humans and has been associated with the development of Guillain-Barré syndrome, a post-infectious polyneuropathy<sup>[13,14]</sup>. Routine detection of *Campylobacter* species in the clinical setting is currently based on culture-based detection and subsequent phenotypic identification. Its detection is difficult because of its special growth requirements, low infectious doses, and potential for entering a viable, but not cultivable, state<sup>[15,16]</sup>. The traditional “gold standard” diagnostic methods for *Campylobacter* infection include bacterial culture and culture-based biochemical tests. These methods are both time-consuming and laborious. In addition to the fastidious culture requirements, the discrimination and differentiation of *Campylobacter spp.* are complicated and error prone. For example, distinguishing *C. jejuni* from *C. coli* is usually done on the basis of the biochemistry test for hippurate hydrolysis. Only *C. jejuni* gives a positive reaction, but previous studies have found that 10% of *C. jejuni* isolates failed to hydrolyze hippurate under laboratory conditions<sup>[17,18]</sup>. The accurate identification of *Campylobacter* up to species level is an essential prerequisite for many epidemiological studies.

In the present study, we developed a real-time PCR assay based on the specific DNA fragment of the *hipO* gene from *C. jejuni*, which could identify *C. jejuni* directly and differentiate it from other pathogens, especially from other *Campylobacter spp.*, in stool specimens from diarrheal patients. The specificity of the PCR system was



**Figure 2** Stability tests. A: Curves constructed based dilutions of the genomic DNA of *Campylobacter jejuni* (C. jejuni) NCTC11168; B: Curves constructed based on the dilution of the genomic DNA of *C. jejuni* ATCC33560; C: Curves generated from the serial dilution of the genomic DNA of *C. jejuni* 81116, ATCC49349 and ICDCCJ07001. Ct: Threshold cycle.

verified with 27 reference enteric pathogens (Table 1). The detection limit of this PCR assay is less effective for stool samples than for pure culture. Loss of template during DNA extraction or inhibitors present in the stool specimens could reduce the efficiency<sup>[19]</sup>. Two hundred and forty-two stool specimens from patients with diarrhea were tested by the direct culture and by the PCR assay. All the culture-positive samples gave positive PCR results and samples containing other pathogens tested negative by PCR (Ct > 40). The DNA sequence of the

positive PCR products indicated the presence of *C. jejuni* in the specimens. The sensitivity of this PCR assay (100%, 41/41) was much higher than that of direct bacterial culture (49%, 20/41). The result from the mixed infection sample indicated that this PCR assay is considerably accurate. The results presented in this study contrast with those reported by certain previous studies, in which PCR results were found to have the same or less sensitivity compared with the direct culture method for the detection of *C. jejuni* from stool specimens<sup>[20,21]</sup>. However, Maher *et al*<sup>[22]</sup> used a DNA probe-based PCR assay and were able to identify 94% (17/18) and 32% (35/109) of *Campylobacter* infections from culture-positive and culture-negative stool specimens, respectively. Furthermore, Bessède *et al*<sup>[23]</sup> proved the weakness of the culture methods compared with PCR and immunoenzymatic methods of detection of *Campylobacter*. Notably, many aspects of the culture-based method, such as the choice of the selective media used for culture, need to be improved<sup>[24]</sup>. Although the sensitivity of the PCR system was high in this study, false-negative PCR results may still occur because of the presence of inherent inhibitors or because of very low numbers of organisms.

Interestingly, when *C. jejuni* were inoculated into stool specimens, the detection limit by the PCR assay was 10<sup>3</sup> CFU/g. The PCR detection limit from stool samples was similar to that reported in a previous study, but the detection limit of the direct culture methods mostly depended on the culture methods used, particularly the choice of plating medium, which may influence the efficiency of *C. jejuni* isolation during direct plating of fecal samples<sup>[25,26]</sup>. Instant plating of the inoculated samples in this study might increase the laboratory isolation capacity. Meanwhile, the non-uniformity of the clinical stool specimens in terms of physical matter, target organisms, the associated fecal flora and transportation status would result in variability of the culture results. In this study, although we used selective medium under specific microaerobic atmosphere conditions, some samples were contaminated with *Proteus* bacilli, and the suspected colonies could not be picked because of the rapid spread of the contaminants. *Proteus* bacilli may be one of the agents that reduced culture sensitivity, and the use of cotton swabs rather than inoculating loops for streaking on plates might have increased this contamination. In this study, we used QIAamp DNA stool mini kits to extract the DNA; the QIAamp kit has the highest sensitivity and requires the least manipulation of template DNA prior to PCR. More research is required to develop an in-house procedure for DNA extraction from different specimens and for optimizing the extraction process to increase the desired yield.

In conclusion, the real-time PCR assay developed in this study is sufficiently sensitive and accurate for *C. jejuni* detection from stool specimens. Although the PCR results do not permit its use as the standard method of diagnosing *C. jejuni* infection in the clinical setting, this newly developed method will benefit large-scale epidemiology

investigations and pre-screening for bacterial isolation.

## COMMENTS

### Background

*Campylobacter jejuni* (*C. jejuni*) is a major food-borne pathogen. In addition to enteritis, *C. jejuni* infection can also cause Guillain-Barré syndrome, an autoimmune neuropathy in humans. The "gold standard" diagnostic methods for *C. jejuni* infection include bacterial culture and culture-based biochemical tests. Primary *in vitro* culture of *C. jejuni* is difficult because of its special growth requirements, low infectious doses, and potential for entering a viable, but not cultivable, state. In addition to its fastidious culture conditions, current methods for discrimination and differentiation of *Campylobacter* spp. are complicated and fallible. Specific DNA detection, particularly real-time polymerase chain reaction (PCR), is a promising tool for the rapid, reliable, and more sensitive detection and diagnosis of *C. jejuni* infection. In this study, the authors developed and validated a real-time PCR assay method for the detection of *C. jejuni* from stool specimens.

### Research frontiers

*C. jejuni* is a fastidious organism and requires microaerophilic conditions for *in vitro* culture. Nucleic acid-based methods for the detection of *C. jejuni* infection are research hotspots, particularly PCR methods, which are promising tools for the rapid, reliable, and more sensitive detection of *C. jejuni* for laboratory-based diagnosis.

### Innovations and breakthroughs

In the present study, the authors developed a sensitive and specific real-time PCR assay using a DNA fragment of the *hipO* gene in *C. jejuni*, which can directly differentiate *C. jejuni* from other pathogens and other *Campylobacter* spp. from stool specimens obtained from diarrheal patients.

### Applications

The real-time PCR assay developed in this study is sensitive and accurate for the detection of *C. jejuni* from stool specimens. This method will benefit both large-scale epidemiology investigations and the clinical diagnosis of *C. jejuni* infection in humans.

### Peer review

This is an excellent and well-written manuscript. The authors are to be congratulated on developing and validating their own in-house PCR-based technique to detect *C. jejuni* in stool specimens. The *hipO* gene is specific to *C. jejuni*; therefore, by definition, this is a highly specific test.

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**P- Reviewers** Bourke B, Louwen R, Rossignol JF, Salazar-Lindo E  
**S- Editor** Wen LL **L- Editor** Stewart GJ **E- Editor** Li JY



## Prediction of risk factors for lymph node metastasis in early gastric cancer

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Supported by Shanghai Jiaotong University Medical School for Scientific Research, No. 09XJ21013; Shanghai Health Bureau for Scientific Research, No. 2010029; Shanghai Science and Technology Commission for Scientific Research, No. 124119a0300

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Received: November 27, 2012 Revised: December 19, 2012

Accepted: March 8, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To explore risk factors for lymph node metastases in early gastric cancer (EGC) and to confirm the appropriate range of lymph node dissection.

**METHODS:** A total of 202 patients with EGC who underwent curative gastrectomy with lymphadenectomy in the Department of Surgery, Xinhua Hospital and Ruijin Hospital of Shanghai Jiaotong University Medical School between November 2003 and July 2009, were retrospectively reviewed. Both the surgical procedure and the extent of lymph node dissection were based on the recommendations of the Japanese gastric cancer treatment guidelines. The macroscopic type was

classified as elevated (type I or II a), flat (II b), or depressed (II c or III). Histopathologically, papillary and tubular adenocarcinomas were grouped together as differentiated adenocarcinomas, and poorly differentiated and signet-ring cell adenocarcinomas were regarded as undifferentiated adenocarcinomas. Univariate and multivariate analyses of lymph node metastases and patient and tumor characteristics were undertaken.

**RESULTS:** The lymph node metastases rate in patients with EGC was 14.4%. Among these, the rate for mucosal cancer was 5.4%, and 8.9% for submucosal cancer. Univariate analysis showed an obvious correlation between lymph node metastases and tumor location, depth of invasion, morphological classification and venous invasion ( $\chi^2 = 122.901, P = 0.001; \chi^2 = 7.14, P = 0.008; \chi^2 = 79.523, P = 0.001; \chi^2 = 8.687, P = 0.003$ , respectively). In patients with submucosal cancers, the lymph node metastases rate in patients with venous invasion (60%, 3/5) was higher than in those without invasion (20%, 15/75) ( $\chi^2 = 4.301, P = 0.038$ ). Multivariate logistic regression analysis revealed that the depth of invasion was the only independent risk factor for lymph node metastases in EGC [ $P = 0.018, \text{Exp (B)} = 2.744$ ]. Among the patients with lymph node metastases, 29 cases (14.4%) were at N1, seven cases were at N2 (3.5%), and two cases were at N3 (1.0%). Univariate analysis of variance revealed a close relationship between the depth of invasion and lymph node metastases at pN<sub>1</sub> ( $P = 0.008$ ).

**CONCLUSION:** The depth of invasion was the only independent risk factor for lymph node metastases. Risk factors for metastases should be considered when choosing surgery for EGC.

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**Key words:** Gastric neoplasm; Lymph node metastasis; Risk factors; Gastrectomy; Lymphadenectomy

**Core tip:** Early gastric cancer (EGC) is defined as a lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases. In this study, we retrospectively evaluated the distribution of metastatic nodes in a two-center cohort of 202 patients with EGC. To assess nodal status in EGC, we applied an index calculated by the multiplication of the incidence of metastases in the respective node stations. Univariate and multivariate analyses were applied to confirm the clinicopathological factors associated with lymph node metastases, and to provide a basis for choosing the optimal surgical treatment and for determining the appropriate range of lymph node dissection.

Ren G, Cai R, Zhang WJ, Ou JM, Jin YN, Li WH. Prediction of risk factors for lymph node metastasis in early gastric cancer. *World J Gastroenterol* 2013; 19(20): 3096-3107 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3096.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3096>

## INTRODUCTION

Early gastric cancer (EGC) is defined as a lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases<sup>[1]</sup>. Five-year survival rates in EGC tend to be greater than 90%, with lymph node status being the most important prognostic factor<sup>[2,3]</sup>. In patients with EGC and lymph node metastases, a 5-year survival rate of 87.3% has been reported, compared to 94.2% in those without nodal involvement<sup>[4]</sup>. Considering the low rate of lymph node metastasis in EGC<sup>[5,6]</sup>, the Japanese guidelines recommend endoscopic mucosal resection, reduction surgery D1 plus No. 7 and 8a and D1 plus No. 7, and 8a and 9 lymph node resection, for the treatment of patients with phase T1 disease. However, this approach is controversial outside Japan. At present, the general consensus is that endoscopic mucosal resection (EMR) or distal gastrectomy plus limited lymph node resection can be undertaken in most patients with mucosal cancer, and a distal gastrectomy plus D2 lymph node resection should be performed in most patients with submucosal cancer<sup>[2,3]</sup>.

Recently, in order to reduce operative and post-operative complications, and to improve quality of life, less invasive surgical alternatives, such as EMR, endoscopic submucosal dissection, laparoscopy-assisted gastrectomy and limited surgery, are used for the treatment of EGC. Although there has been substantial research on the prediction of risk factors for lymph node metastases in EGC, no definitive criteria are available. In addition, controversy surrounds the indications for local treatment in EGC, and limited surgery and the appropriate extent of lymphadenectomy.

In this study, we retrospectively evaluated the distribution of metastatic nodes in a two-center cohort of 202

patients with EGC. To assess nodal status in EGC, we applied an index calculated by the multiplication of the incidence of metastases in the respective node stations. Univariate and multivariate analyses were performed to confirm the clinicopathological factors associated with lymph node metastases, and to provide a basis for choosing the optimal surgical treatment and for determining the appropriate range of lymph node dissection.

## MATERIALS AND METHODS

### Ethics

This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Institutional Review Board of Shanghai Jiaotong University. All patients provided written informed consent.

### Patients

A total of 202 patients with EGC, as defined by the Japanese Classification of Gastric Carcinoma<sup>[7]</sup>, who underwent curative gastrectomy with lymphadenectomy in the Department of Surgery, Xinhua Hospital and Ruijin Hospital of Shanghai Jiaotong University Medical School between November 2003 and July 2009, were retrospectively reviewed. Of these, there were 132 men and 70 women, ranging in age from 25 to 87 years (mean 58.1 ± 12.9 years). Mucosal tumors were found in 122 patients (60.4%) and submucosal tumors in 80 (39.6%). Lymph node involvement was detected in 29 patients (Table 1).

### Surgery

All operations were performed with curative intent. Curative surgery was defined as the removal of all gross tumors and the demonstration of tumor-negative surgical margins by microscopic examination of the entire circumference. Surgical procedures comprised 171 distal gastrectomies, 24 proximal gastrectomies and seven total gastrectomies. Proximal gastrectomy involved resection of the proximal half of the stomach via an abdominal approach, with an intraabdominal esophago-gastric anastomosis. Following a total gastrectomy with D2 lymph node dissection, an esophagojejunostomy was used routinely for Roux-en-Y reconstruction. Proximal and distal resection margins were evaluated intraoperatively to confirm freedom from disease. Both the surgical procedure and the extent of lymph node dissection were based on the recommendations of the Japanese gastric cancer treatment guidelines<sup>[7]</sup>. A total of 2926 lymph nodes (LNs), with a median of 14.5 LNs per patient, were removed. No patient received neoadjuvant therapy before surgery.

### Pathological examination

In both hospitals, the surgical team immediately examined the lymph nodes macroscopically, which were then divided and classified into lymph node stations, as defined by the Japanese Classification of Gastric Carcinoma. No

**Table 1** Demographics of 202 patients with early gastric cancer

|                       | Patients (n = 202) |
|-----------------------|--------------------|
| Age                   |                    |
| < 60 yr               | 109                |
| ≥ 60 yr               | 93                 |
| Sex                   |                    |
| Male                  | 132                |
| Female                | 70                 |
| Tumor site            |                    |
| Upper                 | 25                 |
| Middle                | 98                 |
| Lower                 | 79                 |
| Size of tumor         |                    |
| ≤ 2 cm                | 97                 |
| > 2 cm                | 105                |
| No. of resected nodes |                    |
| < 15                  | 114                |
| ≥ 15                  | 88                 |
| Tumor depth           |                    |
| Mucosal               | 122                |
| Submucosal            | 80                 |
| Macroscopic type      |                    |
| Elevated              | 25                 |
| Flat                  | 21                 |
| Depressed             | 156                |
| Histologic type       |                    |
| Differentiated        | 121                |
| Undifferentiated      | 81                 |
| Lymphovessel embolus  |                    |
| +                     | 2                  |
| -                     | 200                |
| Vessel embolus        |                    |
| +                     | 5                  |
| -                     | 197                |
| Nerve invasion        |                    |
| +                     | 0                  |
| -                     | 202                |
| CEA                   |                    |
| +                     | 1                  |
| -                     | 201                |

CEA: Carcinoembryonic antigen.

size limitation was imposed for lymph node harvesting. Specimens were fixed in formalin, stained with hematoxylin and eosin, and sent for histopathological evaluation, following which the number of histologically confirmed lymph nodes was recorded for each lymph node station. Each lymph node was embedded in paraffin and at least two sections were performed. Immunohistochemistry for micrometastasis was not performed.

Tumor size was recorded as the maximum diameter. The depth of infiltration was measured at the deepest point of penetration of the cancer cells. The macroscopic type was classified as elevated (type I or II a), flat (II b), or depressed (II c or III), according to the Japanese Classification of Gastric Carcinoma<sup>[7]</sup>. Of the 202 patients, 26 (12.9%), 21 (10.4%) and 155 (76.7%) were elevated, flat or depressed, respectively. Histopathologically, papillary and tubular adenocarcinomas were grouped together as differentiated adenocarcinomas, and poorly differentiated and signet-ring cell adenocarcinomas were regarded as undifferentiated adenocarcinomas. Overall, tumors were

differentiated in 121 patients and undifferentiated in 81.

The relationship between various clinicopathological factors and the presence or absence of lymph node metastases was then examined. Clinicopathological parameters included patient age (< 60 years or ≥ 60 years), sex, tumor location (U = upper third, M = middle third, or L = lower third of the stomach), tumor size (maximum dimension ≤ 20 mm or > 20 mm), macroscopic type (elevated, flat, or depressed), depth of invasion (mucosal or submucosal), histological type (differentiated or undifferentiated), carcinoembryonic antigen levels (CEA, < 5 ng/mL or ≥ 5 ng/mL), lymphatic invasion (present or absent) and venous invasion (present or absent). Evaluation of these factors was undertaken according to the Japanese Classification of Gastric Carcinoma established by the Japanese Research Society for Gastric Cancer.

### Statistical analysis

Descriptive data are presented as the mean ± SD. For between group comparisons, continuous variables were analyzed using the Student's *t* test, and categorical variables with the  $\chi^2$  test. Factors found to be significant ( $P < 0.05$ ) in univariate analysis were included in subsequent multivariate logistic regression analysis, in order to identify independent variables associated with lymph node metastases. All statistical analyses were undertaken using the Statistical Package for the Social Sciences (SPSS) for Windows, Version 17.0 (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Univariate analysis of lymph node metastases in EGC and clinicopathological factors

Univariate analysis was performed on the relationship between lymph node metastases and clinicopathological factors. The findings revealed a close relationship between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases ( $\chi^2 = 122.901$ ,  $P = 0.001$ ;  $\chi^2 = 7.14$ ,  $P = 0.008$ ;  $\chi^2 = 79.523$ ,  $P = 0.001$ ;  $\chi^2 = 8.687$ ,  $P = 0.003$ , respectively). There was no correlation between lymph node metastases and sex, age, tumor size, number of retrieved lymph nodes, histological type, lymphatic invasion, nervous invasion, and serum levels of carcinoembryonic antigen (CEA) (Table 2).

In patients with mucosal cancers, no significant differences in the occurrence of lymph node metastases were found in relation to sex, age, tumor location, tumor size, number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, venous invasion, nervous invasion and CEA levels (Table 2).

In patients with submucosal cancers, there was no significant difference in the occurrence of lymph node metastases in relation to sex, age, tumor location, tumor size, number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion and CEA levels. However, the lymph node

**Table 2 Univariate analysis of lymph node metastases in early gastric cancer and clinicopathological factors *n* (%)**

| Clinicopathological factors        | LN (+)    | LN (-)     | <i>P</i> <sup>1</sup> |
|------------------------------------|-----------|------------|-----------------------|
| <b>The entire study population</b> |           |            |                       |
| Age                                |           |            | 0.887                 |
| < 60 yr                            | 16 (14.7) | 93 (85.3)  |                       |
| ≥ 60 yr                            | 13 (14.0) | 80 (86.0)  |                       |
| Sex                                |           |            | 0.213                 |
| Male                               | 16 (12.1) | 116 (87.9) |                       |
| Female                             | 13 (18.6) | 57 (81.4)  |                       |
| Tumor site                         |           |            | 0.001                 |
| Upper                              | 2 (8.0)   | 23 (92.0)  |                       |
| Middle                             | 15 (15.3) | 83 (84.7)  |                       |
| Lower                              | 12 (15.2) | 67 (84.8)  |                       |
| Size of tumor                      |           |            | 0.240                 |
| ≤ 2 cm                             | 11 (11.3) | 86 (88.7)  |                       |
| > 2 cm                             | 18 (17.1) | 87 (82.9)  |                       |
| No. of resected nodes              |           |            | 0.580                 |
| < 15                               | 15 (13.2) | 99 (86.9)  |                       |
| ≥ 15                               | 14 (15.9) | 74 (84.1)  |                       |
| Tumor depth                        |           |            | 0.008 <sup>a</sup>    |
| Mucosal                            | 11 (9.0)  | 111 (91.0) |                       |
| Submucosal                         | 18 (22.5) | 62 (77.5)  |                       |
| Macroscopic type                   |           |            | 0.001 <sup>a</sup>    |
| Elevated                           | 4 (16)    | 21 (84)    |                       |
| Flat                               | 3 (14.3)  | 18 (85.7)  |                       |
| Depressed                          | 22 (14.1) | 134 (85.9) |                       |
| Histologic type                    |           |            | 0.332                 |
| Differentiated                     | 15 (12.4) | 106 (87.6) |                       |
| Undifferentiated                   | 14 (17.3) | 67 (82.7)  |                       |
| Lymphovessel ambolus               |           |            | 0.149                 |
| +                                  | 1 (50.0)  | 1 (50.0)   |                       |
| -                                  | 28 (14.0) | 172 (86.0) |                       |
| Vessel ambolus                     |           |            | 0.003 <sup>a</sup>    |
| +                                  | 3 (60.0)  | 2 (40.0)   |                       |
| -                                  | 26 (13.2) | 171 (86.8) |                       |
| Nerve invasion                     |           |            | N                     |
| +                                  | 0 (0)     | 0 (0)      |                       |
| -                                  | 29 (14.4) | 173 (85.6) |                       |
| CEA                                |           |            | 0.681                 |
| +                                  | 0 (0)     | 1 (100)    |                       |
| -                                  | 29 (14.4) | 173 (85.6) |                       |
| <b>Mucosa cancer</b>               |           |            |                       |
| Age                                |           |            | 0.234                 |
| < 60 yr                            | 8 (11.8)  | 60 (88.2)  |                       |
| ≥ 60 yr                            | 3 (5.6)   | 51 (94.4)  |                       |
| Sex                                |           |            | 0.578                 |
| Male                               | 6 (7.9)   | 70 (92.1)  |                       |
| Female                             | 5 (10.9)  | 41 (89.1)  |                       |
| Tumor site                         |           |            | 0.976                 |
| Upper                              | 1 (10.0)  | 9 (90.0)   |                       |
| Middle                             | 6 (9.4)   | 58 (90.6)  |                       |
| Lower                              | 4 (8.3)   | 44 (91.7)  |                       |
| Size of tumor                      |           |            | 0.142                 |
| ≤ 2 cm                             | 3 (5.1)   | 56 (94.9)  |                       |
| > 2 cm                             | 8 (12.7)  | 55 (87.3)  |                       |
| No. of resected nodes              |           |            | 0.580                 |
| < 15                               | 7 (10.3)  | 61 (89.7)  |                       |
| ≥ 15                               | 4 (7.4)   | 50 (92.6)  |                       |
| Macroscopic type                   |           |            | 0.539                 |
| Elevated                           | 2 (11.1)  | 16 (88.9)  |                       |
| Flat                               | 0 (0)     | 11 (100)   |                       |
| Depressed                          | 9 (9.7)   | 84 (90.3)  |                       |
| Histologic type                    |           |            | 0.073                 |
| Differentiated                     | 4 (5.3)   | 71 (94.7)  |                       |
| Undifferentiated                   | 7 (14.9)  | 40 (85.1)  |                       |
| Lymphovessel ambolus               |           |            | N                     |
| +                                  | 0 (0)     | 0 (0)      |                       |
| -                                  | 11 (9.0)  | 111 (91.0) |                       |

|                          |           |            |                    |
|--------------------------|-----------|------------|--------------------|
| Vessel ambolus           |           |            | N                  |
| +                        | 0 (0)     | 0 (0)      |                    |
| -                        | 11 (9.0)  | 111 (91.0) |                    |
| Nerve invasion           |           |            | N                  |
| +                        | 0 (0)     | 0 (0)      |                    |
| -                        | 11 (9.0)  | 110 (91.0) |                    |
| CEA                      |           |            | 0.752              |
| +                        | 0 (0)     | 1 (100)    |                    |
| -                        | 11 (9.1)  | 110 (90.9) |                    |
| <b>Submucosal cancer</b> |           |            |                    |
| Age                      |           |            | 0.512              |
| < 60 yr                  | 8 (19.5)  | 33 (80.5)  |                    |
| ≥ 60 yr                  | 10 (25.6) | 29 (74.4)  |                    |
| Sex                      |           |            | 0.129              |
| Male                     | 10 (17.9) | 46 (82.1)  |                    |
| Female                   | 8 (33.3)  | 16 (66.7)  |                    |
| Tumor site               |           |            | 0.265              |
| Upper                    | 1 (6.7)   | 14 (93.3)  |                    |
| Middle                   | 9 (26.5)  | 25 (73.5)  |                    |
| Lower                    | 8 (25.8)  | 23 (74.2)  |                    |
| Tumor size               |           |            | 0.768              |
| ≤ 2 cm                   | 8 (21.1)  | 30 (78.9)  |                    |
| > 2 cm                   | 10 (23.8) | 32 (76.2)  |                    |
| No. of resected nodes    |           |            | 0.203              |
| < 15                     | 8 (17.4)  | 38 (82.6)  |                    |
| ≥ 15                     | 10 (29.4) | 24 (70.6)  |                    |
| Macroscopic type         |           |            | 0.742              |
| Elevated                 | 2 (28.6)  | 5 (71.4)   |                    |
| Flat                     | 3 (30.0)  | 7 (70.0)   |                    |
| Depressed                | 13 (20.6) | 50 (79.4)  |                    |
| Histologic type          |           |            | 0.725              |
| Differentiated           | 11 (23.9) | 35 (76.1)  |                    |
| Undifferentiated         | 7 (20.6)  | 27 (79.4)  |                    |
| Lymphovessel ambolus     |           |            | 0.346              |
| +                        | 1 (50.0)  | 1 (50.0)   |                    |
| -                        | 17 (21.8) | 61 (78.2)  |                    |
| Vessel ambolus           |           |            | 0.038 <sup>a</sup> |
| +                        | 3 (60.0)  | 2 (40.0)   |                    |
| -                        | 15 (20.0) | 60 (80.0)  |                    |
| Nerve invasion           |           |            | N                  |
| +                        | 0 (0)     | 0 (0)      |                    |
| -                        | 18 (22.5) | 62 (77.5)  |                    |
| CEA                      |           |            | N                  |
| +                        | 0 (0)     | 0 (0)      |                    |
| -                        | 18 (22.5) | 62 (77.5)  |                    |

<sup>1</sup>Statistically significant. Tumor site: middle and lower *vs* upper; Tumor depth: submucosa *vs* mucosa; Macroscopic type: flat and depressed *vs* elevated; Vessel ambolus: + (present) *vs* - (absent), <sup>a</sup>*P* < 0.05. In patients with mucosal cancers, no significant differences in the occurrence of lymph node metastases were found in relation to the clinicopathological factors. CEA: Carcinoembryonic antigen.

metastases rate in patients with venous invasion (60%, 3/5) was higher than in those without invasion (20%, 15/75), and the difference was significant ( $\chi^2 = 4.301$ , *P* = 0.038) (Table 2). Venous invasion, as a source variable, was therefore used in the logistic regression model. This revealed that it was not an independent risk factor for lymph node metastases in submucosal cancer [B = 1.792, SE = 0.957, Wals = 3.502, *P* = 0.061, Exp (B) = 6.000] (Table 2).

#### Multivariate analysis of lymph node metastases in EGC

Multivariate analysis revealed that the depth of invasion was an independent risk factor for lymph node metastases [*P* = 0.018, Exp (B) = 2.744]. Venous invasion was

also an important influencing factor [ $P = 0.116$ , Exp (B) = 4.147, Table 3]. Tumor location, depth of invasion, morphological classification, and venous invasion had no significant impact on nodal involvement rates.

#### Relationship between depth of invasion and number of metastatic lymph nodes

There was no significant difference between mucosal and submucosal tumors in terms of number of retrieved lymph nodes, using the independent sample  $t$  test ( $t = 0.350$ ,  $df = 200$ ,  $P = 0.727$ , mean difference = 0.534). The number of metastatic lymph nodes in those with mucosal tumors was slightly higher than in those with submucosal tumors. The results of dissected nodes were as follows: mucosa ( $n = 122$ ),  $14.70 \pm 9.894$ ; submucosa ( $n = 80$ ),  $14.16 \pm 11.656$ ,  $P = 0.727$ ; for the metastasis nodes, mucosa ( $n = 122$ ),  $3.91 \pm 5.576$ ; submucosa ( $n = 80$ ),  $3.72 \pm 3.102$ ,  $P = 0.908$ . The difference was not significant.

#### Number of retrieved lymph nodes and lymph node metastasis ratios for involved lymph nodes at each nodal station in EGC

The metastatic ratio is the ratio of metastatic nodes to the total number of dissected nodes and was recorded for each nodal station for all regional lymph nodes. There were 110 metastatic lymph nodes, an incidence of 3.8%. Among them, more lymph nodes were retrieved in stations No. 3, 4, 6, 7, 1, 9, 8 and 12. The number of retrieved lymph nodes in above stations was between 108 and 861, and the metastatic ratio was between 0% and 5.9%. Fewer lymph nodes were retrieved in stations No. 5, 13, 2, 11, 14, 10 and 15. The retrieved number was between 13 and 78, and the metastatic ratio was between 0% and 34.8%. Only one patient received station No. 16 lymph node dissection, and no metastasis was found. According to the Japanese Classification of Gastric Carcinoma<sup>[7]</sup>, 29 cases (14.4%) were at N1, seven cases were at N2 (3.5%), and two cases were at N3 (1.0%). A direct skip to N2, without moving through N1, occurred in two cases (1.0%). There were no skips to N3 without going through N2. The incidence of lymph node metastases in each station, from high to low, was as follows: station No. 14 (34.8%), No. 2 (11.1%), No. 6 (5.9%), No. 3 (4.8%), No. 11 (3.7%), No. 4 (3.4%), No. 5 (2.6%), No. 8 (2.5%), No. 9 (2.2%), No. 7 (1.5%), and No. 1 (0.6%). There were no station No. 10 and No. 11 lymph node metastases in two patients who had undergone a total gastrectomy combined with a splenectomy (Table 4).

#### Lymph node metastasis ratios and incidence at each station in upper third, middle third and lower third gastric cancers

The extent of metastases in 25 cases with upper third gastric cancer was as follows: four cases were at N1 (2.0%), an incidence of 3.4%, and a metastatic rate of 16.0%; one case was at N2 (0.5%), an incidence of 4.5%, and a metastatic rate of 4.0%; and no metastases was

**Table 3** Multivariate analysis of lymph node metastases in early gastric cancer for the entire study population

|                  | RR    | 95%CI       | P <sup>1</sup>     |
|------------------|-------|-------------|--------------------|
| Tumor site       | 1.159 | 0.84-1.478  | 0.644              |
| Invasion depth   | 2.744 | 2.316-3.172 | 0.018 <sup>a</sup> |
| Macroscopic type | 0.864 | 0.57-1.158  | 0.620              |
| Vessel ambloous  | 4.147 | 3.242-5.052 | 0.116              |
| Constant term    | 0.037 | -2.568      | 0.010              |

<sup>1</sup>Statistically significant, invasion depth: submucosa *vs* mucosa, <sup>a</sup> $P < 0.05$ .

**Table 4** Number of retrieved lymph nodes and lymph node metastasis ratios for involved lymph nodes at each nodal station in early gastric cancer

| Node group | No. of dissected nodes | No. of metastasis nodes | Incidence of lymphnode metastasis (%) |
|------------|------------------------|-------------------------|---------------------------------------|
| No. 1      | 165                    | 1                       | 0.6                                   |
| No. 2      | 27                     | 3                       | 11.1                                  |
| No. 3      | 861                    | 41                      | 4.8                                   |
| No. 4      | 670                    | 23                      | 3.4                                   |
| No. 5      | 78                     | 2                       | 2.6                                   |
| No. 6      | 358                    | 21                      | 5.9                                   |
| No. 7      | 263                    | 4                       | 1.5                                   |
| No. 8      | 120                    | 3                       | 2.5                                   |
| No. 9      | 134                    | 3                       | 2.2                                   |
| No. 10     | 16                     | 0                       | 0.0                                   |
| No. 11     | 27                     | 1                       | 3.7                                   |
| No. 12     | 108                    | 0                       | 0.0                                   |
| No. 13     | 58                     | 0                       | 0.0                                   |
| No. 14     | 23                     | 8                       | 34.8                                  |
| No. 15     | 13                     | 0                       | 0.0                                   |
| No. 16     | 5                      | 0                       | 0.0                                   |
| Total      | 2926                   | 110                     | 3.8                                   |

found at N3. At N1, lymph node metastases occurred in stations No. 2, 3, and 4, but not in station No. 1. The incidence of metastases was 5.3%, 5.5%, and 1.6% in stations No. 2, 3, and 4, respectively, and the metastatic rate was 4.0%, 8.0%, and 4.0%, respectively. At N2, lymph node metastases occurred only in station No. 8, and the incidence of metastases was 16.7%, and the metastatic rate was 4.0% (Table 5).

The extent of metastases in middle third gastric cancers was as follows: 18 cases (8.9%) occurred at N1, an incidence of 3.5% and a rate of 18.4%; five cases occurred at N2 (2.5%), an incidence of 1.4%, and a rate of 5.1%; and two cases occurred at N3 (1.0%), an incidence of 14.3%, and a rate of 2.0%. At N1, station No. 1, 3, 4, 5, and 6 had lymph node metastases, a rate of 1.0%, 9.2%, 5.1%, 1.0% and 2.0%, respectively. At N2, station No. 8, 9 and 11 had lymph node metastases, a rate of 1.0%, 3.1%, and 1.0%, respectively. At N3, only station No. 2 and 14 had lymph node metastases, a rate of 1.0%. No distal lymph node metastasis was identified (Table 5).

The extent of metastases in lower third gastric cancers was: 20 cases (9.9%) had metastases at N1: an incidence of 5.7%, and a rate of 25.3%; two cases (1.0%) had metastases at N2: an incidence of 1.8%, and a rate of 2.5%; there were no cases of metastases at N3 or at

**Table 5** Lymph node metastasis ratios and incidence at each station in upper third, middle third and lower third gastric cancers

| Node group | Upper       |             |            | Middle      |              |            | Lower       |               |            |
|------------|-------------|-------------|------------|-------------|--------------|------------|-------------|---------------|------------|
|            | pN category | Incidence   | Ratio      | pN category | Incidence    | Ratio      | pN category | Incidence     | Ratio      |
| No. 1      | pN1         | 0.0 (0/52)  | 0.0 (0/25) | pN1         | 7.4 (1/73)   | 1.0 (1/98) | pN2         | 0.0 (0/40)    | 0.0 (0/79) |
| No. 2      | pN1         | 5.3 (1/19)  | 4.0 (1/25) | pN3         | 28.6 (2/7)   | 1.0 (1/98) | M           | 0.0 (0/1)     | 0.0 (0/79) |
| No. 3      | pN1         | 5.5 (7/128) | 8.0 (2/25) | pN1         | 3.8 (17/445) | 9.2 (9/98) | pN1         | 5.9 (17/288)  | 8.9 (7/79) |
| No. 4      | pN1         | 1.6 (1/62)  | 4.0 (1/25) | pN1         | 4.3 (15/347) | 5.1 (5/98) | pN1         | 2.7 (7/261)   | 6.3 (5/79) |
| No. 5      | pN3         | 0.0 (0/6)   | 0.0 (0/25) | pN1         | 2.6 (1/38)   | 1.0 (1/98) | pN1         | 2.9 (1/34)    | 1.3 (1/79) |
| No. 6      | pN3         | 0.0 (0/21)  | 0.0 (0/25) | pN1         | 2.2 (4/182)  | 2.0 (2/98) | pN1         | 11.0 (17/155) | 8.9 (7/79) |
| No. 7      | pN2         | 0.0 (0/21)  | 0.0 (0/25) | pN2         | 0.0 (0/141)  | 0.0 (0/98) | pN2         | 4.0 (4/101)   | 1.3 (1/79) |
| No. 8      | pN2         | 16.7 (2/12) | 4.0 (1/25) | pN2         | 1.6 (1/63)   | 1.0 (1/98) | pN2         | 0.0 (4/45)    | 0.0 (0/79) |
| No. 9      | pN2         | 0.0 (0/5)   | 0.0 (0/25) | pN2         | 3.5 (3/85)   | 3.1 (3/98) | pN2         | 0.0 (4/44)    | 0.0 (0/79) |
| No. 10     | pN2         | 0.0 (0/2)   | 0.0 (0/25) | pN3         | 0.0 (0/3)    | 0.0 (0/98) | M           | 0.0 (0/11)    | 0.0 (0/79) |
| No. 11     | pN2         | 0.0 (0/4)   | 0.0 (0/25) | pN2         | 7.1 (1/14)   | 1.0 (1/98) | pN2         | 0.0 (0/9)     | 0.0 (0/79) |
| No. 12     | pN3         | 0.0 (0/6)   | 0.0 (0/25) | pN2         | 0.0 (0/61)   | 0.0 (0/98) | pN2         | 0.0 (0/41)    | 0.0 (0/79) |
| No. 13     | M           | 0.0 (0/10)  | 0.0 (0/25) | pN3         | 0.0 (0/31)   | 0.0 (0/98) | pN3         | 0.0 (0/17)    | 0.0 (0/79) |
| No. 14     | M           | 0.0 (0/2)   | 0.0 (0/25) | pN3         | 41.2 (7/17)  | 1.0 (1/98) | pN2         | 25.0 (1/4)    | 1.3 (1/79) |
| No. 15     | M           | 0.0 (0/1)   | 0.0 (0/25) | M           | 0.0 (0/9)    | 0.0 (0/98) | M           | 0.0 (0/3)     | 0.0 (0/79) |
| No. 16     | M           | 0.0 (0/0)   | 0.0 (0/25) | pN3         | 0.0 (0/5)    | 0.0 (0/98) | pN3         | 0.0 (0/0)     | 0.0 (0/79) |

**Table 6** Correlation between lymph node metastases at pN1 and pN2 and clinicopathological factors

|                         | pN1        |           |          | pN2        |         |          | <i>P</i> |
|-------------------------|------------|-----------|----------|------------|---------|----------|----------|
|                         | -          | +         | <i>P</i> | -          | +       | <i>P</i> |          |
| Tumor size (cm)         |            |           | 0.24     |            |         | 0.295    |          |
| ≤ 2.0                   | 86 (88.7)  | 11 (11.3) |          | 95 (97.9)  | 2 (2.1) |          |          |
| > 2.0                   | 87 (82.9)  | 18 (17.1) |          | 100 (95.2) | 5 (4.8) |          |          |
| Macroscopic type        |            |           | 0.969    |            |         | 0.259    |          |
| Elevated                | 21 (84.0)  | 4 (16.0)  |          | 24 (96.0)  | 1 (4.0) |          |          |
| Flat                    | 18 (85.7)  | 3 (14.3)  |          | 19 (90.5)  | 2 (9.5) |          |          |
| Depressed               | 134 (85.9) | 22 (14.1) |          | 152 (97.4) | 4 (2.6) |          |          |
| Invasion depth          |            |           | 0.008    |            |         | 0.334    |          |
| Mucosal                 | 111 (91.0) | 11 (9.0)  |          | 119 (97.5) | 3 (2.5) |          |          |
| Submucosal              | 62 (77.5)  | 18 (22.5) |          | 76 (95.0)  | 4 (5.0) |          |          |
| Differentiate           |            |           | 0.332    |            |         | 0.349    |          |
| Differentiated          | 106 (87.6) | 15 (12.4) |          | 118 (97.5) | 3 (2.5) |          |          |
|                         | 67 (82.7)  | 14 (17.3) |          | 77 (95.1)  | 4 (4.9) |          |          |
| Undifferentiated        |            |           | 0.126    |            |         | 0.804    |          |
| Lymphovascular invasion |            |           |          |            |         |          |          |
| No                      | 174 (87.0) | 26 (13.0) |          | 194 (97.0) | 6 (3.0) |          |          |
| Yes                     | 1 (50.0)   | 1 (50.0)  |          | 2 (100.0)  | 0 (0.0) |          |          |

distal lymph nodes. In N1, the metastatic rate, from high to low, was 8.9%, 8.9%, 6.3% and 1.3% respectively, and incidence was 5.9%, 11.0%, 2.7%, and 2.9%, respectively in stations No. 3, 6, 4, and 5. In N2, lymph nodes in stations No. 7 and 14 were most frequently involved, while no metastases occurred in stations No. 1, 8, 9, 11, and 12. At N2, lymph node metastases occurred in station No. 7, in a depressed type and differentiated submucosal tumor with a diameter 3.0 cm. Lymph node metastases occurred in a mucosal tumor in station No. 14 with a diameter 6.0 cm. It was a type 0-III differentiated cancer (Table 5).

#### Correlation between lymph node metastases at pN1 and pN2 and clinicopathological factors

Univariate analysis of variance revealed a close relationship between the depth of invasion and lymph node metastases at pN1 ( $P = 0.008$ ). There was no obvious rela-

tionship between the depth of invasion and lymph node metastases at pN2 ( $P = 0.334$ ). There was no significant correlation between tumor size, morphological classification, differentiation, lymphatic invasion and the presence of lymph node metastases at pN1 and pN2 (Table 6).

## DISCUSSION

Five-year survival rates in EGC tend to be greater than 90%, with lymph node status the most important prognostic factor. In those with lymph node metastases, a 10-year survival rate of 72% has been reported, compared to 92% for those without nodal involvement<sup>[2]</sup>. Although research has explored the issue of predicting risk factors for lymph node metastases in EGC, as yet there are no definitive criteria. In addition, controversy surrounds indications for local treatment and a limited surgical approach, and the range of lymph node dissection. Hence, in this study, we aimed to explore the risk factors for the development of lymph node metastases in EGC, to confirm the optimal range of lymph node dissection, and to provide a basis for a rational approach to surgical management.

Reported rates of lymph node metastases in EGC range from 5.7% to 20%<sup>[8-16]</sup>. However, Hayes *et al*<sup>[17]</sup> reported a rate over 40% during surgery. Depending on the depth of invasion, EGC can be classified as mucosal or submucosal, with a rate of lymph node involvement of 0%-21%<sup>[11,18,19]</sup> for mucosal tumors, and 16.5%-30%<sup>[2,3,19-22]</sup> for submucosal tumors. Based on anatomical and histological characteristics, there is a close relationship between the depth of tumor invasion and lymph node metastases in EGC. Once the tumor has invaded the submucosal layer, the rate of lymph node metastases increases significantly. In our group of 202 patients with EGC, lymph node metastases occurred in 29. Among these, there were 11 cases of mucosal and 18 cases of submucosal cancer. The rate of involved lymph nodes was higher in those with submucosal cancer than

in mucosal cancer (22.5% *vs* 9.0%,  $\chi^2 = 7.14$ ,  $P = 0.008$ ).

In our group, male patients predominated, accounting for 65.3%, while was similar to other reports<sup>[18,23-26]</sup>. Studies to date suggest that EGC frequently occurs in the lower third of the stomach<sup>[18,22,26]</sup>. In our study, 48.5% of cases originated in the middle third, which was similar to the report from Fujimoto *et al*<sup>[14]</sup>. Also in our group, 52.0% of patients had tumors greater than 2 cm. Larger tumors have higher rates of lymph node metastases. Of the 29 cases with lymph node metastases, the tumor size in 18 was greater than 2 cm, accounting for 62.1% of all metastases. Morphological classification was mainly of the depressed type (77.2%). Among these, type 0-IIc and type 0-III accounted for approximately 50%. Histologically, most tumors were differentiated, accounting for 59.9%, which was similar to that reported by Abe *et al*<sup>[27]</sup>.

Although the lymph node metastatic rate in EGC is relatively low, it has been shown that the presence of lymph node metastases predicts a poor prognosis<sup>[28-32]</sup>. Thus, many researchers have attempted to investigate the relationship between nodal involvement and clinicopathological factors. The size of the primary tumor, undifferentiated histopathological characteristics, lymphatic or venous invasion, and a cancerous ulcer are associated with nodal metastases in EGC<sup>[8,9,12,14,32-42]</sup>. Univariate analysis confirmed a correlation between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases in EGC in our study ( $\chi^2 = 122.901$ ,  $P = 0.001$ ;  $\chi^2 = 7.14$ ,  $P = 0.008$ ;  $\chi^2 = 79.523$ ,  $P = 0.001$ ;  $\chi^2 = 8.687$ ,  $P = 0.003$ , respectively), while nodal metastases were not associated with sex, age, tumor size, number of retrieved lymph nodes, histological type, lymphatic invasion, nervous invasion, and CEA levels. Boku *et al*<sup>[12]</sup> reported an association between lymph node metastases and the tumors arising from the distal third of the stomach, indicating that cancers arising from this section have a worse prognosis. In our study, tumors originating in the upper third of the stomach had a nodal metastatic rate of 8%, compared to 15.3% for the middle third and 15.2% for the lower third, which was similar to that reported by Boku *et al*<sup>[12]</sup>. Reports have suggested both a lack of association between the occurrence of lymph node metastases and mucosal tumors of the upper and middle third of the stomach<sup>[19]</sup>, and a marked increase in nodal involvement with distal gastric cancers<sup>[43]</sup>. Data from our study revealed a lymph node metastases rate in mucosal and submucosal tumors of 9.0% and 22.5%, respectively, which was similar to other reports<sup>[19,44]</sup>. At present, endoscopic submucosal resection is often used for patients with mucosal cancer, while data from Hölscher contradicts this approach<sup>[19]</sup>. Our data indicates that, prior to surgery, determination of the depth of invasion is important for predicting lymph node metastases. Preoperative staging technology, particularly endoscopic ultrasonography, can determine the depth of invasion, but there can be errors of judgment<sup>[11,16,45,46]</sup>. In Japan, the morphological classification of EGC is summarized as elevated, flat and depressed type. The rate of

lymph node metastases in the elevated subtype was relatively high, being 16% in our study. The rate of lymph node metastases tends to be markedly higher in patients with venous or lymphatic invasion<sup>[46]</sup>. However, our results did not concur with this, which might be explained by the fact that the number of specimens varied, and the quantity of venous or lymphatic invasion that we included was low.

It is generally acknowledged that tumor size is closely related to lymph node metastases. Hölscher<sup>[19]</sup> found no evidence of lymph node metastases in mucosal cancers less than 1 cm, and in submucosal cancers. Moreover, lymph node metastases have not been identified in patients with mucosal cancers less than 2 cm<sup>[19]</sup>. Tumors greater than 2 cm appear to be independent risk factors for lymph node metastases<sup>[19,22,47,48]</sup>. In our group, although the rate of lymph node metastases in patients with tumors greater than 2 cm (17.1%) was clearly greater than that observed with smaller tumors (11.3%), the difference was not significant, which is similar to the results from Lee *et al*<sup>[18]</sup>.

Histological type is also closely related to nodal status<sup>[9,18,35,41,42]</sup>. In our group, the rate of lymph node metastases in non-differentiated tumors was higher than in differentiated cancer, 17.3% and 12.4%, respectively. However, a non-differentiated tumor was not an independent risk factor for lymph node metastases. Abe *et al*<sup>[49]</sup> suggest that, apart from tumor size, submucosal invasion and lymphatic invasion, a correlation also exists between females and the occurrence of lymph node metastases. In females, the biological behavior of gastric cancer tends to be more invasive. Moreover, it has also been shown that the extent of the invasiveness of gastric cancer cannot be fully explained by tumor size, depth of invasion or lymphatic invasion<sup>[49]</sup>. A possible explanation for the fact that gastric cancer tends to be more invasive in females could be related to endogenous estrogen levels, which might promote tumor growth.

In recent years, endoscopic surgery has become one of the standard procedures, and is indicated for patients with mucosal tumors, tumors less than 2 cm in size, and those without lymph node metastases. Our results suggest that the rate of lymph node metastases is markedly higher in those with cancers of the middle and lower third of the stomach, and those with submucosal tumors, who are therefore not suitable for endoscopic surgery.

Multivariate analysis found that only depth of invasion was an independent risk factor for lymph node metastases in EGC [ $P = 0.018$ , Exp (B) = 2.744], which is consistent with previous studies<sup>[18,26,31,32,35,37,50-55]</sup>. Multivariate analysis by Kim *et al*<sup>[3]</sup> on 748 cases of EGC, indicated that tumor size, poorly differentiated tumors and submucosal cancers were all independent risk factors for lymph node metastases. Hyung *et al*<sup>[56]</sup> suggest that poorly differentiated tumors, submucosal cancer, tumor size, and venous or lymphatic invasion are independent risk factors for nodal metastases. Many other studies have reached similar conclusions, namely that tumor

size, depth of invasion, histological type, morphological classification, venous or lymphatic invasion, are all independent risk factors for lymph node metastases in EGC<sup>[3,14,22,26,27,47,48,50,56-62]</sup>.

A consideration of the number of lymph node is related to the extent of surgical intervention, which is also related to the depth of tumor invasion. Thus the depth of tumor invasion is also associated with the number of lymph nodes: as the depth increases, the number of lymph nodes also increases. In our group, all 202 cases of EGC had a radical gastrectomy. Post-operatively, 2892 regional lymph nodes were located. In 122 cases, where tumor invasion was limited to the mucosal layer, 1790 lymph nodes were found, with an average of 14.67 per case. In 80 cases with tumor invading the submucosa, 1102 lymph nodes were detected, with an average of 13.77 per case. The difference between the number of lymph nodes in mucosal and in submucosal cancer was not significantly different ( $P = 0.727$ ). Five hundred and sixty lymph nodes were found in 29 cases with lymph node metastases, an average of 19.31 per case. In our study, there was a relationship between the number of lymph nodes and the presence of lymph node metastases. In 29 patients with metastases, the number of involved nodes with tumor depth limited to the mucosa was similar to that observed for submucosal tumors, with no significant difference between the two groups.

Our results showed that there was no obvious correlation between lymph node metastases in mucosal cancer, and sex, age, tumor location, tumor size, the number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion and CEA levels. Currently, there is much interest in the optimal management approach for patients with submucosal tumors and involved lymph nodes<sup>[36-40]</sup>. In a univariate analysis, An *et al*<sup>[22]</sup> demonstrated that tumor size, histological type, the Lauren classification, the depth of tumor invasion, lymphatic invasion and nervous invasion, were all relevant in terms of the risk of lymph node metastases in submucosal cancer. Of these factors, tumor size and lymphatic invasion are independent risk factors for nodal involvement<sup>[22,36-39]</sup>. In our study, there was no obvious correlation between the presence of lymph node metastases in patients with submucosal cancer, and sex, age, tumor location, tumor size, the number of retrieved lymph nodes, morphological classification, histological type, lymphatic invasion, nervous invasion, and CEA levels. Nonetheless, the lymph node metastases rate (60%, 3/5) in those with venous invasion was higher than that observed in patients without invasion (20%, 15/75), and the difference was significant. However, an analysis of venous invasion, as a source variable, in the logistic regression model, did not confirm it as an independent risk factor for lymph node metastases. An *et al*<sup>[22]</sup> considered that, for 19.4% of patients with submucosal cancer and lymph node metastases, laparoscopic subtotal distal gastrectomy plus lymph node dissection improved both the resection rate and quality of life. Controversy continues

as to which surgical approach is the optimal for the management of patients with submucosal cancer<sup>[40]</sup>.

Most lymph node metastases in EGC are limited to N1 and/or N2. In line with previous reports<sup>[34,63]</sup>, and our own experience, approximately 80%-90% of nodal metastases in EGC are limited to the N1. The proportion of involved N2 and N3 lymph nodes is lower at 10%-19% and 0%-1%, respectively<sup>[22,34,63,64]</sup>. This appears to support R2 resection<sup>[65]</sup>. In our group, of 29 patients with lymph node metastases, all were N1, seven were N2 (24.1%), and two were N3 (6.9%). These results are all higher than those reported by An *et al*<sup>[22]</sup>. Among them, the two N3 patients had the following clinicopathological features: both were mucosal cancers; the tumor size was relatively large, at 6 cm and 2.5 cm, respectively; and both tumors were depressed subtypes. Both patients underwent a radical gastrectomy plus a D2 lymph node dissection. Even in EGC, extensive lymph node metastases can occur. Surgeons should avoid conventional limited surgery, and assess the range of possible surgical resections<sup>[22]</sup>. Kuni-saki *et al*<sup>[55]</sup> believe that, for most cases of mucosal cancer, the routine D2 lymph node dissection might also be too invasive. Therefore, the method and range of lymph node dissection should be based on clinicopathological features before and after surgery. However, Yoshikawa *et al*<sup>[44]</sup> suggest that, during surgical treatment of mucosal cancers, apart from perigastric lymph node dissection, it is necessary to perform lymph node dissection at the coeliac trunk and beside the common hepatic artery. Our results support this recommendation. In our group, there was one case of mucosal cancer with a station No. 8 lymph node metastasis.

Since the mid 1900s, D2 lymph node dissection has been the standard procedure worldwide for the management of EGC, particularly for submucosal tumors. Studies have shown that the rate of lymph node metastases for N1 is 9%-16%, 4%-6% for N2, and 0.3%-1% for N3<sup>[49,56,59,66]</sup>. Accordingly, if D2 surgery is performed in all patients with EGC, 70%-80% would undergo unnecessary lymph node evacuation, and analysis suggests that these patients do not necessarily benefit as a result<sup>[40]</sup>. Consequently, indications for standard D2 lymph node dissection should be reconsidered. Our study revealed that, for cancers originating in the middle third of the stomach, the lymph node metastasis rate at N2 was 5.1%, mainly station No. 9, whereas there were no metastases in stations No. 7 and No. 12. Similarly, the metastasis rate in lower third tumors was 2.5%, mainly station No. 7 and No. 14. There was one case of metastasis (1.3%) in each of these. In upper third cancers, the rate was 4.0%, predominately station No. 8, with one case of metastasis (4.0%). Thus, when performing D2 surgery, patients with station No. 7 and No. 12 lymph nodes in tumors from the middle third of the stomach, stations No. 1, 8, 9, 11, and 12 lymph nodes in those from the lower third, and stations No. 7, 9-11 lymph nodes in the upper third should not be referred for routine dissection. In tumors of the middle third of the stomach, there was only one

case of N3 lymph node metastasis in both stations No. 2 and No. 14, and the metastasis rate was 1.0%. There were no N3 and distal lymph node metastases in tumors from both the upper and the lower third of the stomach. The benefit of performing D3 dissection in EGC is extremely low, with a long operative time and relatively more complications, which adversely affect quality of life. Therefore, D3 dissection should be avoided in EGC. There were no distal lymph node metastases in our study, and thus expanded lymph node dissection was not required.

Investigating the correlation between pN1 and pN2 lymph node metastases and clinicopathological factors, we found no significant associations between nodal status at pN1 and pN2 and tumor diameter, morphological classification, tumor differentiation and lymphatic invasion. The lymph node metastasis rate (22.5%, 18/80) at N1 in patients with submucosal cancers was higher than in those with mucosal cancers (9.0%, 11/122), and the difference was significant ( $\chi^2 = 7.144$ ,  $P = 0.008$ ). A histological study performed by Asao *et al.*<sup>[67]</sup> on 417 patients with gastric cancer who had routine gastrectomy plus D2 lymph node dissection, reported a rate of lymph node metastases in submucosal cancer of 1.3% (2/154), and in mucosal cancer distal to the perigastric part of 18% (17/96). These nodal metastases were mainly concentrated around the common hepatic artery and the coeliac trunk. In submucosal cancer, there was no nodal metastases elsewhere<sup>[67]</sup>. However, our study highlighted that tumors with N2 lymph node metastases were usually submucosal, with a diameter greater than 2.0 cm and were of the depressed subtype. Thus, we suggest that submucosal cancers, with a diameter greater than 2.0 cm, and of the depressed subtype are risk factors lymph node metastases. Patients with these factors should be identified during surgery.

There are limitations to this study. Firstly, it was retrospective, based on the examination of morphological samples after surgery. Prospective studies are needed to confirm whether our approach could be applied to endoscopic surgery based on biopsies. It is essential to define the acute T stage before surgery, in order to safely implement limited lymph node dissection. Although the accuracy of endoscopic ultrasonography is relatively high, overestimation or underestimation occurs<sup>[46]</sup>. Secondly, according to a previous Japanese study based on different depths of tumor invasion, mucosal cancer can be further subdivided into m1, m2, and m3 cancer, and submucosal cancer into sm1, sm2 and sm3 cancer. Moreover, there were obvious differences between the subgroups, with m1 and m2 cancers not usually associated with lymph node metastases, and the rate of nodal metastases in m3 cancers varies from 0% to 12.8%. At present, most endoscopic specialists agreed that all patients with mucosal cancers are suitable for EMR, while Hölischer *et al.*<sup>[19]</sup> oppose this view. Unfortunately, our two hospitals do not routinely perform sub-classifications for mucosal and submucosal cancers, which we plan to redress in future studies. Thirdly, the number of patients in our study was

lower than in the Japanese study. In a seminal study by Gotoda<sup>[46]</sup>, 5000 patients with EGC were enrolled. Results from our study need to be confirmed in larger studies.

In summary, our research revealed a lymph node metastasis rate of 14.4% in patients with EGC, with a rate of 5.4% for mucosal tumors and 8.9% for submucosal tumors. The occurrence of lymph node metastasis in EGC is related to tumor location, depth of invasion, morphological classification and venous invasion, with depth of invasion identified as the only independent risk factor for nodal involvement. Lymph node metastases should be considered when deciding on the surgical management of EGC.

## COMMENTS

### Background

Five-year survival rates in early gastric cancer (EGC) tend to be greater than 90%, with lymph node status the most important prognostic factor. Recently, in order to reduce operative and post-operative complications, and to improve quality of life, less invasive surgical alternatives, such as endoscopic mucosal resection (EMR), endoscopic submucosal dissection (ESD), laparoscopy-assisted gastrectomy and limited surgery, are used for the treatment of EGC. Although there has been substantial research on the prediction of risk factors for lymph node metastases in EGC, no definitive criteria exist.

### Research frontiers

The presence of lymph node metastases in EGC predicts a poor prognosis. In the area of the appropriate extent of lymphadenectomy in EGC, the research hotspot is to investigate the relationship between nodal involvement and clinicopathological factors, predict risk factors for lymph node metastases in EGC and determine the indications for local treatment in EGC, and limited surgery and the appropriate extent of lymphadenectomy.

### Innovations and breakthroughs

The presence of lymph node metastases predicts a poor prognosis, univariate analysis confirmed a correlation between tumor location, depth of invasion, morphological classification, venous invasion and lymph node metastases in EGC in this study. The results suggest that the rate of lymph node metastases is markedly higher in those with cancers of the middle and lower third of the stomach, and those with submucosal tumors, who are therefore not suitable for endoscopic surgery. When performing D2 surgery, patients with station No. 7 and No. 12 lymph nodes in tumors from the middle third of the stomach, stations No. 1, 8, 9, 11, and 12 lymph nodes in those from the lower third, and stations No. 7, 9, 10, and 11 lymph nodes in the upper third should not be referred for routine dissection. D3 dissection should be avoided in EGC. There were no distal lymph node metastases in this study, and thus expanded lymph node dissection was not required. The authors suggest that submucosal cancers, with a diameter greater than 2.0 cm, and of the depressed subtype, are risk factors for lymph node metastases. Patients with these factors should be identified during surgery.

### Applications

The depth of invasion is the only independent risk factor for lymph node metastases. Risk factors for metastases should be considered when choosing surgery for EGC.

### Terminology

EGC: A lesion confined to the mucosa or the submucosa, irrespective of the presence of regional lymph node metastases; EMR: A technique used for providing accurate histological staging of superficial gastrointestinal neoplasms and providing a minimally invasive technique for removal of superficial malignancies; ESD: A technique for the resection of early gastrointestinal neoplasia, whose main advantage is that lesions can be resected without almost any size limit.

### Peer review

The paper is good and complex. The authors thoroughly explained the problem of prediction of risk factors for lymph node metastasis in early gastric cancer.

They have described, in detail, the research results, as well as, the discussion. The conclusion was a logical consequence of the research results.

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**P-Reviewer** Stanojevic GZ **S-Editor** Song XX  
**L-Editor** Ma JY **E-Editor** Zhang DN



## Rectal gastrointestinal stromal tumors: Imaging features with clinical and pathological correlation

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Received: February 26, 2013 Revised: April 1, 2013

Accepted: April 10, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To investigate computed tomography (CT) and magnetic resonance imaging (MRI) manifestations of rectal gastrointestinal stromal tumors (GISTs) in order to enhance the recognition of these rare tumors.

**METHODS:** Fourteen patients with pathologically proven rectal GISTs were retrospectively reviewed. Patient histories were retrospectively reviewed for patient age, gender, presenting symptoms, endoscopic investigations, operation notes and pathologic slides. All tumors were evaluated for CD117, CD34 expression, and the tumors were stratified according to current criteria of the National Institutes of Health (NIH). In all cases the first pre-operation imaging findings (CT and MRI,  $n = 3$ ; MRI only,  $n = 8$ ; CT only,  $n = 3$ ) were analyzed by two experienced radiologists by consensus, which include:

tumor size, shape, CT density (hypodense, isodense and hyperdense), MRI signal intensity (hypointense, isointense and hyperintense), epicenter (intraluminal or extraluminal), margin (well-defined or ill-defined), internal component (presence of calcifications, necrosis, hemorrhage or ulceration), pattern and degree of enhancement, invasion into adjacent structures. After review of the radiologic studies, clinical and pathological findings were correlated with radiological findings.

**RESULTS:** The patients, 13 men and 1 woman, were aged 31-62 years (mean =  $51.5 \pm 10.7$  years). The most common initial presentation was hematochezia ( $n = 6$ ). The mean tumor diameter was  $5.68 \pm 2.64$  cm (range 1.5-11.2 cm). Eight lesions were round or oval, and 6 lesions were irregular. Eleven lesions were well-defined and 3 had ill-defined margins. Ten tumors were extraluminal and 4 were intraluminal. The density and MR signal intensity of the solid component of the lesions were similar to that of muscle on unenhanced CT ( $n = 6$ ) and T1-weighted images ( $n = 11$ ), and hyperintense on T2-weighted MR images. Calcification was detected in 2 tumors. Following intravenous injection of contrast media, 3 lesions had mild enhancement and 11 lesions had moderate enhancement. Enhancement was homogenous in 3 lesions and heterogeneous in 11. In 1 of 11 patients who underwent both CT and MRI, the tumor was homogenous on CT scan and heterogeneous on MRI. Eight patients were classified as high risk according to the modified recurrent risk classification system of NIH.

**CONCLUSION:** Rectal GISTs usually manifest as large, well-circumscribed, exophytic masses with moderate and heterogeneous enhancement on CT and MRI. The invasion of adjacent organs, bowel obstruction and local adenopathy are uncommon.

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**Key words:** Gastrointestinal stromal tumors; Rectum;

## Computed tomography; Magnetic resonance imaging

**Core tip:** Rectal gastrointestinal stromal tumors (GISTs) are so rare that little information has been reported on their radiological features. This study describes the computed tomography (CT) and magnetic resonance imaging (MRI) features of rectal GISTs with clinical and pathological correlation, in order to better understand this rare disease. Rectal GISTs usually manifest as large, well-circumscribed, exophytic masses with moderate and heterogeneous enhancement on CT and MRI. The presence of invasion of adjacent organs, bowel obstruction and local adenopathy is uncommon.

Jiang ZX, Zhang SJ, Peng WJ, Yu BH. Rectal gastrointestinal stromal tumors: Imaging features with clinical and pathological correlation. *World J Gastroenterol* 2013; 19(20): 3108-3116 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3108.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3108>

## INTRODUCTION

Gastrointestinal stromal tumors (GISTs), which arise from the interstitial cell of Cajal or its precursor, the intestinal mesenchymal precursor cell, are the most common mesenchymal neoplasms of the gastrointestinal tract. They are defined by their expression of CD117 (KIT), a tyrosine kinase growth factor receptor, which distinguishes them from other mesenchymal neoplasms such as leiomyomas, leiomyosarcomas, schwannomas, and neurofibromas and which determines the appropriateness of CD117-inhibitor therapy. Throughout the length of the gastrointestinal tract, GISTs arise most commonly in the stomach (60%-70%) followed by the small bowel (20%-25%), however, GISTs in the rectum are extremely rare (5%)<sup>[1]</sup>. It was reported that GISTs account for 0.6% of all malignant rectal tumors<sup>[2]</sup>. The imaging features of rectal GISTs are unclear due to their rarity and have only been described in small series<sup>[3-6]</sup>. Surgical resection remains the initial treatment for localized rectal GISTs<sup>[7]</sup>, familiarity of these imaging features may permit preoperative diagnosis and improve the surgical management of patients. The purpose of our study was to describe the computed tomography (CT) and magnetic resonance imaging (MRI) features of rectal GISTs with clinical and pathological correlation, in order to enhance the understanding of this rare disease.

## MATERIALS AND METHODS

The database of the Department of Surgery at our hospital was reviewed in order to identify patients presenting between January 2000 and June 2012 with histologically and immunohistochemically confirmed GISTs arising in the rectum. Fourteen patients were identified during this

period. Patient histories were retrospectively reviewed for patient age, gender, presenting symptoms, endoscopic investigations, and surgical notes. Pathologic slides of the specimens and mitotic activity (number of mitoses per 50 consecutive high-power fields) were reviewed by an experienced pathologist, and the tumors were stratified according to the current criteria of the National Institutes of Health (NIH)<sup>[8]</sup>. All tumors were evaluated for CD117 and CD34 expression.

In all cases, the first pre-operative CT or MRI was reviewed. Of the 14 patients, 3 underwent both CT and MRI examinations, 8 underwent only MRI examinations and 3 underwent only CT examinations. The CT and MRI technique varied somewhat due to the different imaging equipment and the retrospective nature of the study. However, intravenous contrast-enhanced images had been obtained for studies in all patients. A retrospective review of CT and MR images was implemented by two radiologists by consensus. The following imaging features of each mass were assessed: tumor size, shape, CT density (hypodense, isodense and hyperdense), MRI signal intensity (hypointense, isointense and hyperintense), epicenter (intraluminal or extraluminal), margin (well-defined or ill-defined), internal component (presence of calcifications, necrosis, hemorrhage or ulceration), pattern of enhancement (homogeneous or heterogeneous), and invasion into adjacent structures. The degree of enhancement of the lesion was assessed subjectively and categorized as follows: mild, when the enhancement was similar to that of adjacent muscle; moderate, when the enhancement was higher than that of muscle, but lower than that of blood vessels; and marked, when the enhancement was approaching that of blood vessels. High signal intensity in the necrosis on T1WI was defined as hemorrhage. The adjacent structures were also assessed for the presence of invasion. Findings on CT and MRI for abdominal adenopathy and hepatic metastasis were also evaluated. After review of the radiologic studies, surgical notes and excised specimens were correlated with radiological findings.

## RESULTS

### Clinical findings

Our patient cohort included 13 men and 1 woman with a mean age of  $51.5 \pm 10.7$  years (range, 31-62 years), and the clinical findings are summarized in Table 1. Digital colorectal examination was performed in 7 patients. Colonoscopy showed a mass protruding from the rectal wall with intact overlying mucosa in 6 patients (Figure 1), while a central mucosal ulceration was revealed in 1 patient. Two of these 7 patients underwent additional endoscopic ultrasonography, which showed hypoechoic masses that were contiguous with the muscularis propria of the rectal wall (Figure 1). All masses were found in the lower part of the rectum, with a distance from the anal verge ranging from 2.0 to 5.5 cm. Twelve patients

**Table 1 Summary of the presenting complaints of 14 patients with rectal gastrointestinal stromal tumors**

| Presenting symptoms or signs           | <i>n</i> |
|--|----------|
| Incidental finding on an imaging study | 2        |
| Change of defecation habit             | 2        |
| Hematochezia                           | 6        |
| Pelvic pain                            | 1        |
| Narrow stools                          | 1        |
| Difficult defecation                   | 1        |
| Sense of anal falling inflation        | 1        |

underwent radical resection, including abdominoperineal resection (*n* = 9) and low anterior resection (*n* = 3) depending on the extent and localization of the tumors. The other 2 patients underwent transanal excision. At the time of surgical exploration, none of the patients had evidence of remote metastasis. Tumor rupture was not found during surgery. No lymph node metastases were identified pathologically in all 12 patients who underwent lymphadenectomy.

### Pathological findings

Immunohistochemical staining showed that all tumors were positive for CD117 (14/14) (Figure 2), while 13 patients were positive for CD34 (13/14). The masses were solid and grayish-white or dark-red with areas of hemorrhage or necrosis on the cut sections (Figure 3), and showed a spindle cell pattern under light microscope (Figure 2). According to the current NIH classification scheme, 8 tumors (8/14) had high risk, 3 (3/14) had intermediate risk, 3 (3/14) had low risk for aggressive behavior.

### Imaging findings

The diameter of the tumors ranged from 1.5 to 11.2 cm (mean, 5.68 ± 2.64 cm). The tumor was smaller than 5 cm in 4 patients and larger than 5 cm in 10 patients. Eight masses were either round (*n* = 2) or oval (*n* = 6), and 6 were irregular. The tumors showed definite intraluminal tumor growth in 4 patients, whereas extraluminal tumor growth was seen in the majority of our patients (10/14) (Figures 1-8). Eleven tumors showed a well-defined margin, and 3 tumors showed contour irregularity or blurring.

On unenhanced CT images, the density of the 6 tumors was 33-45 HU (mean, 38 HU) which was similar to that of muscle. Flecks of calcification were detected in 2 extraluminal tumors (Figures 4, 5). Following intravenous administration of contrast media, the majority (4/6) of rectal GISTs were moderately enhanced masses with areas of unenhanced low attenuation on CT scan. A homogeneous pattern of enhancement was less common and was present in 2 of the patients who underwent CT studies (Figure 6); in 1 patient who underwent both CT and MRI, the tumor was homogenous on CT scan, but demonstrated heterogeneous enhancement with foci of fluid signal on MR images. All the heterogeneous tumors

showed areas of fluid density in keeping with cystic changes on cut sections.

In the 11 cases examined with MRI, the solid component of all the tumors was isointense to skeletal muscle on T1-weighted images and hyperintense on T2-weighted images. There were some intratumoral high-intensity foci on T1WI representing hemorrhage (*n* = 1) (Figure 3) and hyperintensity on T2WI corresponding to necrosis (*n* = 8) (Figure 7). Nine of these 11 tumors were heterogeneous, with non-enhancing components showing fluid signal. After intravenous administration of gadolinium, 7 lesions had moderate enhancement and 4 lesions had mild enhancement on fat-suppressed T1WI. Ulceration to the rectal lumen was seen in 1 patient (Figure 8). Bowel obstruction, abdominal adenopathy and remote metastasis were not seen in any of our patients.

## DISCUSSION

Although an increasing amount of literature concerning GISTs has been published, there is little information reported on the radiological features of rectal GISTs due to their rarity. To our knowledge, this article is the largest study of rectal GISTs in the radiological literature.

### Clinical characteristics

It was reported that rectal GISTs occur in adults between the fifth and sixth decades with a significant male predominance (71%)<sup>[9]</sup>. In this study, there was a marked male predominance with a male: female ratio of 13:1, and the mean age at onset was 51.5 years, in line with the pathological literature. The clinical manifestations of rectal GISTs depend on the location and size of the tumors and are often nonspecific<sup>[10-12]</sup>. A GIST can remain clinically silent and present late when the tumor is large. Two of our patients were asymptomatic and the tumors were incidental findings, perhaps due to their small size (less than 3.0 cm in maximum diameter) and tendency to grow exophytically. The exophytic growth pattern may also explain why rectal GISTs rarely cause intestinal obstruction even when they are large. Miettinen *et al*<sup>[9]</sup> reported that rectal bleeding was the most common symptom in patients with a large rectal GIST more than 5 cm. In this study, the most frequent symptom was also hematochezia.

### Imaging features

Rectal GISTs generally manifest as large eccentric masses growing beyond the rectal wall<sup>[3-6]</sup>. Our series showed a similar growth pattern. Although larger tumors have a higher rate of malignancy, size does not predict benignity, and small GISTs have been known to behave in a malignant fashion<sup>[13-16]</sup>. Since most rectal GISTs arise within the muscularis propria of the intestinal wall, they most commonly have an exophytic growth pattern with the epicenter located well outside the rectum<sup>[17,18]</sup>. GISTs arising from the anterior rectal wall in male patients can even mimic tumors of prostatic origin on CT<sup>[19,20]</sup>. Thus,

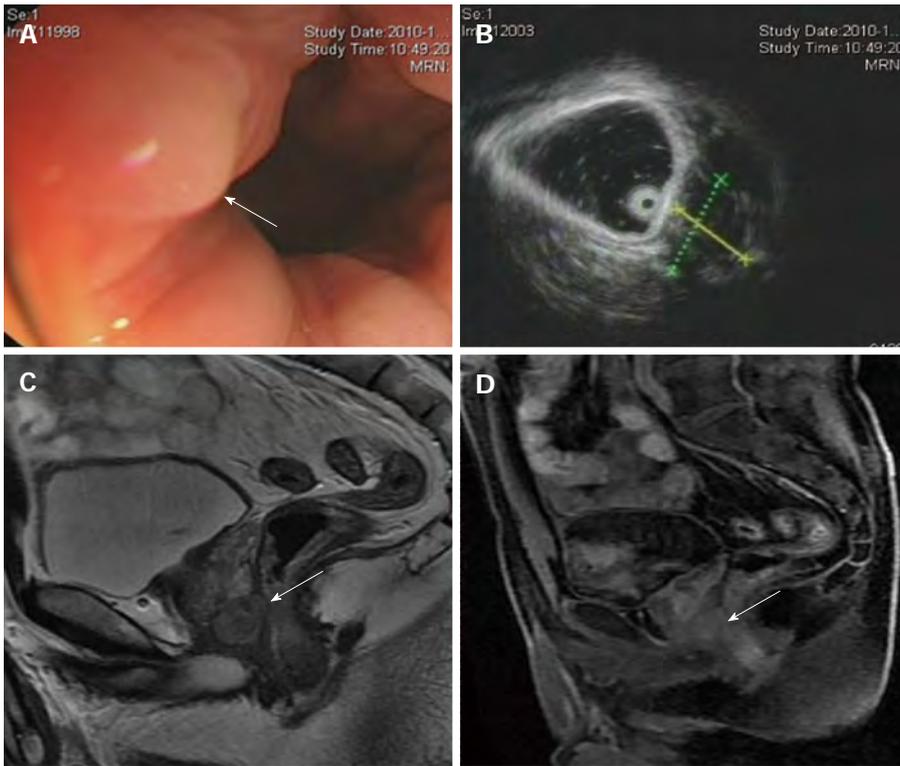


Figure 1 A 40-year-old man with a rectal gastrointestinal stromal tumor. A: Colonoscopy shows a mass protruding from the rectal wall with intact overlying mucosa (arrow); B: Endoscopic ultrasonography shows a well-defined hypoechoic mass located along the right anterior aspect of the rectal wall; C: Sagittal T2-weighted magnetic resonance imaging shows an oval, homogenous, hyperintense mass with a sharp margin bordering the anterior rectal wall. A small area of anatomical continuity between the tumor and the anterior rectal wall is observed (arrow); D: Postcontrast T1-weighted image shows a slightly homogeneously enhancing mass (arrow).

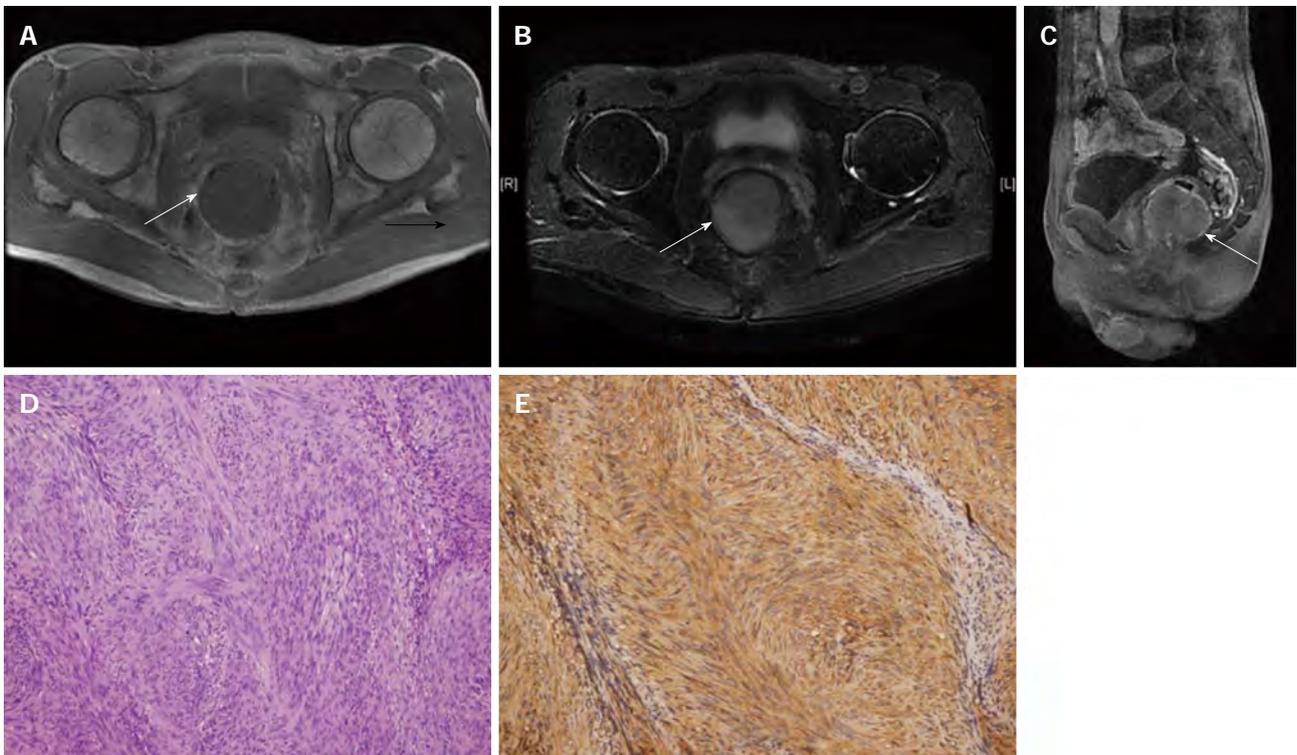
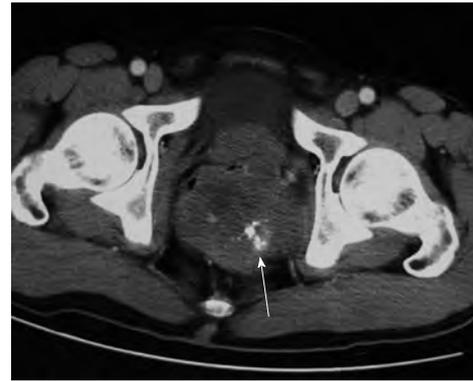


Figure 2 A 56-year-old man with a rectal gastrointestinal stromal tumor. A: Axial T1WI shows the lesion as a round, intraluminal, homogenous, hypointense mass with a sharp margin (arrow); B: It shows homogenous hyperintense on T2WI (arrow); C: Sagittal enhanced T1WI shows homogenous moderate enhancement (arrow); D: Photomicrograph shows fascicular proliferation of spindle-shaped tumor cells (hematoxylin and eosin,  $\times 200$ ); E: The tumor cells were strongly and diffusely positive for CD117 staining (immune-histochemistry,  $\times 200$ ).

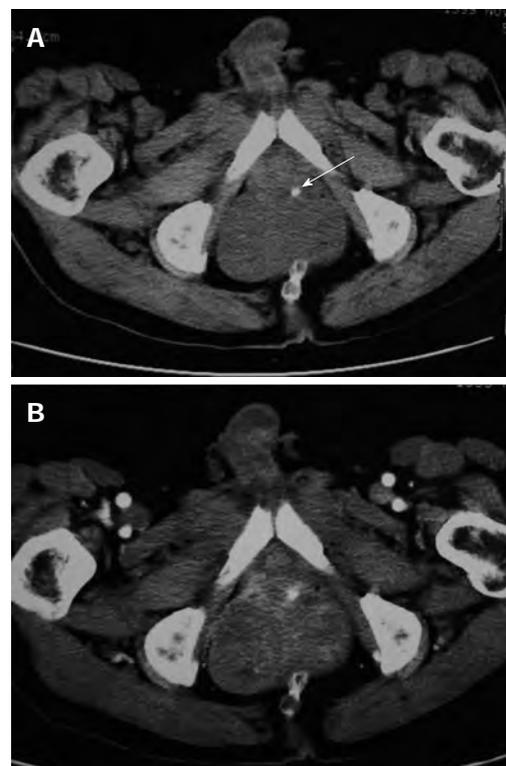


**Figure 3** A 62-year-old man with a rectal gastrointestinal stromal tumor. A: The mass is located between the prostate and the anterior rectal wall and its epicenter is well outside the rectum. Axial T1-weighted MR image shows a high signal (arrow) within the mass, in keeping with hemorrhage; B: Sagittal T2WI shows a heterogenous mixed-intense mass with blurring contour (arrow); C: Postcontrast T1WI shows the solid component of the mass enhanced heterogeneously (arrow); D: Macroscopic cross section shows a pale yellow, tan and solid mass with hemorrhage (arrow).

the classic rule regarding determination of the organ of origin based on the location of the epicenter of a



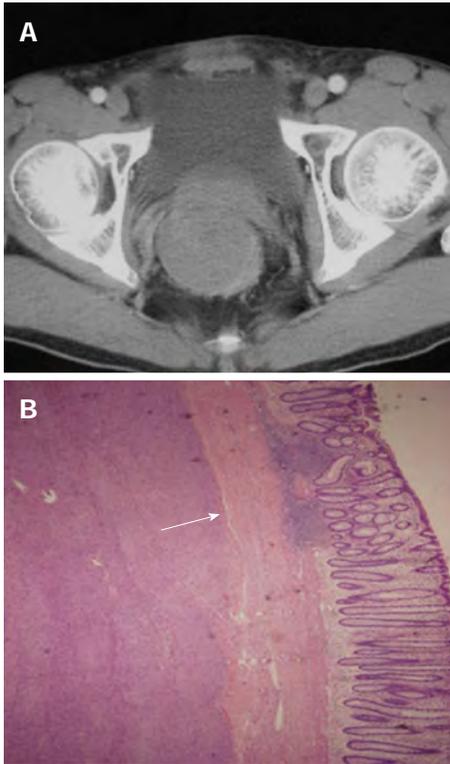
**Figure 4** A 52-year-old man with a rectal gastrointestinal stromal tumor. The mass is located in the left posterior wall of the rectum with scattered calcification (arrow).



**Figure 5** A 61-year-old man with a rectal gastrointestinal stromal tumor. A: The mass located in the left wall of the rectum with fleck of calcification at the tumor margin (arrow); B: The mass enhanced heterogeneously following intravenous administration of contrast media.

tumor is often not applicable. In such cases, enhanced MRI should be performed. MRI with direct multiplanar capability is definitely more useful in determining the exact tumor origin, delineating the spatial relation to adjacent structures, and outlining the pelvic floor surgical anatomy. In our study, tumor-rectal wall continuity was revealed in the sagittal plane, which suggested the diagnosis of a rectal mass. On the other hand, invasion of adjacent organs was better seen on MRI compared with CT examination.

In this study, most of the tumors were round or oval and smooth with well-defined margins. On unenhanced CT, rectal GISTs appear as isodense with normal muscle



**Figure 6** A 53-year-old man with a rectal gastrointestinal stromal tumor. A: Computed tomography scan shows a round intraluminal mass with a sharp margin; B: Photomicrograph shows the tumor originating from the muscularis propria (arrow) of the rectum (hematoxylin and eosin,  $\times 20$ ).

as the standard for comparison. On unenhanced MRI, rectal GISTs appear as isointense to skeletal muscle on T1-weighted images and hyperintense on T2-weighted images, and moderately or mildly enhanced on CT and/or MR studies. A heterogeneous pattern of enhancement is more common on CT and/or MR studies, and was present in 11 of our cases. Heterogeneity corresponds to intralesional necrosis or hemorrhage, which was confirmed on cut sections. It is likely that MRI may be superior to CT in detecting the internal component. Only one of our patients underwent both CT and MRI, and the tumor was homogenous on CT scan, but was heterogeneous with fluid signal on MR images.

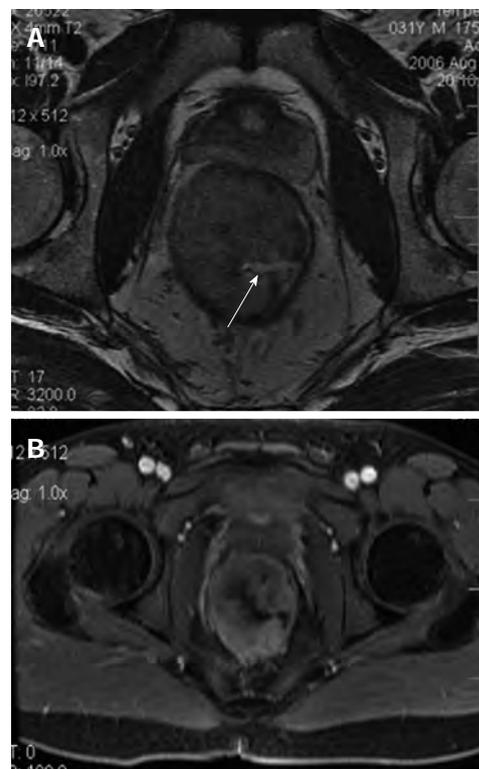
Although calcification is not a usual clinicopathologic feature of GISTs, it has been reported in previous studies<sup>[21-23]</sup>. Most calcifications within GISTs are circumscribed and patchy. In our study, one of the two calcified tumors showed focal calcification and the other was mottled. Previous episodes of bleeding or tumor necrosis with cystic degeneration may cause calcification<sup>[24,25]</sup>. Because these tumors are submucosal, the overlying mucosa can be intact. However, overlying mucosal ulcerations are often present and cause bleeding, and are more common in high risk GISTs<sup>[26-28]</sup>.

### Differential diagnosis

Unfortunately, there is significant overlap between the imaging appearances of rectal GISTs and other rectal diseases, such as epithelial neoplasms, lymphoma or



**Figure 7** A 59-year-old woman with a rectal gastrointestinal stromal tumor. The mass is located between the uterus and the anterior rectal wall with focal fluid signal on T2WI corresponding to necrosis (arrow).



**Figure 8** A 31-year-old man with a rectal gastrointestinal stromal tumor. A: Axial T2WI shows the lesion as an intraluminal mass with ulceration (arrow) of the rectal lumen; B: The mass is enhanced moderately heterogeneously following intravenous administration of gadolinium.

carcinoids. Most of these tumors may be differentiated from rectal GISTs by the presence of the following features: well-demarcated margins, prominent extraluminal location and no surrounding adenopathy, and the lack of bowel lumen constriction despite the large size of the rectal GIST. This behavior is unlike most cases of adenocarcinoma which has a propensity for luminal obstruction. The appearance of a smooth regular border is a feature that allows these tumors to be differentiated from malignant epithelial neoplasms. Signs of intratumoral degeneration, such as cystic change, hemorrhage, and calcification, should exclude lymphoma from the

differential diagnosis.

Due to the submucosal origin of the tumors, endoscopy is only of use when the tumor infiltrates the mucosa and can be detected<sup>[29]</sup>. In addition to endoscopy, endoscopic ultrasonography is a valuable technique in the diagnosis of these tumors because it can reliably distinguish intramural lesions from extrinsic compression. Enhanced MRI with an endoluminal coil has been performed to determine the tumor origin in some reported cases<sup>[30-32]</sup>, but these procedures are invasive, and are rarely used in routine examinations.

### Pathological features

CD117 is the most specific and important diagnostic molecular marker of GISTs. Most GISTs (more than 95%) express CD117, which can be detected immunohistochemically<sup>[9,33]</sup>. Although CD117-positive expression is very common in GISTs and a major defining characteristic of GISTs, it is not absolutely necessary for the diagnosis of GISTs. CD34 is also commonly expressed in GISTs, which is less specific compared with CD117 and is not considered a requirement for the diagnosis of GISTs either<sup>[34-36]</sup>. The review of pathology in our patients showed that the tumors were strongly positive for CD117 and in most cases also CD34, consistent with previous reports.

The pathologic differentiation and biologic behavior of GISTs have been continuing topics of controversy for many years. A small number of GISTs recur or metastasize despite a histologically benign appearance. Therefore, some authors support stratifying GISTs into very low-, low-, intermediate-, and high-risk categories rather than classifying them as benign or malignant. The NIH risk classification system, which consists of tumor size, mitotic count (number of mitoses per 50 consecutive high-power fields), anatomic location, and tumor rupture, is recommended as a valuable tool for estimating the clinical behavior of GISTs<sup>[8]</sup>. Rectal GISTs have a high-risk tendency<sup>[23]</sup> which was observed in the majority of our patients.

Attempts to predict the potential high-risk behavior of GISTs from their imaging features is difficult. It was reported that GISTs with irregular margins, size larger than 10 cm, central necrosis, ulceration, and heterogeneous contrast enhancement are normally regarded as denoting aggression<sup>[37,38]</sup>. These signs are mainly derived from a study population of stomach and small intestine GISTs and seldom from rectal GISTs. However, our series is small and no correlation between radiologic appearance and risk levels could be established with regard to the degree of necrosis, hemorrhage, ulceration, or contrast material enhancement.

There are several limitations in this study. The study was retrospective and the reviewers of the imaging studies knew that all patients had a pathologically confirmed rectal GIST, which may have increased the sensitivity for detecting each imaging sign. In addition, different CT and MRI equipment and techniques were used. How-

ever, these problems are simply unavoidable due to the rarity of this type of tumor, and this should not have significantly affected the imaging features studied.

In summary, we present the largest series of radiological studies of rectal GISTs to date. These tumors often present with hematochezia. They are normally seen as large, bulky, exophytic rectal masses with heterogeneous enhancement on CT and MRI. Cross-sectional imaging, which allows better visualization of the origin of the mass, its internal components, and other organ involvement, is indicated for surgical planning.

## COMMENTS

### Background

Rectal gastrointestinal stromal tumors (GISTs) are very rare, which accounts for 0.6% of all rectal malignant tumors. Only a few reports concerning the radiological features of rectal GIST has been published due to its rarity. Surgical resection remains the mainstay of therapy for localized rectal GISTs, familiarity of their radiological features may permit preoperative diagnosis and improves surgical management of patients. Thus, it is very important to enhance the understanding of the imaging features of this rare tumor.

### Research frontiers

The current imaging knowledge of rectal GISTs is based on a few case reports. Grassi *et al* reported a 4-cm mass in a 70-year-old man that showed marked, irregular, eccentric thickening of the lateral left wall of the lower third of the rectum. Hama *et al* reported a 9.8-cm mass in a 50-year-old man that was contiguous with the prostate and enhanced on both computed tomography (CT) and magnetic resonance imaging (MRI). Levy *et al* reported six anorectal gastrointestinal stromal tumors, and found that anorectal GISTs were typically large, well-demarcated anorectal masses containing hemorrhage.

### Innovations and breakthroughs

This study contained a relative large cohort of cases of rectal GISTs confirmed by histology and immunochemistry. All the patients had a complete medical records including age, gender, presenting symptoms, endoscopic examinations, surgical notes and pre-operative cross-sectional imaging studies. The authors focused on the correlation of imaging features of this rare tumor with clinical and pathological characteristics.

### Applications

Rectal GISTs are normally seen as large, bulky, exophytic rectal masses with heterogeneous enhancement on CT and MRI. Cross-sectional imaging, which allows better visualization of the origin of the mass, its internal components, and other organ involvement, is indicated for surgical planning.

### Terminology

GIST is the most common mesenchymal neoplasm of the gastrointestinal tract, which arises from the interstitial cell of Cajal or its precursor. The rectum is the rare primary site involving about 5% cases. Due to the submucosal origin of the rectal GISTs, the presence of the following features allows these tumors to be differentiated from malignant epithelial neoplasms: well-demarcated margins, prominent extraluminal location and no surrounding adenopathy, and the lack of bowel lumen constriction despite the large size of the tumor.

### Peer review

The authors reported imaging features of rectal gastrointestinal stromal tumors with clinical and pathological correlation. The main strength of this study is the relatively large number ( $n = 14$ ) of patients and broader spectrum of imaging findings in this rare tumor. The findings are instructive to both radiologists and physicians.

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**P- Reviewers** Chen F, Nough MR **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Zhang DN



## Synchronous adenocarcinoma and gastrointestinal stromal tumors in the stomach

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Supported by Shanghai Jiaotong University Medical School for Scientific Research, No. 09XJ21013; Shanghai Health Bureau for Scientific Research, No. 2010029; Shanghai Science and Technology Commission for Scientific Research, No. 124119a0300

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Received: January 6, 2013 Revised: April 19, 2013

Accepted: May 9, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To review the clinicopathological characteristics of concurrent gastrointestinal stromal tumors (GISTs) and gastric adenocarcinoma.

**METHODS:** We retrospectively analyzed eight cases of synchronous adenocarcinoma and GIST in the stomach that had been surgically resected with curative intent between March 2003 and December 2008 in Xinhua hospital and Ruijin hospital. The adenocarcinoma was determined to be the primary tumor based on the histological features. The GIST cells were diffusely and strongly positive for CD34 and CD117.

**RESULTS:** The patients were six men and two women aged 47-80 years (average, 68.6 years). GIST was pre-operatively detected in only one patient. The average sizes of the gastric adenocarcinomas and GISTs were

6.000 ± 2.6186 cm and 1.825 ± 1.4370 cm, respectively. All GISTs were very low- or low-risk lesions that were detected during evaluation, staging, operation or follow-up for gastric adenocarcinoma.

**CONCLUSION:** We hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

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**Key words:** Gastric adenocarcinoma; Gastrointestinal stromal tumor; Synchronous occurrence; Gastrectomy

**Core tip:** We retrospectively analyzed eight cases of synchronous adenocarcinoma and gastrointestinal stromal tumors (GISTs) in the stomach that had been surgically resected with curative intent between March 2003 and December 2008 in Xinhua hospital and Ruijin hospital. The average sizes of the gastric adenocarcinomas and GISTs were 6.000 ± 2.6186 cm and 1.825 ± 1.4370 cm, respectively. All GISTs were very low- or low-risk lesions that were detected during evaluation, staging, operation or follow-up for gastric adenocarcinoma. We hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

Cai R, Ren G, Wang DB. Synchronous adenocarcinoma and gastrointestinal stromal tumors in the stomach. *World J Gastroenterol* 2013; 19(20): 3117-3123 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3117.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3117>

### INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are mesen-

chymal tumors of the digestive tract and have various clinical and biological characteristics. The expression of c-Kit distinguishes GISTs from true leiomyomas, leiomyosarcomas and other mesenchymal tumors of the gastrointestinal tract<sup>[1-3]</sup>. The stomach (60%-70%) and small intestine (20%-30%) are the most common sites of GISTs<sup>[1,2]</sup>. GISTs are composed of spindle (70%) or epithelioid (30%) cells and are positive for c-Kit (CD117), CD34 and occasionally for actin, but almost always negative for desmin and S-100 protein<sup>[4]</sup>, in contrast to other mesenchymal tumors. Benign GISTs are far more common than malignant ones (10%-30%)<sup>[1]</sup>, and all GISTs are often found incidentally at surgery and excised in the same session<sup>[5]</sup>.

Collision tumors of the stomach are uncommon; however, several cases have been reported. Most collision tumors of the stomach are composed of an adenocarcinoma intermixed with a gastric lymphoma<sup>[6-9]</sup>. Some are composed of an adenocarcinoma intermixed with a carcinoid tumor<sup>[8,10,11]</sup>. However, gastric collision tumors composed of a GIST and an adenocarcinoma are exceedingly rare. Ruka *et al.*<sup>[12]</sup> found that 10% of their GIST patients had an associated non-GIST neoplasm, usually, a carcinoma. Furthermore, Maiorana *et al.*<sup>[15]</sup> found that of 52 patients with gastric GISTs, six patients (11.5%) had an associated, second gastric tumor (five adenocarcinomas and one carcinoid tumor).

Most of these publications were reports of single cases. Here, we present a series of eight patients with synchronously occurring GISTs and gastric adenocarcinomas. The aim of this study was to evaluate the clinicopathological characteristics of GISTs occurring concomitantly with gastric adenocarcinomas and to provide an English literature review<sup>[14,15]</sup>.

## MATERIALS AND METHODS

### Patients

We analyzed the clinicopathological findings in eight patients with CD117-immunopositive GISTs (six men and two women) who underwent surgery with a curative intent for a primary, resectable tumor without detectable metastases, between March 2003 and December 2008. Their clinicopathological data were supplemented by a review of all available medical and histopathological records from Xinhua hospital and Ruijin hospital. Selected were the patients with postoperative pathological diagnosis of primary gastric GIST, and patients who didn't receive chemotherapy and Imatinib as adjuvant treatment. Excluded were patients who had synchronous gastrointestinal stromal tumors in stomach and extra-stomach neoplasms, patients with initial surgery performed in other hospital, and patients with the pathology diagnostic data insufficiency. None of the patients had a family history of gastrointestinal carcinoma or GIST. Prior to the commencement of the present study, our ethics committee deemed that approval of the committee and informed consent were not required for the retrospective analysis

of clinical data.

### Methods

GISTs were defined as primary spindle cell and/or epithelioid neoplasms of the tubular gastrointestinal tract with CD117 overexpression, with or without CD34 expression according to well-established criteria for the diagnosis of GISTs<sup>[16,17]</sup>. The risk of aggressive GISTs was assessed using criteria derived from the 2002 Fletcher classification. For each patient with a primary tumor and no metastatic disease at the time of diagnosis, a TGM system<sup>[18]</sup> was used for staging: T: T1, localized and < 5 cm; T2, localized and  $\geq$  5 cm; T3, contiguous organ invasion or peritoneal implants; T4, tumor rupture. G: G1, low grade; G2, high grade. M: M0, no metastases; M1, metastases present. The final staging system was determined as follows: Stages I (< 5 cm) and II ( $\geq$  5 cm) are localized lesions with low histologic grade. Stage III lesions are either high-grade tumors of any size or tumors with regional involvement (contiguous organ invasion or peritoneal implants). Stage IVA refers to patients with systemic metastases or unresectable tumor. Stage IVB is designated when tumor rupture has occurred despite resection of all macroscopic disease. The gastric adenocarcinomas were staged according to the TNM system devised by the International Union Against Cancer<sup>[19]</sup>.

Representative hematoxylin and eosin-stained slides of archival tumor specimens were prepared from formalin-fixed, paraffin-embedded tissue blocks. To confirm the histogenesis of GISTs, immunohistochemistry (IHC) panels were obtained with the following markers: CD117, CD34, smooth muscle actin (SMA) and S-100. The IHC studies were performed using formalin-fixed, paraffin-embedded blocks and primary antibodies to the above markers, on a standard, automated, streptavidin-biotin peroxidase-detection system (EnVision™ Autostainer Visualization System; DakoCytomation, Glostrup, Denmark) equipped with a microwave antigen-retrieval step. Parallel positive controls were run for each antibody. A rabbit or mouse, universal, negative-control monoclonal antibody was used for each specific antibody.

### Ethics committee approval

This study was approved by the Bioethics Committees at each hospital.

### Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences, version 11.0 (SPSS Inc., Chicago, IL). Descriptive data are presented as the mean  $\pm$  SD.

## RESULTS

The study consisted of eight patients, including six men (75%) and two women (25%). The median age of the patients at the time of presentation was 68.6 years (range, 47-80 years). The most common presenting features were

**Table 1** Size and histological characteristics of gastrointestinal stromal tumors in eight patients

| Patient No. | Age (yr) | Sex | Localization     | Size (cm) | Growth pattern | Type    | Grade    | Staging    | Surgical resection | Origin     |
|-------------|----------|-----|------------------|-----------|----------------|---------|----------|------------|--------------------|------------|
| 1           | 47       | M   | Cardia           | 2.0       | Extraluminal   | Spindle | Very low | T1G1M0, I  | Complete           | Subserous  |
| 2           | 80       | M   | Cardia           | 1.5       | Extraluminal   | Spindle | Very low | T1G1M0, I  | Complete           | Subserous  |
| 3           | 60       | M   | Antrum           | 0.6       | Extraluminal   | Spindle | Very low | T1G1M0, I  | Complete           | Subserous  |
| 4           | 67       | F   | Anterior wall    | 0.8       | Extraluminal   | Spindle | Very low | T1G1M0, I  | Complete           | Subserous  |
| 5           | 78       | M   | Posterior wall   | 2.5       | Extraluminal   | Spindle | Low      | T1G1M0, I  | Complete           | Submucosal |
| 6           | 78       | M   | Body             | 1.4       | Extraluminal   | Spindle | Low      | T1G1M0, I  | Complete           | Subserous  |
| 7           | 59       | F   | Anterior wall    | 0.8       | Extraluminal   | Spindle | Low      | T1G1M0, I  | Complete           | Subserous  |
| 8           | 80       | M   | Lesser curvature | 5.0       | Extraluminal   | Spindle | Low      | T2G1M0, II | Complete           | Subserous  |

M: Male; F: Female.

**Table 2** Immunohistochemical characteristics of gastrointestinal stromal tumors in eight patients

| Patient No. | CD117 | CD34 | SMA | VIM | S-100 | DES |
|-------------|-------|------|-----|-----|-------|-----|
| 1           | +     | +    | ++  | +   | +     | ++  |
| 2           | +     | +    | +   | +   | -     | +   |
| 3           | +     | +    | -   | +   | -     | -   |
| 4           | +     | +    | -   | +   | +     | +   |
| 5           | +     | +    | -   | +   | +     | -   |
| 6           | +     | +    | -   | -   | ±     | -   |
| 7           | +     | +    | -   | -   | -     | -   |
| 8           | +     | +    | -   | +   | -     | -   |

SMA: Smooth muscle actin; VIM: Vimentin; DES: Desmin.

abdominal discomfort ( $n = 3$ ), gastrointestinal bleeding ( $n = 2$ ), difficulty eating ( $n = 1$ ), nausea and vomiting ( $n = 1$ ) and weight loss ( $n = 1$ ). Some patients had more than one of these symptoms. The median duration of disease was 1.5 mo (range, 0.5-6 mo). Total ( $n = 1$ ) or subtotal gastrectomies ( $n = 7$ ) were performed in the patients with gastric malignancy.

All patients underwent preoperative gastroscopy, which revealed an ulcerative lesion in four patients, a diffuse infiltrative lesion in two patients and an infiltrative ulcerative lesion in two patients. All lesions were diagnosed as adenocarcinomas on biopsy examination. Body computer tomography (CT) scans and chest images were available for review in all eight patients. In one patient, preoperative CT revealed a soft tissue lesion with a diameter of 5.0 cm in the lesser curvature, this lesion was considered a GIST.

All patients underwent simultaneous, radical resection of the gastric adenocarcinoma and GIST. In most cases, the stromal tumors were an incidental finding during operation. Detailed clinicopathological data for all GISTs are shown in Tables 1 and 2. According to the American Joint Committee on Cancer staging, 87.5% of patients had stage I tumors, and 12.5% of patients had stage II tumors. The mean GIST size was  $1.825 \pm 1.4370$  cm (range, 0.6-5.0 cm). Seven GISTs were located in the serosal layer, and one was present in the muscular layer. All GISTs were of the spindle type, and were strongly and diffusely positive for CD117 and CD34. Six GISTs were also positive for vimentin (VIM) (75.0%), four for S-100 (50.0%), three for desmin (37.5%) and two for SMA

(25.0%).

The synchronous gastric adenocarcinomas were located in different parts of the stomach (Table 3). The mean sizes of the primary adenocarcinomas were  $6.000 \pm 2.6186$  cm (range, 2.0-8.0 cm). No patients had distant metastases at the time of diagnosis. Half of the tumors were poorly differentiated; in the case of the other half, differentiation was not recorded because there is no standard for pathological recording. One of the patients had early gastric cancer, and the other seven had advanced gastric cancers; two patients each had stage I and II disease, three had stage III disease and one had stage IV disease.

## DISCUSSION

The term gastric stromal tumor was originally coined by Mazur *et al*<sup>[20]</sup>. Most gastric stromal tumors exhibit variable differentiation; these tumors tend to originate from primitive mesenchymal cells<sup>[21]</sup>, rather than from smooth muscle cells. GISTs are rare neoplasms that are most commonly located in the stomach (39%-60%), small bowel (30%-42%), colon-rectum (5%-10%) and esophagus (5%)<sup>[22]</sup>. The most common symptoms of the patients in the current study were abdominal masses, pain and bleeding<sup>[23,24]</sup>. The symptoms of these tumors depend on their size and location<sup>[24]</sup>. GISTs are generally found within the deeper stroma and the submucosa, and therefore, in most of the reported cases of synchronous gastric adenocarcinoma and GIST, the preoperative biopsy specimens have shown only adenocarcinoma. GISTs are often incidentally detected during imaging studies, operation or examination of the resected specimens<sup>[25]</sup>.

In this report, only one case of GIST was detected preoperatively, on a CT scan; the others were found incidentally during or after operation, upon general pathological examination. Owing to the variability and specific characteristics of GISTs, the coexistence of these tumors with other gastric tumors should be considered in the treatment of gastric cancer. To avoid missed diagnosis or misdiagnosis of GISTs, imaging studies such as ultrasonography, CT and magnetic resonance imaging should be performed in patients in whom gastric cancer is detected on gastroscopy. Moreover, surgical exploration in these patients should be careful and comprehensive. If suspi-

**Table 3** Clinical characteristics, treatments and outcomes of gastric adenocarcinoma in eight patients

| Patient No. | Primary site         | Size (cm) | TNM status    | Surgical resection     | Gross appearance             | Histology                              | Clinical presentation    |
|-------------|----------------------|-----------|---------------|------------------------|------------------------------|--|--------------------------|
| 1           | Cardia               | 8         | T3N1M0, III B | Radical operation      | Ulcerative infiltrative type | The low differentiation adenocarcinoma | Difficulty in swallowing |
| 2           | Antrum               | 2         | T1N0M0, I A   | Radical operation      | Ulcerative                   | The low differentiation adenocarcinoma | Haematemesis             |
| 3           | Antrum               | 8         | T3N0M0, II    | Radical operation      | Ulcerative                   | Adenocarcinoma                         | Black stool              |
| 4           | Antrum               | 4         | T3N1M0, III A | Radical operation      | Ulcerative                   | Adenocarcinoma                         | Abdominal discomfort     |
| 5           | Cardia               | 6         | T4N2M0, IV    | Palliative gastrectomy | Ulcerative                   | Adenocarcinoma                         | Abdominal discomfort     |
| 6           | The lesser curvature | 10        | T3N1M0, III A | Radical operation      | Infiltrative type            | Tubular adenocarcinoma                 | Emaciation               |
| 7           | The posterior wall   | 4         | T2N1M0, II    | Radical operation      | Infiltrative type            | The low differentiation adenocarcinoma | Nausea, vomiting         |
| 8           | Antrum               | 6         | T2N0M0, I B   | Radical operation      | Uplift ulcer type            | The low differentiation adenocarcinoma | Abdominal discomfort     |

TNM: Tumor, nodes, metastasis-classification.

cious lesions are found, these should not be assumed to be metastases of gastric cancer. Physicians should consider the possibility of other types of tumors, radically resect the suspicious lesion, obtain frozen sections for pathological examination, determine the histological origin of the lesion and apply appropriate surgical techniques. In addition, any nodule on the walls of the digestive tract should be carefully assessed so that no small GIST is overlooked during the postoperative pathological examination.

Liszka *et al*<sup>[26]</sup> retrospectively analyzed the clinicopathological characteristics of 22 cases of concurrent GISTs and other tumors, including two cases of GISTs accompanied by gastric cancers, and 60 cases of only GISTs. They found that the risk of invasion was much lower and the tumor diameter was smaller in patients with concurrent GISTs and other tumors than in patients with only GISTs ( $P < 0.05$ ), which is consistent with our results. These findings may be attributable to the following factors: the risk of malignant invasion of GISTs is relatively low, and the biological behavior of GISTs might have been inhibited by the gastric cancer. However, definitive evidence for this theory is lacking at present.

The morphology of GIST cells is usually spindle shaped (70%), epithelioid (20%) or mixed. GISTs are immunohistochemically positive for Kit expression (90%-95%) and often for Bcl-2 (80%), CD34 (70%), SMA (35%), S-100 (10%) and desmin (5%) expression. In this study, all the GISTs were strongly and diffusely positive for CD117 and CD34. Six GISTs were also positive for VIM (75.0%), four for S-100 (50.0%), three for desmin (37.5%) and two for SMA (25.0%). Fletcher *et al*<sup>[17]</sup> proposed a classification for malignancies that was based on tumor size and the number of mitotic divisions. According to this classification, all the tumors in our study were classified as low risk or very low risk; nevertheless, careful follow-up is mandatory.

Collision tumors rarely develop in the stomach. The frequency of secondary malignancies in GIST patients has been reported to be 4.5%-35% in different series<sup>[15,27-33]</sup>. The most common GIST-associated malig-

nancies were gastrointestinal carcinomas (47%), prostate cancer (9%), lymphoma/leukemia (7%) and breast cancer (7%)<sup>[27]</sup>. Single case reports have described the occurrence of adenocarcinoma admixed with gastric lymphoma, carcinoid tumor, leiomyosarcoma<sup>[13,34-37]</sup> or rhabdomyosarcoma<sup>[35,36]</sup>, as well as adenoma admixed with a sarcomatous stromal component<sup>[38]</sup>. Thus far, only a few case reports of gastric collision tumors consisting of adenocarcinoma and leiomyoma have been documented<sup>[39,40]</sup>. Rare cases of concurrent presentation of gastric adenocarcinoma and GIST have been reported in the literature<sup>[13,15,24,37,41-46]</sup>. Maiorana *et al*<sup>[13]</sup> found that 6 of 52 (11.5%) patients with gastric GISTs had an associated second gastric tumor (five adenocarcinomas and one carcinoid tumor), which considering the restriction of both tumor types to the stomach, indicates a high incidence. In 2000, Maiorana *et al*<sup>[13]</sup> found that GISTs were the most common non-epithelial tumor that could simultaneously occur with epithelial tumors (carcinomas and carcinoid tumors); they also reported five cases of GIST combined with gastric cancer.

The admixture of gastric epithelial and stromal tumors raises the question of whether such an occurrence is a simple incidental association or whether the two lesions are connected by a causal relationship. Some researchers claim that this simultaneous presentation is coincidental; however, others hypothesize that some unknown carcinogens induce the simultaneous proliferation and oncogenesis of epithelial and stromal cells<sup>[13-15,24,41,42,44,45]</sup>. Theoretically, genetic mutations could play an important role in the pathogenesis of gastric synchronous tumors; however, no available evidence supports a common genetic mutation underlying gastric adenocarcinoma and GIST<sup>[27,47,48]</sup>. Another hypothesis suggested in experimental models is that the same unknown carcinogenic agent may interact with two adjacent tissues, causing the simultaneous development of tumors of different histological types<sup>[13,41,49-52]</sup>. Cohen *et al*<sup>[51]</sup> reported that exposure to both acetylsalicylic acid and nitrosoguanidine causes synchronous development of both gastric cancer and leiomyosarcoma. An interesting hypothesis is that a single

carcinogenic agent may interact with two neighboring tissues, inducing the development of tumors of different histological types in the same organ<sup>[51]</sup>. *Helicobacter pylori* has been found to be related to the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tumor<sup>[53,54]</sup> or GIST<sup>[24]</sup>. Liu *et al.*<sup>[42]</sup> hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in the simultaneous proliferation of different cell lines (epithelial and stromal cells).

Our study has some limitations: it was retrospective and the number of patients was small. This limited the validity of significant statistical evidence that can be extrapolated from our study. In addition, our study does not answer the question of whether a causal relationship exists between gastric adenocarcinoma and GIST, since we did not perform genetic and molecular ancillary tests. This study, however, highlights an interesting observation of a possible association between gastric adenocarcinoma and GIST that should encourage further statistically validated and genetically based studies.

In conclusion, the synchronous occurrence of GISTs and other gastrointestinal malignancies is more common than it has been considered. The concomitant GIST is usually discovered incidentally during endoscopy, imaging study or operation performed because of the other malignancy. We have reported eight cases of GIST combined with gastric cancer. All the GISTs were positive for CD117 and CD34. The cause of the association between GISTs and adenocarcinoma is difficult to determine. In the majority of cases, this association is most likely coincidental. Surgical excision is the mainstay of therapy, and further research is required for explaining this simultaneous tumor development.

## COMMENTS

### Background

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors of the digestive tract and have various clinical and biological characteristics. The expression of c-Kit distinguishes GISTs from true leiomyomas, leiomyosarcomas and other mesenchymal tumors of the gastrointestinal tract.

### Research frontiers

GISTs are generally found within the deeper stroma and the submucosa, and therefore, in most of the reported cases of synchronous gastric adenocarcinoma and GIST, the preoperative biopsy specimens have shown only adenocarcinoma. GISTs are often incidentally detected during imaging studies, operation or examination of the resected specimens.

### Innovations and breakthroughs

The authors hypothesized that the stomach was influenced by the same unknown carcinogen, resulting in a simultaneous proliferation of different cell lines (epithelial and stromal cell).

### Applications

In the majority of cases, this association is most likely coincidental. Surgical excision is the mainstay of therapy, and further research is required for explaining this simultaneous tumor development.

### Peer review

Synchronous gastric adenocarcinoma and GIST were not common but can be found sometimes in practice. Usually, intraoperatively serendipitous GISTs are of very low risk and with their max diameters less than 1 cm. Carefully preoperative imaging evaluation might be helpful to find the > 1 cm GIST. This topic is interesting and lack of relevant literatures, especially the data of the epidemiological incidence. If possible, the author should report the data of its proportion

in the consecutive series.

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**P- Reviewers** Hu JK, Timothy RK **S- Editor** Zhai HH  
**L- Editor** A **E- Editor** Zhang DN



## Risk factors for proton pump inhibitor refractoriness in Chinese patients with non-erosive reflux disease

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Received: January 14, 2013 Revised: March 20, 2013

Accepted: April 9, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To analyze risk factors for refractoriness to proton pump inhibitors (PPIs) in patients with non-erosive reflux disease (NERD).

**METHODS:** A total of 256 NERD patients treated with the PPI esomeprazole were enrolled. They were classified into symptom-free and residual symptoms groups according to Quality of Life in Reflux and Dyspepsia (QoLRad) scale. All subjects completed questionnaires on psychological status (self-rating anxiety scale; self-rating depression scale) and quality of life scale (Short Form 36). Multivariate analysis was used to determine the predictive factors for PPI responses.

**RESULTS:** According to QoLRad, 97 patients were confirmed to have residual reflux symptoms, and the remaining 159 patients were considered symptom free. There were no significant differences between the two groups in lifestyle factors (smoking and alcohol consumption), age, *Helicobacter pylori* infection, and hiatal hernia. There were significant differences between the two groups in relation to sex, psychological distress including anxiety and depression, body mass index (BMI), and irritable bowel syndrome (IBS) ( $P < 0.05$ ). Logistic regression analysis found that BMI  $< 23$ , comorbid IBS, anxiety, and depression were major risk factors for PPI resistance. Symptomatic patients had a lower quality of life compared with symptom-free patients.

**CONCLUSION:** Some NERD patients are refractory to PPIs and have lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low BMI.

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**Key words:** Risk factors; Refractoriness; Proton pump inhibitors; Non-erosive reflux disease

**Core tip:** Non-erosive reflux disease (NERD) is significantly more refractory to proton pump inhibitor (PPI) treatment than erosive esophagitis is, although the reason is unclear at present. Here, we investigated the risk factors for refractoriness to PPI treatment in patients with NERD. Our results demonstrate that some NERD patients are refractory to standard doses of PPIs and have a lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low body mass index. Recognition of this might hold the key to improving long-term management of NERD.

Niu XP, Yu BP, Wang YD, Han Z, Liu SF, He CY, Zhang GZ,

Wu WC. Risk factors for proton pump inhibitor refractoriness in Chinese patients with non-erosive reflux disease. *World J Gastroenterol* 2013; 19(20): 3124-3129 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3124.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3124>

## INTRODUCTION

Gastroesophageal reflux disease (GERD) is caused by abnormal reflux of gastric contents into the esophagus and is characterized by specific symptoms such as heartburn and acid regurgitation. An epidemiological survey has found that GERD is a common condition with a prevalence of 10%-20% in Western Europe and North America<sup>[1]</sup>. The prevalence of GERD in China is lower than that in Western countries, but appears to be increasing<sup>[2,3]</sup>. However, only about one-third to one-half of patients with GERD has endoscopically positive findings such as erosions and ulcers, whereas others with GERD symptoms have no obvious mucosal breaks during endoscopic examination. Therefore, GERD includes erosive esophagitis (EE) and endoscopy-negative reflux disease, which is also known as non-erosive reflux disease (NERD)<sup>[4,5]</sup>. At present, the most effective drug therapy for GERD is proton-pump inhibitors (PPIs)<sup>[6]</sup>. PPI treatment results in sustained acid reduction for symptom control in the majority of patients. However, 17%-32% of patients with GERD in primary care trials have experienced persistent, troublesome heartburn or regurgitation despite standard-dose PPI treatment, and the majority of them have even experienced refractory symptoms at higher doses<sup>[7-10]</sup>. Studies have shown that EE and NERD have different responses to PPIs because their pathogenesis is distinct<sup>[11-13]</sup>. In addition, NERD is significantly more refractory than EE to PPI treatment<sup>[14,15]</sup>. However, a recent meta-analysis has reported that NERD has the same response rate to PPIs as EE has, and the previously reported low response rate in patients with NERD was likely the result of inclusion of patients with upper gastrointestinal symptoms who did not have reflux disease<sup>[16,17]</sup>.

PPI failure has become a common clinical dilemma in gastrointestinal clinics and has been increasingly encountered at the primary care level as well. It is likely to be an expensive clinical problem because patients tend to utilize health care resources repeatedly, such as clinic visits, diagnostic studies, and prescription medication. A previous observation has shown that non-acid reflux contributes to poor effectiveness of PPIs in both NERD and EE patients<sup>[18]</sup>. However, most previous studies regarding PPI responses and the natural course of EE and NERD were based on Western countries; there have been few reports from Eastern Asian countries, including China<sup>[14,19]</sup>. In addition, the risk factors that affect the response of patients with NERD to PPIs are unclear at present<sup>[20,21]</sup>. To the best of our knowledge, there has been no report on the risk factors for response to PPIs in patients with NERD

in China. Here, we investigated the risk factors for refractoriness to PPI treatment in patients with NERD and propose a potential treatment strategy for them.

## MATERIALS AND METHODS

### Patient selection

Patients with NERD receiving PPI (esomeprazole) maintenance treatment were consecutively enrolled from May 2008 to August 2010. We enrolled 256 patients with reflux symptoms who were assessed by a locally validated GERD questionnaire, the Chinese GerdQ<sup>[22]</sup>. All patients were positive for ambulatory 24-h esophageal pH monitoring (DeMeester score > 14.27). All patients had undergone endoscopy at his/her first visit to exclude erosive reflux disease. All patients with NERD recruited for our study were using standard-dose esomeprazole for at least 6 mo.

### Exclusion criteria

According to the recent Rome III Criteria, patients with functional heartburn whose typical symptoms were associated with neither abnormal pH testing nor a positive symptom index were excluded<sup>[23,24]</sup>. Patients were excluded if there was a history of gastrointestinal surgery, Barrett's esophagus, peptic ulcer, or gastroduodenal cancer, and if they could not accurately express their condition or were unwilling to accept the scale survey.

### Assessments

The patients' medical records were screened for gastrointestinal morbidity, years since the first episode, and comorbidity (unclear what to deliver). Information was obtained regarding age, sex, smoking, alcohol use, *Helicobacter pylori* (*H. pylori*) infection, body mass index (BMI), comorbid irritable bowel syndrome (IBS), and hiatal hernia. The BMI was categorized using 23 and 25 kg/m<sup>2</sup> as a cut-off point in accordance with the WHO recommendation for Asia. IBS was diagnosed using a questionnaire based on the Rome III Criteria. All patients were asked to complete the following questionnaires.

### Quality of life in reflux and dyspepsia

The reflux version of quality of life in reflux and dyspepsia (QoLRad) is a disease-specific instrument, including 25 items combined into five dimensions: emotional distress, sleep disturbance, vitality, food/drink problems, and physical/social functioning. The recall period refers to the last week. QoLRad outcome has been shown to reflect treatment response and impact of symptoms<sup>[25]</sup>. A 7-point scale was used to assess item severity or frequency (1 = a great deal/all of the time; 2 = a lot/most; 3 = a moderate amount/quite a lot; 4 = some; 5 = a little; 6 = hardly any; 7 = none). The lower the scores were, the more severe the impact on daily functioning. Patients scoring  $\geq 6$  on all dimensions were considered symptom free, and those scoring < 6 on at least one dimension as having residual symptoms.

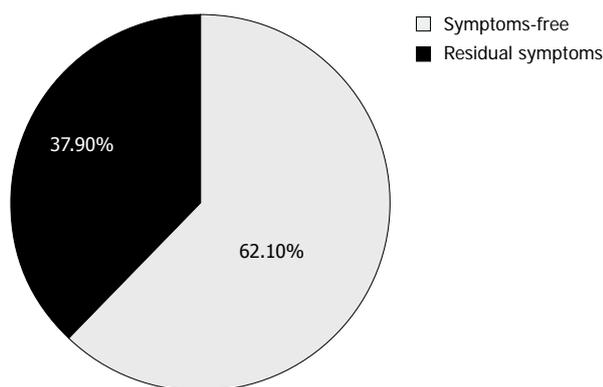


Figure 1 Effectiveness of proton pump inhibitor in 256 patients with non-erosive reflux disease.

**Table 1 Self-rating anxiety scale and self-rating depression scale scores in the two groups of patients**

| Group             | n   | SAS score    | SDS score    | SAS%            | SDS%           |
|-------------------|-----|--------------|--------------|-----------------|----------------|
| Residual symptoms | 97  | 42.68 ± 6.21 | 52.36 ± 6.93 | 46.39% (45/97)  | 50.52% (49/97) |
| Symptom-free      | 159 | 31.17 ± 6.15 | 43.13 ± 5.27 | 10.06% (16/159) | 8.18% (13/159) |
| Control           | 52  | 30.74 ± 8.18 | 35.32 ± 6.71 | 0.00% (0/20)    | 1.90% (1/52)   |

Self-rating anxiety scale (SAS) grades:  $F = 104.54$ ,  $P < 0.001$ ; Self-rating depression scale (SDS) grades:  $F = 143.91$ ,  $P < 0.001$ ; SAS%:  $\chi^2 = 52.30$ ,  $P < 0.001$ ; SDS%:  $\chi^2 = 79.58$ ,  $P < 0.001$ .

### Zung self-rating anxiety scale and Zung self-rating depression scale

The scores of the 20 items in the self-rating depression scale (SDS) and self-rating anxiety scale (SAS) were added and multiplied by 1.25. The nearest integer was taken as the standard score. An SDS standard score  $\geq 53$  indicated the presence of depression. An SAS standard score  $\geq 50$  indicated the presence of anxiety<sup>[26]</sup>.

### Quality of life scale (Short Form 36, SF-36)

This 36-question survey measured generic quality of life in eight dimensions<sup>[27]</sup>: physical functioning (PF), role limitations-physical (RP), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role limitations-emotional (RE), and mental health (MH). Summary physical component score (PCS) and mental component score (MCS) were also calculated from patient responses. Raw scores were converted to a scale of 0 to 100, with higher scores indicating higher levels of health or wellbeing.

### Data analysis

The ages of the patients were categorized into deciles. Descriptive statistics (mean and SD) and additional analyses were calculated using SPSS version 14.0. Mean values were compared using Student's *t* test and analysis of variance, and two-sided *P* values of 0.05 were considered statistically significant. Ninety-five percent confidence intervals and two-tailed *P* values were calculated for the

**Table 2 Lifestyle characteristics of two groups of patients**

| Variables                           | Residual symptoms (n = 97) | Symptom-free (n = 159) | t/ $\chi^2$ | P value |
|-------------------------------------|----------------------------|------------------------|-------------|---------|
| Age (yr)                            | 59.3 ± 11.2                | 57.1 ± 12.7            | 1.28        | 0.203   |
| Sex (male/female)                   | 38/59                      | 97/62                  | 0.92        | 0.016   |
| Alcohol consumption                 | 19%                        | 26%                    | 0.44        | 0.509   |
| Smoking                             | 37%                        | 32%                    | 9.93        | 0.062   |
| <i>Helicobacter pylori</i> positive | 59%                        | 62%                    | 11.52       | < 0.001 |
| BMI (kg/m <sup>2</sup> )            |                            |                        | 6.19        | 0.045   |
| $\geq 25$                           | 37                         | 86                     |             |         |
| 23–24.9                             | 30                         | 38                     |             |         |
| < 23                                | 30                         | 35                     |             |         |
| Comorbid IBS                        | 37%                        | 12%                    | 36.44       | < 0.001 |
| Hiatal hernia                       | 9%                         | 8%                     | 1.75        | 0.186   |

BMI: Body mass index; IBS: Irritable bowel syndrome.

ORs. Multivariate logistic regression analysis was carried out using determinants with *P* values  $\leq 0.1$ .

## RESULTS

A total of 256 patients with NERD on PPI treatment were included in the study (mean age 58 years, 53% male). Residual reflux symptoms were investigated by analyzing their impact on QOLRad score. They were divided into the residual symptoms ( $n = 97$ ) and symptom-free ( $n = 159$ ) groups according to QOLRad scores (Figure 1). Scores for SAS and SDS were significantly higher in the residual symptoms than symptom-free group (Table 1).

Demographics, clinical and laboratory findings is summarized in Table 2. We found that there was no significant difference between the two groups with regard to age, smoking, alcohol consumption, *H. pylori* infection, and hiatal hernia. A significant sex difference was observed in the residual symptoms and symptom-free groups. There were more women than men in the residual symptoms than symptom-free group ( $P < 0.05$ ). There were significantly more patients in the residual symptoms group with BMI < 23 and comorbid IBS than in the symptom-free group (Table 2). Multivariate analysis of patient characteristics indicated that the risk factors for residual symptoms were: BMI < 23, comorbid IBS, and mental health problems (anxiety or depression) (Table 3). The SF-36 scores indicated that symptomatic patients had a lower quality of life than those who were symptom free (Table 4).

## DISCUSSION

Patients with NERD experience typical GERD symptoms caused by acid and non-acid reflux, but they do not have visible esophageal injury<sup>[28,29]</sup>. NERD is a more common type of GERD in Asian than in Western populations<sup>[30]</sup>. Patients with NERD are a heterogeneous group including various subpopulations with different mechanisms for their main symptoms: reflux of acidic and non-acidic

**Table 3** Multivariate logistic regression of patient characteristics (*n* = 256)

| Variables    | $\beta$ | SE   | Z     | P value | B     | OR   | 95%CI     |
|--------------|---------|------|-------|---------|-------|------|-----------|
| Women        | -0.14   | 0.10 | 6.25  | < 0.001 | -0.19 | 0.66 | 0.31-0.89 |
| BMI < 23     | 0.09    | 0.03 | 14.12 | < 0.001 | 0.32  | 1.56 | 1.37-2.81 |
| Comorbid IBS | 0.21    | 0.06 | 12.30 | < 0.006 | 0.37  | 1.33 | 1.26-2.55 |
| SDS score    | 0.46    | 0.29 | 5.95  | < 0.026 | 0.53  | 1.56 | 1.13-2.39 |
| SAS score    | 1.05    | 0.32 | 9.26  | < 0.001 | 0.85  | 2.17 | 1.57-2.76 |

BMI: Body mass index; IBS: Irritable bowel syndrome; SDS: Self-rating depression scale; SAS: Self-rating anxiety scale.

gastric contents, mucosal hypersensitivity, intraesophageal distension by gas, intraduodenal infusion of fat, muscle contractions, and psychological abnormalities. Some studies have reported that the proportion of patients with NERD that responds to a standard dose of PPI is 20%-30%, which is lower than the proportion of patients with EE<sup>[15,17]</sup>. Some NERD patients even use high doses of PPI but cannot completely control the symptoms. For a long time, ineffective drug maintenance treatment has become a common problem in primary care<sup>[31,32]</sup>. These patients tend to utilize repeatedly healthcare resources, such as frequent consultations, referrals, diagnostic tests, and repeat prescriptions, which consume a large amount of medical resources. These patients also bear physical suffering and economic pressure, which seriously affect their quality of life<sup>[33]</sup>. Several studies have demonstrated that the proportion of patients with NERD that respond to a standard dose of PPI is 60%-70%, which is lower than that of patients with EE<sup>[34]</sup>. A recent study has suggested that, in well-defined NERD patients, the estimated complete symptom response rate after PPI therapy is comparable to the response rate in patients with EE. The previously reported low response rate in studies with patients classified as NERD was probably the result of inclusion of patients with upper gastrointestinal symptoms who did not have reflux disease. In the present study, we found that only 62.1% of patients with NERD treated with PPI were symptom free, and 37.9% of patients had residual symptoms and had a significantly lower quality of life.

The reason why NERD is more refractory than EE to PPIs is unclear at present. Previous observations have shown that the pathogenesis of NERD is associated with age, sex, lifestyle, *H. pylori* infection, BMI, comorbid IBS, and hiatal hernia<sup>[31]</sup>. It has also been shown that patients with NERD often have more psychological problems than those with EE<sup>[35,36]</sup>. A large number of epidemiological investigations have found that anxiety, depression, and chronic stress can lead to NERD<sup>[37]</sup>. Conventional treatment for NERD depends on PPI applications, but it cannot resolve the underlying psychological problems. Patients with NERD are often not satisfied with the treatment. Zerbib *et al.*<sup>[38]</sup> have reported that a no-reflux pattern demonstrated by 24-h pH-impedance monitoring is associated with response to PPIs in patients with

**Table 4** Social functioning-36 scores in residual symptoms and symptom-free groups

| Dimensionality | Residual symptoms          | Symptom-free  | Control       | F     | P value  |
|----------------|----------------------------|---------------|---------------|-------|----------|
| PF             | 91.31 ± 8.73               | 92.03 ± 7.62  | 92.31 ± 8.26  | 0.81  | 0.3926   |
| RP             | 80.01 ± 19.12 <sup>1</sup> | 89.93 ± 18.93 | 94.10 ± 12.15 | 3.90  | 0.0117   |
| BP             | 59.00 ± 10.06 <sup>1</sup> | 86.22 ± 11.13 | 89.31 ± 14.81 | 31.24 | < 0.0001 |
| GH             | 61.25 ± 16.12 <sup>1</sup> | 85.28 ± 15.26 | 83.15 ± 11.32 | 30.27 | < 0.0001 |
| VT             | 64.46 ± 17.92 <sup>1</sup> | 81.30 ± 19.21 | 90.61 ± 20.13 | 12.93 | < 0.0001 |
| SF             | 61.53 ± 11.46 <sup>1</sup> | 79.23 ± 19.73 | 80.16 ± 17.23 | 10.25 | < 0.0001 |
| RE             | 57.64 ± 10.11 <sup>2</sup> | 68.17 ± 23.55 | 82.72 ± 18.19 | 11.75 | < 0.0001 |
| MH             | 50.96 ± 13.13 <sup>2</sup> | 66.21 ± 12.46 | 89.15 ± 16.28 | 23.29 | < 0.0001 |

<sup>1</sup>Significant difference between residual symptoms and symptom-free groups and control group; <sup>2</sup>Significant difference among the three groups. PF: Physical functioning; RP: Role limitations-physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role limitations-emotional; MH: Mental health.

GERD symptoms. In contrast, absence of esophagitis, presence of functional digestive disorders, and BMI ≤ 25 kg/m<sup>2</sup> are strongly associated with PPI failure.

To date, there has been no report on the risk factors that affect the response of NERD patients to PPI therapy in China. In the present study, we found that there was no significant difference between the symptom-free and residual symptoms groups with regard to age, smoking, alcohol consumption, *H. pylori* infection, and hiatal hernia. However, symptomatic patients differed from symptom-free patients in relation to sex, BMI, comorbid IBS, and psychological distress. These variables were analyzed by multivariate analysis and we found that anxiety, depression, comorbid IBS, and BMI < 23 were independent risk factors associated with residual symptoms, but sex was not a risk factor for residual symptoms. We used the modified BMI criteria as proposed by the WPRO, which considers the smaller body frame of Asians and provides a more accurate reflection of body fat stores, thus avoiding a false perception of not being overweight<sup>[39]</sup>.

In conclusion, our results demonstrate that some NERD patients are refractory to standard doses of PPIs and have a lower quality of life. Residual symptoms are associated with psychological distress, intestinal disorders, and low BMI. Recognition of this might hold the key to improving long-term management of NERD.

## COMMENTS

### Background

Non-erosive reflux disease (NERD) is significantly more refractory than erosive esophagitis to proton pump inhibitor (PPI) treatment, although the reason is unclear at present.

### Research frontiers

Here, the authors report a study of the risk factors for PPI refractoriness in Chinese patients with NERD. The majority of studied cases were sporadic.

### Innovations and breakthroughs

Although a previous observation has shown that non-acid reflux contributes to poor effectiveness of PPIs in NERD patients, there has been no report on the risk factors for response to PPIs in patients with NERD in China. Here, the authors investigated the risk factors for refractoriness to PPI treatment in patients

with NERD and propose a potential treatment strategy for them.

### Applications

The authors found that some NERD patients were refractory to standard doses of PPIs and had a lower quality of life. Residual symptoms were associated with psychological distress, intestinal disorders, and low body mass index. Recognition of this might hold the key to improving long-term management of NERD.

### Terminology

There is no specific, unique terminology that will not be familiar to the majority of readers.

### Peer review

This was a qualitative study with an original approach to establishing the risk factors for refractoriness to PPIs in patients with NERD. This is an important problem in the treatment of these patients. The study was well designed and the results are clearly described.

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**P- Reviewers** Pimanov SI, Savarino E **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Zhang DN



## Expression of huCdc7 in colorectal cancer

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Received: March 7, 2013 Revised: March 29, 2013

Accepted: April 3, 2013

Published online: May 28, 2013

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**Key words:** huCdc7; Semiquantitative reverse transcription-polymerase chain reaction; Colorectal cancer

**Core tip:** huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor with a gradually increasing incidence. We found that huCdc7 may play an important role in the development and progression of colorectal cancer.

Chen HJ, Zhu Z, Wang XL, Feng QL, Wu Q, Xu ZP, Wu J, Yu XF, Qian HL, Lu Q. Expression of huCdc7 in colorectal cancer. *World J Gastroenterol* 2013; 19(20): 3130-3133 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3130.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3130>

### Abstract

**AIM:** To detect the expression of huCdc7 in colorectal cancer.

**METHODS:** The mRNA and protein expression of huCdc7 in 39 colorectal cancer tissue specimens and matched tumor-adjacent normal colorectal tissue specimens was detected by reverse transcription-polymerase chain reaction and immunohistochemistry, respectively.

**RESULTS:** The relative expression level of huCdc7 mRNA in colorectal cancer was significantly higher than that in tumor-adjacent normal colorectal tissues ( $0.03675 \pm 1.00$  vs  $0.01199 \pm 0.44$ ,  $P < 0.05$ ). huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissues contained many huCdc7-positive cells, whereas normal colorectal tissues contained very few positive cells.

**CONCLUSION:** huCdc7 may play an important role in the development and progression of colorectal cancer.

### INTRODUCTION

huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor with a gradually increasing incidence. This study aimed to detect the expression of huCdc7 in colorectal cancer to provide a basis for the diagnosis and treatment of this malignancy.

### MATERIALS AND METHODS

#### Specimen collection

Tissue specimens were collected from 39 patients with colorectal cancer who were surgically treated at our hospital. Colorectal cancer tissue specimens and matched

tumor-adjacent normal colorectal tissue specimens (5 cm away from tumor tissue) were placed in liquid nitrogen immediately after surgical specimens were removed. The final diagnosis of colorectal cancer and tumor-adjacent normal colorectal tissues was made pathologically.

### Main reagents

M-MLV reverse transcriptase (Promega, United States), Taq DNA polymerase (Takara, Japan), and dNTPs (Takara, Japan) were obtained commercially. The primers for the amplification of a 525-bp CDC7 gene fragment were: forward, 5'-GCT CAG CAG GAA AGG TGT TC-3' and reverse, 5'-AGT TTG ATT GGG GCA CTT TG-3'. The primers for amplification of a 420-bp glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene fragment were: forward, 5'-GTC AGT GGT GGA CCT GAC CT-3' and reverse, 5'-AGG GGT CTA CAT GGC AAC TG-3'.

### Reverse transcription-polymerase chain reaction

**Preparation of total RNA:** Total RNA was extracted using TRIzol reagent (GIBCO/BRL, United States). All centrifuge tubes, plastic and glasswares and water used for RNA extraction were treated to create an RNase-free environment. A homogenizer was baked at 200 °C for 4 h to remove RNase and then cooled. Tissue samples were frozen in liquid nitrogen, pulverized into powder, and placed in the homogenizer containing TRIzol reagent. After homogenization for several minutes, the homogenized sample was transferred to an RNase-free centrifuge tube. Following the addition of chloroform, the tube was centrifuged at 4 °C. The upper aqueous phase was transferred to an RNase-free centrifuge tube, and isopropanol was added to precipitate the RNA. The tube was centrifuged at 4 °C, and the pellet was washed twice with 75% ethanol and dissolved in RNase-free deionized water. The purity of extracted RNA was assessed by measuring the *A*<sub>260</sub>/*A*<sub>280</sub> ratio (1.7-2.0) using an ultraviolet spectrophotometer. 3-morpholinopropanesulfonic acid-formaldehyde denaturing agarose gel electrophoresis was performed to check if the extracted RNA was degraded. To remove the contamination of genomic DNA, RNA sample was treated with RNase-free DNA enzyme I (Ambion, United States).

**cDNA synthesis:** Two micrograms of total RNA were mixed with 1 μL of random primers (50 pmol/L, 1 μL) and 15 μL of diethylpyrocarbonate-treated water, placed at 70 °C for 3 min and then immediately placed on ice for 5 min. The following components were then added: 5 μL of 5 × buffer, 1 μL of 10 mmol/L dNTP, 1 μL of M-MLV reverse transcriptase (200 U/μL), and 0.75 μL of RNase inhibitor (40 U/μL, TaKaRa). The mixture was incubated at 42 °C for 2 h.

**Semiquantitative reverse transcription-polymerase chain reaction:** The *huCdc7* gene was amplified using cDNA as the template. *GAPDH* was used as a control.

Polymerase chain reaction (PCR) conditions were as follows: denaturation at 94 °C for 3 min; 35 cycles (25 cycles for *GAPDH*) of denaturation at 94 °C for 30 s, annealing at 58.5 °C for 30 s, and extension at 72 °C for 40 s; and a final extension at 72 °C for 5 min. PCR products were resolved by 2% agarose gel electrophoresis. Band densities were analyzed using the FR-980 bio-electrophoresis image analysis system.

**Real-time PCR:** The CFX96 real-time PCR detection system (BIO-RAD, United States) was used to detect the expression of genes of interest in tumor tissues and matched tumor-adjacent normal tissues. PCR reaction was performed in a 20-μL system consisting of 10 μL of SYBR Premix EX Taq, 0.4 μL of each primer (10 μM), 2 μL of DNA, and 7.2 μL of ddH<sub>2</sub>O. *GAPDH* was used as an internal control. The experiment was repeated three times to ensure the reliability of the results. The expression level of the *huCdc7* gene was calculated using the following formulas:  $Cdc7 \Delta Ct = \text{mean } Cdc7Ct - \text{mean } GAPDHCt$ , and  $Cdc7 \Delta \Delta Ct = Cdc7 \Delta Ct \text{ cancer tissue} - Cdc7 \Delta Ct \text{ tumor-adjacent tissue}$ . Relative expression level of the *huCdc7* gene was calculated using the  $2^{-Cdc7 \Delta \Delta Ct}$  method.

### Immunohistochemistry

Streptavidin-peroxidase immunohistochemical staining was performed using a commercial kit according to the manufacturer's instructions.

### Statistical analysis

Statistical analysis were performed using SPSS 10.0 software. Means between two groups were compared using the *t* test. *P* values < 0.05 were considered statistically significant.

## RESULTS

### huCdc7 mRNA expression

The relative expression levels of huCdc7 mRNA in colorectal cancer and tumor-adjacent normal colorectal tissues were  $0.03675 \pm 1.00$  and  $0.01199 \pm 0.44$ , respectively. Statistical analysis indicated that the expression level of huCdc7 mRNA was significantly higher in colorectal cancer than in tumor-adjacent normal colorectal tissues (*P* < 0.05) (Figure 1).

### huCdc7 protein expression

huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissues contained many huCdc7-positive cells, whereas normal colorectal tissues contained very few positive cells (Figure 2).

## DISCUSSION

*Cdc7* is a serine/threonine kinase, and huCdc7 is expressed in all human tissues<sup>[1]</sup>. By forming complex with other molecules in the nucleus, huCdc7 can phosphorylate and activate chromosome-binding minichromosome



Figure 1 Semiquantitative reverse transcription-polymerase chain reaction determination of huCdc7 and glyceraldehyde-3-phosphate dehydrogenase mRNA expression in colorectal cancer (1, 3, 5, 7, 9 and 11) and tumor-adjacent normal colorectal tissues (2, 4, 6, 8, 10 and 12). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

maintenance complex (MCM) proteins. The MCM family has multiple members, including MCM2, MCM4 and MCM6. huCdc7 has the strongest ability to phosphorylate MCM2<sup>[2]</sup>. On one hand, MCMs function as helicase, a component of cell cycle initiation complex<sup>[3]</sup>. On the other hand, MCMs can act as important regulatory factors for S phase checkpoints to control cell cycle progression.

It is still unclear how huCdc7 mediates these processes. The expression of huCdc7 is tightly controlled by some factors and auxiliary proteins in normal cell cycle and maintained in a dynamic equilibrium state. In tumor cells, huCdc7 is abnormally expressed and excessively activated due to cell cycle disturbances. Hess *et al*<sup>[4]</sup> found that huCdc7 was overexpressed in tumor cells and excessive expression of huCdc7 promoted excessive MCM2 activation and abnormal proliferation of tumor cells. In addition, they found that huCdc7 was overexpressed in metastatic tumor cells, suggesting that tumor metastasis may be closely related to abnormal high huCdc7 expression. A previous study has revealed that CDC7 kinase is a predictor of survival and a novel therapeutic target in epithelial ovarian carcinoma<sup>[5]</sup>. Similar findings have also been reported in some studies on lymphoma<sup>[6]</sup>.

In this study, we utilized semi-quantitative reverse transcription-PCR and immunohistochemistry to determine the expression of huCdc7 in colorectal cancer and tumor-adjacent normal colorectal tissues. We found that huCdc7 mRNA expression was significantly higher in colorectal cancer than in normal colorectal tissues. huCdc7 is a conservative serine/threonine kinase that is indispensable for DNA replication initiation. Abnormal high expression of huCdc7 will promote DNA replication, cause abnormal cell proliferation, and thereby lead to the occurrence of tumors.

To investigate the relationship between huCdc7 and tumor development and progression, Montagnoli *et al*<sup>[7]</sup> used the siRNA interference technology to suppress high huCdc7 expression in tumor cells. They found that the phosphorylation levels of MCM2 activation sites were greatly decreased, DNA replication initiation in tumor cells was restrained, and tumor cell growth slowed down. In addition, some researchers believe that high huCdc7 expression in tumor cells is related to p53 inactivation<sup>[8]</sup>.

A study on ovarian cancer revealed that CDC7 kinase can predict survival and serve as a target for cancer treatment. Kim *et al*<sup>[9]</sup> found that siRNA-mediated inhibition

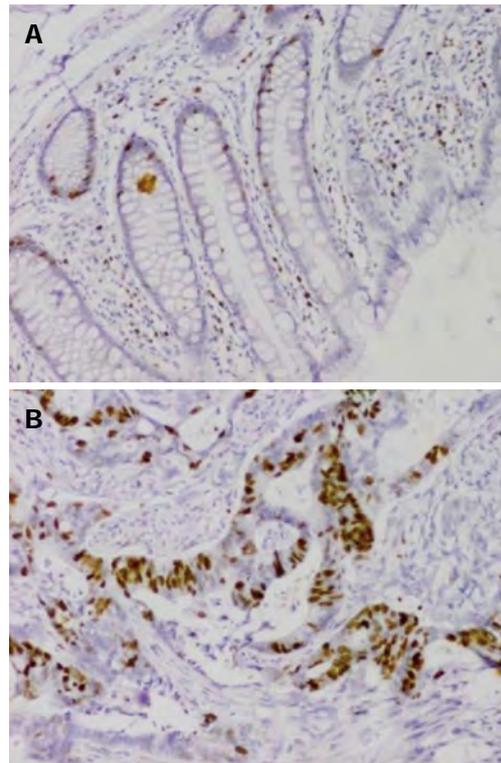


Figure 2 Immunohistochemical staining of huCdc7 in normal colorectal tissues (A) and colorectal cancer (B) (magnification,  $\times 100$ ).

of huCdc7 reduced the phosphorylation level of the N-terminus of MCM4. As a result, Cdc 45 could not be positioned to the chromosome and replication initiation complex could not form. This inhibited, to a certain extent, tumor cell proliferation. Thus, inhibition of huCdc7 activity can effectively inhibit tumor cell growth and promote tumor cell apoptosis, without damage to normal cells. A recent study has identified PHA-767491 as an inhibitor of huCdc7<sup>[10]</sup>, which may be used to down-regulate abnormal huCdc7 expression to inhibit abnormal DNA replication and cell cycle progression in tumors.

Small molecule compounds that can interfere with the activity of CDC7 have been developed and proved to be effective in inhibiting tumor growth in animal models. Unprecedented attention has been paid to the inhibition of CDC7 kinase activity for suppressing DNA replication in tumor cells and inducing tumor cell apoptosis. Further elucidation of the role of huCdc7 in colorectal cancer may improve the treatment of this malignancy.

## COMMENTS

### Background

huCdc7 is ubiquitously expressed in human tissues and can regulate DNA replication initiation. Abnormal expression or excessive activation of huCdc7 can promote excessive cell proliferation and cause tumorigenesis. Colorectal cancer is a common digestive tract tumor and has a gradually increasing incidence.

### Research frontiers

In this study, tissue specimens were collected from 39 surgically treated patients with colorectal cancer. Colorectal cancer tissue specimens and matched tumor-adjacent normal colorectal tissue specimens (5 cm away from tumor tis-

sue) were placed in liquid nitrogen immediately after surgical specimens were removed. The final diagnosis of colorectal cancer and tumor-adjacent normal colorectal tissues was made pathologically.

### Innovations and breakthroughs

The relative expression level of huCdc7 mRNA in colorectal cancer was significantly higher than that in tumor-adjacent normal colorectal tissue. huCdc7-positive cells displayed brown granules in the nucleus. Tumor tissue contained many huCdc7-positive cells, whereas normal colorectal tissue contained very few positive cells.

### Applications

The authors found that huCdc7 may play an important role in the development and progression of colorectal cancer.

### Peer review

In the manuscript, the authors detected the expression of huCdc7 in colorectal cancer. The data is interesting. The manuscript is short, but informative.

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**P- Reviewers** Buyse M, Hoff PM, Mitchell P **S- Editor** Wang JL  
**L- Editor** Ma JY **E- Editor** Li JY



## Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes

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Supported by Natural Science Foundation of China, No. 81270939, No. 30900510 and No. 81170774; and Natural Science Foundation of Fujian Province, No. 2011J01127

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Received: November 28, 2012 Revised: March 6, 2013

Accepted: March 23, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To evaluate the correlation between nonalcoholic fatty liver disease (NAFLD) and microvascular complications in type 2 diabetes mellitus (T2DM).

**METHODS:** Data were obtained from 1217 inpatients with T2DM (757 females, 460 males; aged  $63.39 \pm 12.28$  years). NAFLD was diagnosed by hepatic ultrasonography. Diabetic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR) were diagnosed according to their respective criteria. The prevalence of NAFLD and the independent correlations of clinical characteristics with NAFLD were determined by cross-tabulation and logistic regression, respectively.

**RESULTS:** Approximately 61% of inpatients with T2DM in Qingdao, China had NAFLD, which decreased significantly with increase in age and prolonged course of diabetes. The prevalence of NAFLD in patients presenting with DN, DPN and DR was 49.4%, 57.2% and 54.9%, respectively. These rates were significantly lower than those of patients without DN, DPN and DR (65.9%, 65.6% and 66.1%, respectively,  $P < 0.05$ ). Participants with NAFLD had greater body weight, waist circumference (WC), body mass index (BMI), fasting blood glucose (FBG), hemoglobin A1c, alanine aminotransferase, aspartate aminotransferase,  $\gamma$ -glutamyltransferase, blood pressure, as well as triglyceride (TG) levels and lower high-density lipoprotein (HDL) concentration than those without NAFLD ( $P < 0.05$ ). NAFLD was positively correlated with BMI, WC, TG, FBG, diastolic blood pressure, and systolic blood pressure but negatively correlated with the duration of diabetes, DR, DPN, DN, and HDL.

**CONCLUSION:** Despite the benign nature of NAFLD, efforts should be directed toward early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction.

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**Key words:** Nonalcoholic fatty liver disease; Type 2 diabetes mellitus; Diabetic nephropathy; Diabetic retinopathy; Diabetic neuropathy

**Core tip:** Nonalcoholic fatty liver disease (NAFLD) and diabetic microangiopathy complications represent important burdens for patients with type 2 diabetes mellitus (T2DM). However based on the finding that the prevalence of NAFLD was negatively correlated with age and duration of T2DM, we suggest that NAFLD is benign process and efforts should be directed at strengthening early diagnosis, intensive blood glucose

and pressure control, and effective dyslipidemia correction to prevent and minimize occurrence of NAFLD.

Lv WS, Sun RX, Gao YY, Wen JP, Pan RF, Li L, Wang J, Xian YX, Cao CX, Zheng M. Nonalcoholic fatty liver disease and microvascular complications in type 2 diabetes. *World J Gastroenterol* 2013; 19(20): 3134-3142 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3134.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3134>

## INTRODUCTION

Diabetes is an independent risk factor for the development of nonalcoholic fatty liver disease (NAFLD) and progression to advanced liver disease, including fibrosis, cirrhosis, and hepatocellular carcinoma<sup>[1]</sup>. Cross-sectional studies have reported that the prevalence of NAFLD in patients with type 2 diabetes mellitus (T2DM) ranges from 42.1% to 75.2% in China<sup>[2,3]</sup>. With the rising incidence and prevalence of T2DM in China<sup>[4]</sup>, a close estimate of the prevalence of NAFLD, as well as its clinical risk factors, is important for predicting the number of patients that require monitoring for more advanced liver diseases, or those who may benefit from future disease-modifying agents.

Targher *et al*<sup>[5]</sup> have reported that most individuals with NAFLD are older, more likely to be male, and have a longer duration of diabetes than those without NAFLD. By contrast, Williamson *et al*<sup>[6]</sup> have reported that participants with definite steatosis (grade 3) are significantly younger and have shorter duration of diabetes than the combined normal/probably normal groups (grades 0-2). Zhou *et al*<sup>[3]</sup> have indicated that the prevalence of NAFLD in men aged < 50 years is higher than that in women. However, this finding is contrary for patients > 50 years; that is, the prevalence of NAFLD is higher in women<sup>[3]</sup>. Thus, further studies must be conducted to ascertain the differences in the prevalence of NAFLD between the sexes and the relationship between age, duration of diabetes, and prevalence of NAFLD.

The present cross-sectional study determined the prevalence and some risk factors for NAFLD and evaluated its correlations with microvascular complications in a large cohort of inpatients with T2DM in Qingdao, China. Differences in the prevalence of NAFLD between men and women, as well as the clinical and biochemical characteristics of the condition, are discussed.

## MATERIALS AND METHODS

A total of 1217 T2DM patients (757 women and 460 men) participated in this study. All patients were hospitalized between January 2008 and January 2012 at the Department of Internal Medicine, Affiliated Hospital of Medical College, Qingdao University, China. Most participants abstained from alcohol consumption ( $n = 1107$ ;

91%) or drank minimally (alcohol consumption < 20 g/d;  $n = 110$ ; 9%). Patient information such as sex, birth date, duration of diabetes, daily alcohol consumption, smoking status, and medications (including hepatotoxic drugs such as glucocorticoids, amiodarone, methotrexate, or antineoplastic drugs) were obtained by the questionnaire method. The heights and body weights (BWs) of the patients were measured (*i.e.*, without wearing a heavy coat and shoes). Body mass index (BMI) was calculated by dividing the BW (kg) by the square of the height (m). Waist circumference (WC) was measured in a standing position at the level of the umbilicus. Fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), alanine aminotransferase (ALT), aspartate aminotransferase (AST),  $\gamma$ -glutamyltransferase (GGT), fasting serum triglyceride (TG), high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were determined using standardized methods in our laboratory. Serology for viral hepatitis B and C was also assessed in all participants. Hepatic ultrasonography scanning was performed on patients after an overnight fast by assigned and experienced radiologists, who were blinded to the health conditions of the patients. The liver was graded for markers of hepatic steatosis by using established criteria: a bright hepatic echo pattern (compared with the echo response of the right kidney); increased attenuation of the echo beam; and presence of focal fatty sparing<sup>[6]</sup>. Participants manifesting symptoms of hepatic steatosis or showing abnormal blood tests of liver function tests were further investigated for other parameters such as antinuclear antibody, antismooth muscle antibody, anti-mitochondrial antibody, and ferritin.

This study was approved by the Ethics Committee of the Affiliated Hospital Medical College, Qingdao University, and all participants provided written informed consent.

### Definition of NAFLD

NAFLD was defined as the presence of definite hepatic steatosis on ultrasound scan (*i.e.*, grade 3) in the absence of a secondary cause for hepatic steatosis. Secondary causes were defined as: alcohol consumption  $\geq 14$  U/wk or participant report of alcohol excess; use of hepatotoxic medication (glucocorticoids, isoniazid, methotrexate, amiodarone, and tamoxifen) within 6 mo prior to the study; positive hepatitis B or C serology; ferritin concentration  $\geq 1000$  mg/L (milder hyperferritinemia can be associated with obesity, insulin resistance, and NAFLD); clinically significant positive immunology titers (antismooth muscle antibody titer  $\geq 1:160$  or antimitochondrial antibody titer  $\geq 1:40$ ); or previous diagnosis of a persistent secondary cause for chronic liver disease according to their medical records. Patients were excluded from calculations on the prevalence of NAFLD if their data on the above-mentioned measures were missing, such that a secondary cause could not be excluded.

Diabetic microvascular complications included dia-

**Table 1** Baseline characteristics of the study participants, according to nonalcoholic fatty liver disease status

| Variables                | Without NAFLD  | With NAFLD     | <i>P</i> value |
|--------------------------|----------------|----------------|----------------|
| <i>n</i>                 | 475            | 742            |                |
| Sex (female/male)        | 287/188        | 470/272        | > 0.05         |
| Age (yr)                 | 65.11 ± 11.70  | 62.29 ± 12.52  | < 0.05         |
| Diabetes duration (yr)   | 11.31 ± 7.10   | 8.47 ± 6.87    | < 0.05         |
| Height (cm)              | 163.99 ± 8.49  | 164.20 ± 8.24  | > 0.05         |
| BW (kg)                  | 65.54 ± 9.76   | 74.32 ± 11.64  | < 0.05         |
| WC (cm)                  | 88.75 ± 8.72   | 96.76 ± 10.32  | < 0.05         |
| BMI (kg/m <sup>2</sup> ) | 24.33 ± 2.87   | 27.49 ± 3.37   | < 0.05         |
| Current smokers (%)      | 17.7           | 14.3           | > 0.05         |
| SBP (mmHg)               | 131.43 ± 17.93 | 141.56 ± 16.15 | < 0.05         |
| DBP (mmHg)               | 76.94 ± 8.87   | 83.61 ± 9.57   | < 0.05         |
| FBG (mmol/L)             | 7.72 ± 3.18    | 8.97 ± 3.20    | < 0.05         |
| HbA1c (%)                | 8.45 ± 2.44    | 8.91 ± 2.47    | < 0.05         |
| ALT (mmol/L)             | 17.16 ± 8.19   | 25.72 ± 13.01  | < 0.05         |
| AST (mmol/L)             | 18.23 ± 6.74   | 22.34 ± 9.00   | < 0.05         |
| GGT (mmol/L)             | 19.47 ± 16.32  | 25.05 ± 17.94  | < 0.05         |
| TG (mmol/L)              | 1.45 ± 1.06    | 2.12 ± 1.43    | < 0.05         |
| TC (mmol/L)              | 4.99 ± 1.41    | 5.04 ± 1.22    | > 0.05         |
| LDL (mmol/L)             | 2.95 ± 0.92    | 2.95 ± 0.82    | > 0.05         |
| HDL (mmol/L)             | 1.24 ± 0.29    | 1.17 ± 0.28    | < 0.05         |

Data are expressed as mean ± SD or proportions of the entire cohort of participants. BW: Body weight; WC: Waist circumference; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; HbA1c: Hemoglobin A1c; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT:  $\gamma$ -glutamyltransferase; TG: Triglyceride; TC: Total cholesterol; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

betic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR). DN was diagnosed by a positive persistent proteinuria for at least three consecutive readings per year, serum creatinine > 130  $\mu$ mol/L, and/or glomerular filtration rate < 60 mL/min. DPN was diagnosed in the presence of persistent numbness, paresthesia, loss of hearing of the tuning fork and sense of vibration, and failure to elicit a knee and/or ankle jerk. DR was diagnosed in the presence of retinal hemorrhage, exudates, and macular edema<sup>[7]</sup>.

### Statistical analysis

Analyses were conducted using SPSS version 17.0. Data are presented as means ± SD or proportions, where appropriate. Skewed variables (*e.g.*, TG and HDL) were logarithmically transformed, and genomic means are presented. Independent *t* and  $\chi^2$  tests were used to compare the differences in means or proportions between different subgroups. The independent associations of variables with NAFLD were determined by a binary logistic regression. A value of *P* < 0.05 was considered statistically significant.

## RESULTS

### Clinical and biological characteristics of study population

The mean demographic and clinical characteristics of the patients were as follows: age, 63.39 ± 12.28 years;

duration of diabetes, 9.58 ± 7.09 years; BW, 70.9 ± 11.75 kg; WC, 93.63 ± 10.48 cm; BMI, 26.26 ± 3.54 kg/m<sup>2</sup>; FBG, 8.48 ± 3.25 mmol/L; HbA1c, 8.73% ± 2.47%; TG, 1.85 ± 1.33 mmol/L; total cholesterol (TC), 5.0 ± 1.29 mmol/L; HDL, 1.20 ± 0.29 mmol/L; and LDL, 2.95 ± 0.86 mmol/L.

The baseline characteristics of the study participants according to their NAFLD status are presented in Table 1. The prevalence of NAFLD in patients with T2DM was 61%, and no statistical difference was indicated between men and women (59.1% *vs* 62.1%, *P* > 0.05). Participants with NAFLD had higher BW, WC, BMI, FBG, HbA1c, ALT, AST, GGT, blood pressure and TG levels, and lower HDL concentration than those without NAFLD (*P* < 0.05). Meanwhile, no statistical difference was observed in the smoking history, height, plasma TC, and LDL levels between the groups. However, participants diagnosed with NAFLD were younger and had shorter duration of diabetes than those without NAFLD (*P* < 0.05).

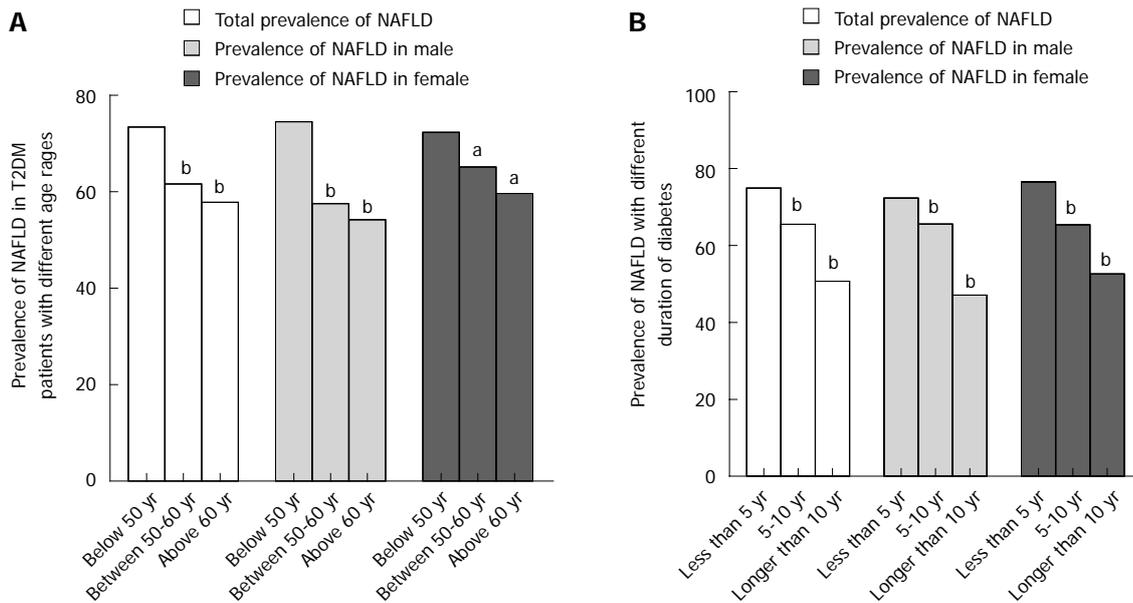
### Prevalence of NAFLD in T2DM patients by age range and duration of diabetes

Patients were divided into three groups according to age: < 50, 50-60, and > 60 years. The prevalence rates of NAFLD for the three age groups were 73.4%, 61.6%, and 57.8%, respectively, indicating that the prevalence of NAFLD decreased significantly as age increased (Figure 1A, *P* < 0.05). This trend persisted even when patients were classified by sex (74.5%, 57.5%, and 54.2% in men and 72.3%, 65.1%, and 59.6% in women, *P* < 0.05). Although the prevalence of NAFLD in women was higher than that in men < 50 years and the reverse was evident at > 50 years, no significant difference was found in the prevalence of NAFLD between men and women at different ages (Figure 1A, *P* > 0.05).

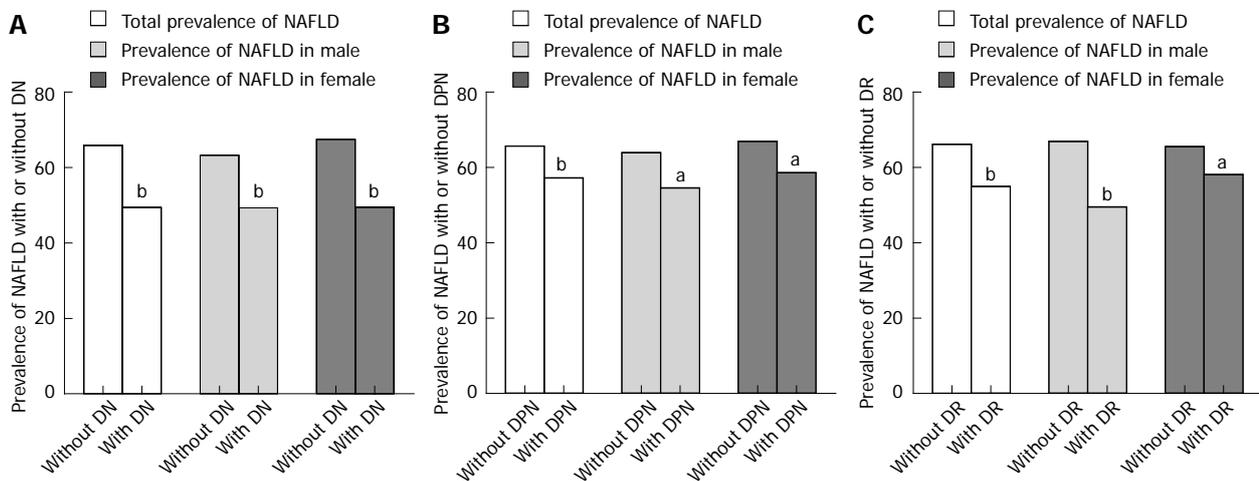
When patients were divided into three groups according to duration of diabetes, that is, < 5 years, 5-10 years, and > 10 years, the prevalence rates of NAFLD were 74.9%, 65.5%, and 50.7%, respectively. This indicates that the prevalence of NAFLD decreased significantly as diabetes was prolonged (Figure 1B, *P* < 0.05). This trend persisted even when patients were classified by sex (72.3%, 65.6%, and 47.1% in men and 76.5%, 65.4%, and 52.6% in females, respectively, *P* < 0.05). No significant difference was indicated in the prevalence rate of NAFLD between men and women with different durations of diabetes (Figure 1B, *P* > 0.05).

### Prevalence of NAFLD in T2DM with or without microangiopathic complications

The incidence of NAFLD was found to correlate negatively with DN, as shown in Figure 2A (*r* = -0.154, *P* < 0.05). The general prevalence of NAFLD in T2DM with and without DN was 49.4% and 65.9%, respectively (*P* < 0.05). This trend persisted even when patients were stratified by sex (*r* = -0.13 in men, *r* = -0.17 in women,



**Figure 1** Prevalence of nonalcoholic fatty liver disease in type 2 diabetes mellitus patients according to different age ranges and durations of diabetes. **A:** Prevalence of nonalcoholic fatty liver disease (NAFLD) in patients aged < 50, 50-60, and > 60 years was 73.4%, 61.6%, and 57.8%, respectively, indicating that the prevalence of NAFLD decreased significantly with increasing age (<sup>b</sup> $P < 0.01$  vs group below 50 years). In men (74.5%, 57.5%, and 54.2%, respectively, <sup>b</sup> $P < 0.01$  vs group below 50 years) and women (72.3%, 65.1%, and 59.6%, respectively, <sup>a</sup> $P < 0.05$  vs group below 50 years), the prevalence of NAFLD decreased significantly with increasing age. The prevalence of NAFLD in men was higher than in women (74.5% and 72.3%, respectively,  $P > 0.05$ ) in the < 50 years age group; however, the reverse was evident in the > 50 years age group (57.5% and 65.1%, respectively, in the 50-60 years age group, and 54.2% and 59.6%, in the > 60 years age group, both  $P > 0.05$ ); **B:** Prevalence of NAFLD, according to duration of diabetes, was 74.9%, 65.5%, and 50.7% for < 5, 5-10, and > 10 years, respectively. Prevalence of NAFLD decreased significantly with prolonged course of diabetes (<sup>b</sup> $P < 0.01$  vs group less than 5 years). This trend persisted even when patients were stratified by sex (72.3%, 65.6%, and 47.1%, respectively, in men, and 76.5%, 65.4%, and 52.6% in women, <sup>b</sup> $P < 0.01$  vs group less than 5 years). There were no significant differences in the prevalence of NAFLD between men and women with different durations of diabetes ( $P > 0.05$ ). T2DM: Type 2 diabetes mellitus.



**Figure 2** Prevalence of nonalcoholic fatty liver disease in T2DM with or without microangiopathy complications. **A:** Incidence of nonalcoholic fatty liver disease (NAFLD) negatively correlated with DN ( $r = -0.154$ ,  $P < 0.05$ ). This trend persisted even when patients were stratified by sex ( $r = -0.13$  in men,  $r = -0.17$  in women, both  $P < 0.05$ ). Prevalence of NAFLD in T2DM with and without DN was 49.4% and 65.9%, respectively (<sup>b</sup> $P < 0.01$  vs group without DN). Prevalence of NAFLD in men without DN was significantly higher than in patients with DN (63.3% and 49.3%, respectively, <sup>b</sup> $P < 0.01$  vs the group without DN). Prevalence of NAFLD in women without DN was significantly higher than in patients with DN (67.5% and 49.5%, respectively, <sup>b</sup> $P < 0.01$  vs the group without DN); **B:** Incidence of NAFLD negatively correlated with DPN ( $r = -0.086$ ,  $P < 0.05$ ). This trend persisted even when patients were stratified by sex ( $r = -0.095$  in men,  $r = -0.084$  in women, both  $P < 0.05$ ). The general prevalence of NAFLD in T2DM with and without DPN was 57.2% and 65.6%, respectively (<sup>b</sup> $P < 0.01$  vs the group without DPN). Prevalence of NAFLD in men without DPN was significantly higher than in patients with DPN (63.9% and 54.5%, respectively, <sup>a</sup> $P < 0.05$  vs the group without DPN). Prevalence of NAFLD in women without DPN was also significantly higher than that in patients with DPN (66.9% and 58.6%, respectively, <sup>a</sup> $P < 0.05$  vs the group without DPN); **C:** Incidence of NAFLD negatively correlated with DR ( $r = -0.114$ ,  $P < 0.05$ ). This trend persisted even when patients were stratified by sex ( $r = -0.176$  in men,  $r = -0.076$  in women, both  $P < 0.05$ ). Prevalence of NAFLD in T2DM with and without DR was 54.9% and 66.1%, respectively (<sup>b</sup> $P < 0.01$  vs the group without DR). Furthermore, the Prevalence of NAFLD in men without DR was significantly higher than that in patients with DR (66.9% and 49.5%, respectively, <sup>b</sup> $P < 0.01$  vs the group without DR). Prevalence of NAFLD in women without DR was also significantly higher than that in patients with DR (65.5% and 58.1%, respectively, <sup>a</sup> $P < 0.05$  vs the group without DR).

**Table 2 Risk factors for nonalcoholic fatty liver disease**

| Variables                 | $\beta$ | SE   | P value | OR                    | 95%CI                                    |
|---------------------------|---------|------|---------|-----------------------|--|
| Total patients with T2DM  |         |      |         |                       |  |
| logBMI                    | 16.33   | 2.24 | 0.00    | $1.238 \times 10^7$   | $154915.06-9.897 \times 10^8$            |
| logWC                     | 5.57    | 2.57 | 0.03    | 261.45                | 1.69-40422.03                            |
| logTG                     | 2.05    | 0.34 | 0.00    | 7.79                  | 3.99-15.19                               |
| FBG                       | 0.079   | 0.03 | 0.01    | 1.08                  | 1.02-1.15                                |
| DBP                       | 0.03    | 0.01 | 0.00    | 1.03                  | 1.01-1.05                                |
| SBP                       | 0.03    | 0.01 | 0.00    | 1.03                  | 1.02-1.04                                |
| Duration of diabetes      | -0.05   | 0.01 | 0.00    | 0.96                  | 0.93-0.98                                |
| DR                        | -0.035  | 0.17 | 0.04    | 0.71                  | 0.51-0.99                                |
| DPN                       | -0.38   | 0.17 | 0.03    | 0.69                  | 0.50-0.95                                |
| DN                        | -1.15   | 0.19 | 0.00    | 0.32                  | 0.22-0.46                                |
| logHDL                    | -1.76   | 0.84 | 0.04    | 0.17                  | 0.03-0.89                                |
| Male patients with T2DM   |         |      |         |                       |  |
| logBMI                    | 24.53   | 3.37 | 0.00    | $4.52 \times 10^{10}$ | $6.128 \times 10^7-3.334 \times 10^{13}$ |
| logTG                     | 3.63    | 0.62 | 0.00    | 37.88                 | 11.34-126.61                             |
| DBP                       | 0.08    | 0.02 | 0.00    | 1.09                  | 1.05-1.12                                |
| Duration of diabetes      | -0.07   | 0.02 | 0.00    | 0.94                  | 0.90-0.98                                |
| logHDL                    | -4.92   | 1.72 | 0.00    | 0.01                  | 0.00-0.21                                |
| Female patients with T2DM |         |      |         |                       |  |
| logBMI                    | 16.91   | 2.05 | 0.00    | $2.21 \times 10^7$    | $395927.04-1.23 \times 10^9$             |
| logTG                     | 1.25    | 0.42 | 0.00    | 3.48                  | 1.54-7.90                                |
| FBG                       | 0.2     | 0.04 | 0.00    | 1.22                  | 1.13-1.31                                |
| SBP                       | 0.04    | 0.01 | 0.00    | 1.04                  | 1.03-1.05                                |
| Duration of diabetes      | -0.04   | 0.01 | 0.00    | 0.96                  | 0.93-0.98                                |
| DPN                       | -0.44   | 0.21 | 0.04    | 0.65                  | 0.43-0.98                                |
| DR                        | -0.49   | 0.21 | 0.02    | 0.61                  | 0.41-0.93                                |
| DN                        | -1.23   | 0.23 | 0.00    | 0.29                  | 0.19-0.46                                |

T2DM: Type 2 diabetes mellitus; WC: Waist circumference; TG: Triglyceride; BMI: Body mass index; FBG: Fasting blood glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HDL: High-density lipoprotein; DR: Diabetic retinopathy; DPN: Diabetic peripheral neuropathy; DN: Diabetic nephropathy.

both  $P < 0.05$ ). The prevalence of NAFLD in men without DN was significantly higher than that of patients with DN (63.3% and 49.3%, respectively,  $P < 0.05$ ). Similarly, the prevalence of NAFLD in women without DN is significantly higher than that of patients with DN (67.5% and 49.5%, respectively,  $P < 0.05$ ).

The incidence of NAFLD was found to correlate negatively with DPN, as shown in Figure 2B ( $r = -0.086$ ,  $P < 0.05$ ). The general prevalence of NAFLD in T2DM with and without DPN was 57.2% and 65.6%, respectively ( $P < 0.05$ ). This trend also persisted when patients were stratified by sex ( $r = -0.095$  in men,  $r = -0.084$  in women, both  $P < 0.05$ ). The prevalence of NAFLD in men without DPN was significantly higher than that of patients with DPN (63.9% and 54.5%, respectively,  $P < 0.05$ ). Similar results were found in women (66.9% and 58.6%, respectively,  $P < 0.05$ ).

The incidence of NAFLD was negatively correlated with DR, as shown in Figure 2C ( $r = -0.114$ ,  $P < 0.05$ ). The general prevalence of NAFLD in T2DM with and without DR was 54.9% and 66.1%, respectively ( $P < 0.05$ ). This trend persisted even when patients

were stratified by sex ( $r = -0.176$  in men,  $r = -0.076$  in women, both  $P < 0.05$ ). The prevalence of NAFLD in men without DR was significantly higher than that of patients with DR (66.9% and 49.5%, respectively,  $P < 0.05$ ). Similar results were determined in women (65.5% and 58.1%, respectively,  $P < 0.05$ ).

### Risk factors for the development of NAFLD in patients with T2DM

With NAFLD as the dependent variable, the variables that were independently associated with NAFLD in T2DM patients were identified by binary stepwise logistic regression. Independent predictors of NAFLD were BMI (OR for log BMI:  $1.238 \times 10^7$ ; 95%CI:  $154915.06-9.897 \times 10^7$ ), WC (OR for log WC: 261.44; 95%CI: 1.691-4.422.03), TG (OR for log TG: 7.79; 95%CI: 3.99-15.19), FBG (OR = 1.08; 95%CI: 1.02-1.15), diastolic blood pressure (DBP) (OR = 1.03; 95%CI: 1.01-1.05), systolic blood pressure (SBP) (OR = 1.03; 95%CI: 1.021-1.04), duration of diabetes (OR = 0.96; 95%CI: 0.93-0.98), DR (OR = 0.71; 95%CI: 0.51-0.99), DPN (OR = 0.69; 95%CI: 0.50-0.95), DN (OR = 0.32; 95%CI: 0.22-0.46), and HDL (OR for log HDL: 0.17; 95%CI: 0.03-0.89) (Table 2).

However, the risk factors for NAFLD in men and women were not identical. Independent predictors of NAFLD in men were BMI (OR for log BMI:  $4.52 \times 10^{10}$ ; 95%CI:  $6.12 \times 10^7-3.33 \times 10^{13}$ ), TG (OR for log TG: 37.88; 95%CI: 11.34-126.61), DBP (OR = 1.09; 95%CI: 1.05-1.12), duration of diabetes (OR = 0.94; 95%CI: 0.90-0.98), and HDL (OR: for log HDL: 0.01; 95%CI: 0-0.21) (Table 2). Independent predictors of NAFLD in women were BMI (OR for log BMI:  $2.21 \times 10^7$ ; 95%CI:  $395927.04-1.23 \times 10^9$ ), TG (OR for log TG: 3.48; 95%CI: 1.54-7.90), FBG (OR = 1.22; 95%CI: 1.13-1.31), SBP (OR = 1.04; 95%CI: 1.03-1.05), duration of diabetes (OR = 0.96; 95%CI: 0.93-0.98), DPN (OR = 0.65; 95%CI: 0.43-0.98), DR (OR = 0.61; 95%CI: 0.41-0.93), and DN (OR = 0.29; 95%CI: 0.19-0.46) (Table 2).

## DISCUSSION

NAFLD represents an important burden of disease for patients with T2DM; however, the magnitude of the problem is currently unknown. In this study, the prevalence of NAFLD in hospitalized Chinese T2DM patients was calculated at 61%. This prevalence is lower than that reported by Lu *et al*<sup>[2]</sup> in 2009 (75.18%) but markedly higher compared with that reported by Zhou *et al*<sup>[3]</sup> conducted 5 years ago (42.1%).

A limitation of this study was that the diagnosis of NAFLD was based on ultrasound imaging. The patients did not undergo liver biopsy and histological examination, which is the gold standard technique for identifying steatosis. The sensitivity of ultrasonography in detecting

steatosis varies between 60% and 94% and is dependent on the degree of steatosis. In particular, sensitivity is significantly low when the degree of steatosis is < 30%<sup>[5,8]</sup>. Furthermore, some participants with normal ultrasound scans may have undiagnosed hepatic fibrosis and thus be considered a severe case of NAFLD<sup>[6]</sup>. Therefore, the cases that were potentially misclassified by ultrasonography might have resulted in an underestimated prevalence of NAFLD. This limitation might have attenuated the magnitude of our effect measures to null. The findings of this study may then be considered conservative estimates of the prevalence of NAFLD in hospitalized Chinese T2DM patients.

This study revealed that the prevalence of NAFLD in male hospitalized T2DM patients was 59.1%, which was slightly lower than that in female patients (62.1%), but without statistical significance. When patients were divided into three groups according to their age (*i.e.*, < 50, 50-60, and > 60 years), the prevalence of NAFLD in women was higher than in men aged < 50 years, whereas the reverse was evident when age was > 50 years. However, no significant difference was indicated in the prevalence of NAFLD between male and female patients at different ages. This finding differs from that of Zhou *et al.*<sup>[3]</sup>, who reported that the prevalence of fatty liver in patients < 50 years old was significantly higher in men than women; however, the reverse was evident when participants were older than 50 years. In the present study, several male patients with NAFLD, especially those aged < 50 years, were excluded for overdrinking. With this factor considered, the prevalence of NAFLD in male hospitalized T2DM patients may be significantly higher than that of female patients.

In our study, patients were assessed according to three age groups. The prevalence rates of NAFLD significantly decreased as age increased. This trend persisted even when the patients were stratified by sex. These observations are inconsistent with those of Targher *et al.*<sup>[5]</sup>, who reported that the prevalence of NAFLD increased with age (*i.e.*, 65.4% among participants aged 40-59 years and 74.6% among those aged  $\geq$  60 years;  $P < 0.001$ ). By contrast, Lu *et al.*<sup>[2]</sup> found that the prevalence of NAFLD in Chinese T2DM patients did not increase with age. In particular, T2DM patients in China aged 40-59 years are more prone to NAFLD. These findings are consistent with those published in a review by Duvnjak *et al.*<sup>[9]</sup>, which reports that the highest prevalence of NAFLD occurs in those aged 40-60 years. A potential explanation for these observations is that middle-aged persons are often too busy to participate in physical exercise and are more likely to dine outside of the home, whereas older and retired persons may have more time for physical exercise and devote more attention to their lifestyle. These behaviors among middle-aged persons may be one reason why Chinese patients with T2DM are more vulnerable to NAFLD, considering that the prevalence

of NAFLD does not increase with age<sup>[2]</sup>.

Patients were also assessed according to the duration of diabetes (*i.e.*, < 5, 5-10, and > 10 years). The prevalence rates of NAFLD decreased significantly with the prolonged course of diabetes. This trend persisted even when patients were stratified by sex. No significant difference was observed in the prevalence of NAFLD between male and female patients with different durations of diabetes. These findings contradict those of Banerjee *et al.*<sup>[10]</sup>, who found that a longer duration of T2DM was significantly associated with NAFLD. The association of a shorter duration of diabetes with liver disease has been previously described. A possible explanation for this association may be that a greater degree of hyperinsulinemia in early T2DM increases the uptake of free fatty acids (FFAs) by hepatocytes<sup>[6]</sup>. Popović *et al.*<sup>[11]</sup> presented a significant negative correlation between the duration of diabetes and fasting insulinemia as well as insulin resistance assessed by homeostatic model assessment-insulin resistance index. Increased fasting insulinemia and hepatic insulin resistance may trigger a reduced catabolism of lipoproteins rich in TGs and an increased hepatic very LDL production via changes in the rate of apolipoprotein B synthesis and degradation, as well as *de novo* lipogenesis or increased FFA flux from adipose tissue into the liver<sup>[12]</sup>.

In this study, we demonstrated that the general prevalence of diabetic microangiopathy complications (*i.e.*, DN, DPN and DR) increased significantly with age increased (data not shown). This trend persisted even when the patients were stratified by sex. When the patients were stratified by duration of T2DM, the prevalence rates of diabetic microangiopathy complications increased significantly with prolonged duration of T2DM (data not shown). In previous reports, the age and duration of diabetes were common risk factors for the three microvascular complications of T2DM<sup>[13-17]</sup>. Thus, chronic microvascular diabetic complications increase gradually with prolonged duration of T2DM, and the clinical symptoms tend to worsen progressively. Consequently, these patients need to pay more attention to their lifestyle and drug therapy. Therefore, the incidence of NAFLD does not increase with a prolonged duration of T2DM.

The development of NAFLD is a multifaceted cascade of physiological and biochemical events, including genetic, environmental, metabolic, and stress-related factors; the exact risk factors for NAFLD have not been clearly identified<sup>[18]</sup>. On the basis of the univariate analysis results, patients diagnosed with NAFLD were significantly younger and had a shorter duration of diabetes than those without NAFLD. The BW, WC, BMI, SBP, DBP, FBG, HbA1c, and TG were significantly higher, and HDL cholesterol was significantly lower in patients with NAFLD (Table 1,  $P < 0.05$ ). Multivariate analysis identified the following as independent pre-

dictors of NAFLD: BMI, WC, TG, FBG, DBP, SBP, duration of diabetes, DR, DPN, DN, and HDL. This observation is consistent with the findings reported in literature that the prevalence of NAFLD is closely associated with obesity, dyslipidemia, hypertension, and glucose intolerance, a cluster of metabolic disorders that is now recognized as metabolic syndrome<sup>[10,18,19]</sup>. However, the negative correlations between the prevalence of NAFLD and the duration of diabetes, DR, DPN, and DN identified in this study are inconsistent with the data reported in previous studies<sup>[10]</sup>. Given the negative correlations between the prevalence of NAFLD as well as the duration of diabetes and diabetic chronic microvascular complications, more attention should be given to lifestyle and drug therapy, particularly in patients presenting an aggravation in any of the clinical symptoms mentioned above.

Despite the difference in the independent risk factors for NAFLD between men (Table 2) and women (Table 2), both positively correlated with BMI, TG, and hypertension and a negative correlation with the duration of diabetes. In men, the prevalence of NAFLD positively correlated with DBP but negatively with HDL (Table 2). In women, the prevalence of NAFLD positively correlated with FBG and SBP but negatively with DPN, DR and DN (Table 2). According to a previous study, SBP represents the most prevalent type in obese women, being a stronger predictor of cardiovascular disease than DBP<sup>[20]</sup>. Therefore, the regulation of SBP is more critical for women.

Although most patients with NAFLD presented with non-progressive bland steatosis, few others developed a histological subtype of nonalcoholic steatohepatitis (NASH), which can develop to cirrhosis, hepatocellular carcinoma, and liver-cancer-related death<sup>[9,21,22]</sup>, especially for NASH patients with T2DM<sup>[21,23,24]</sup>. Thus, specialists have considered that NAFLD may not be a completely benign disorder<sup>[9,25]</sup>. On the contrary, we consider NAFLD a benign condition because of the negative relationship between the prevalence of NAFLD with age and duration of T2DM. No treatment to alleviate NAFLD or prevent its progression has been scientifically proven. However, various therapeutic alternatives aimed at interfering with the risk factors involved in the pathogenesis of this disorder have been applied to prevent the progression of NAFLD to end-stage liver disease. The most important therapeutic measure is to increase insulin sensitivity by motivating patients to change their lifestyle habits, particularly by diet modification and participation in physical activities to lose weight<sup>[26,27]</sup>.

Tushuizen *et al.*<sup>[28]</sup> reported that after 44 wk of exenatide therapy, patients experienced a 73% reduction in hepatic fat content (*i.e.*, from a baseline of 15.8% to 4.3%), as evaluated by proton magnetic resonance spectroscopy. This finding is consistent with that reported by Ding *et al.*<sup>[29]</sup> on the reduced hepatic lipid content in exenatide-treated ob/ob or obese mice compared with

placebo-treated mice. Lazo *et al.*<sup>[30]</sup> reported that a 12-mo intensive lifestyle intervention in patients with T2DM significantly reduced steatosis and NAFLD. In addition, Lo *et al.*<sup>[24]</sup> have reported that diet-induced NASH fibrosis is exacerbated by diabetes and attenuated by insulin therapy. On the basis of these findings, we believe that NAFLD is reversible, and patients may benefit from its early diagnosis and treatment<sup>[31]</sup>.

In conclusion, the results of this study suggest that NAFLD is extremely common in people with T2DM, positively correlates with BMI, WC, TG, FBG, DBP, and SBP, and negatively correlates with the duration of diabetes, DR, DPN, DN, and HDL. Given that hepatic fat content can be reversed with lifestyle changes and drugs, NAFLD should be included in future preventive public health initiatives, and the affected individuals should be motivated to adopt a healthier lifestyle<sup>[27]</sup>. Patients with T2DM should be always assessed for NAFLD to ensure early diagnosis and entry into proper and thorough medical care. Future efforts should be directed toward strengthening early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction to prevent and minimize the occurrence of NAFLD.

## COMMENTS

### Background

Diabetes is an independent risk factor for the development of nonalcoholic fatty liver disease (NAFLD) and its progression to more advanced liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma. NAFLD and diabetic microangiopathy complications represent important burdens of disease for patients with type 2 diabetes mellitus (T2DM). Previous studies have shown that the prevalence of NAFLD and microangiopathy complications in patients with T2DM significantly correlate with age and duration of T2DM. However, few studies have focused on the relationship between NAFLD and diabetic microangiopathy complications.

### Research frontiers

Many studies have reported that individuals with NAFLD are older, more likely to be male, and have a longer duration of diabetes than those without NAFLD. However, recently, conflicting results have shown that participants with definite steatosis are significantly younger and have shorter durations of diabetes than the combined normal/probably normal groups. Further studies are required to ascertain the differences in the prevalence of NAFLD between the sexes, and the relationships between age, duration of diabetes, and prevalence of NAFLD.

### Innovations and breakthroughs

The relatively larger cross-sectional study of inpatients with T2DM in Qingdao, China showed that the prevalence of NAFLD was 61%. However, based on the finding that the prevalence of NAFLD was negatively correlated with age and duration of T2DM, the authors believe that NAFLD is a benign process.

### Applications

Our results suggest that patients with T2DM should be always assessed for NAFLD to ensure early diagnosis and entry into proper and thorough medical care. Future efforts should be directed at strengthening early diagnosis, intensive blood glucose and pressure control, and effective dyslipidemia correction to prevent and minimize the occurrence of NAFLD.

### Terminology

NAFLD is defined as the presence of definite hepatic steatosis on ultrasound scan in the absence of a secondary cause for hepatic steatosis. Diabetic microvascular complications included diabetic nephropathy (DN), diabetic peripheral neuropathy (DPN), and diabetic retinopathy (DR). DN is defined by increased urinary albumin excretion in the absence of urinary tract infection or other renal abnormalities. DPN is diagnosed in the presence of persistent numbness, par-

esthesia, loss of hearing of the tuning fork and sense of vibration, and failure to elicit a knee and/or ankle jerk. DR is diagnosed in the presence of retinal hemorrhages, exudates, and macular edema.

### Peer review

This was a good descriptive study in which the authors evaluated the correlation between NAFLD and microvascular complications in Chinese T2DM patients. The results are interesting and suggest that NAFLD is likely a benign process and efforts should be directed at early diagnosis, intensive blood glucose and blood pressure control, and effective dyslipidemia correction, which is of clinical interest.

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**P- Reviewers** Buechler C, Hsieh PS, Tarantino G  
**S- Editor** Wen LL **L- Editor** Kerr C **E- Editor** Zhang DN



## Value of circulating cell-free DNA in diagnosis of hepatocellular carcinoma

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Received: December 12, 2012 Revised: April 8, 2013

Accepted: April 18, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To investigate the value of combined detection of circulating cell-free DNA (cfDNA),  $\alpha$ -fetal protein (AFP) and  $\alpha$  L-fucosidase (AFU) for diagnosis of hepatocellular carcinoma (HCC).

**METHODS:** Serum samples from 39 HCC patients and 45 normal controls were collected. Branched DNA (bDNA) was used to detect the level of cfDNA, and a receiver operating characteristic curve was employed to evaluate the diagnostic sensitivity, specificity, accuracy, positive predictive value, negative predictive value, positive likelihood ratio, negative likelihood ratio and Youden index, and to assess the diagnostic efficiency and their correlations with the clinicopathological features. AFP and AFU were detected by chemilumines-

cence and colorimetry, respectively. The significance of combined detection of the three biomarkers was discussed.

**RESULTS:** cfDNA level was increased in 22 of the 39 HCC samples and in 2 of the 45 normal controls. cfDNA level in HCC samples was significantly higher than that in normal controls ( $P < 0.05$ ). There were significant differences in sex and extra- and intrahepatic metastasis ( $P < 0.05$ ). There was no significant correlation between cfDNA, AFP and AFU in the detection of HCC. The sensitivity of combined detection of cfDNA with one marker (AFP or AFU) and cfDNA with two markers (AFP and AFU) was 71.8%, 87.2% and 89.7% vs 56.4%, 53.8% and 66.7% for cfDNA, AFP and AFU used alone, respectively, the difference being statistically significant ( $P < 0.05$ ).

**CONCLUSION:** Quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for HCC.

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**Key words:** Alu; Branched DNA; Circulating cell free DNA; Diagnosis; Hepatocellular carcinoma

**Core tip:** Various techniques are available to quantify serum circulating free cell DNA (cfDNA), some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum. This study investigates the value of combined detection of cfDNA,  $\alpha$ -fetal protein (AFP) and  $\alpha$  L-fucosidase (AFU) for diagnosis of hepatocellular carcinoma. Quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for hepatocellular carcinoma.

Chen K, Zhang H, Zhang LN, Ju SQ, Qi J, Huang DF, Li F, Wei Q, Zhang J. Value of circulating cell-free DNA in diagnosis of hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(20): 3143-3149 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3143.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3143>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, with an incidence which ranks third and mortality which ranks second in all malignant tumors. The one-year survival in patients with HCC without surgical ablation is less than 30%, and the 5-year recurrence rate is about 80%, even in patients who undergo radical resection. Therefore, the key to HCC treatment is early detection and diagnosis<sup>[1,2]</sup>. Existing biomarkers for the diagnosis of HCC are alpha-fetal protein (AFP),  $\gamma$ -glutamyltranspeptidase isoenzymes II (GGT-II),  $\alpha$  L-fucosidase (AFU) and acidic isoferritin<sup>[3]</sup>, of which AFP remains the most specific marker due to its recognized effect in the diagnosis of HCC and assessment of the curative effect, prognosis and recurrence. However, not all HCC patients are diagnosed by AFP alone as the false negative rate of AFP is about 30%. Markers like AFU and GGT-II have assisted in diagnosing patients with HCC whose AFP levels are negative, but they can not replace AFP in the diagnosis of HCC. Therefore, there is an urgent need to find a new biomarker which is superior or at least equivalent to AFP for the diagnosis of HCC.

Cell-free fetal DNA (cfDNA) has been extensively studied over the past few decades. Supported by theory and observation, two major sources of cfDNA have been postulated: first, fragmented DNA released as a consequence of cell death (apoptotic/necrotic) and, second, active metabolic secretion of DNA from cells. Considerable research effort has been made on the use of cfDNA as a biomarker in cancer diagnosis. cfDNA from malignancies exhibits characteristic changes such as mutations, deletions, methylations and microsatellite aberrations which are distinct from those in benign conditions, and thus may be useful in the diagnosis of cancer<sup>[4]</sup>.

This study was designed to validate a bDNA-based Alu assay for quantifying human cfDNA in blood, and explore the feasibility of Alu quantification for HCC screening.

## MATERIALS AND METHODS

### Patients and samples

Included in this study were 39 patients randomly selected from those who had been diagnosed with HCC by clinical and radiographic evaluations at the Affiliated Hospital of Nantong University (Nantong, China) between May and October 2011. They included 32 males and 7 females ranging in age from 40 to 85 years. Forty-

five healthy volunteers (36 males and 9 females; age range 28-83 years) who were negative for physical indices following health examination and documented to have normal liver biochemistry served as controls. Fresh blood was collected in serum separator tubes (Vacuette, Austria) coated with a clot activator and barrier gel, and EDTA tubes (Kangshi, China) for plasma, were centrifuged at  $1600 \times g$  for 10 min, and then microcentrifuged at  $16000 \times g$ . The separated serum was immediately stored at  $-80^\circ\text{C}$ . The samples tested were diluted using the standard human genomic DNA qualification kit according to the manufacturer's instructions, with an initial concentration of  $2.73 \times 10^5$ .

### Methods

**Quantitation of cfDNA:** Ten  $\mu\text{L}$  diluted serum was added to lysis solution to a final volume of 100  $\mu\text{L}$  containing 50% Lysis Mixture (Panomics) and 1 g/L proteinase K. Target gene probe sets containing 250, 250 and 250 fmol of capture extender probe (CE), blocking probe and label extender probe (LE) were added to the blood lysate. The lysate was then transferred to an assay well in a 96-well plate (Panomics, Redwood City, CA, United States) covalently coated with capture probe (CP) oligo, incubated at  $55^\circ\text{C}$  for 1 h, washed 3 times with 200  $\mu\text{L}$  wash buffer ( $0.1 \times$  standard 4 saline citrate containing 0.3 g/L lithium lauryl sulfate), sequentially hybridized with 100  $\mu\text{L}$  1:1000 dilution of bDNA preamplifier (amplifier) (Panomics) at  $55^\circ\text{C}$  for 30 min and then with 100  $\mu\text{L}$  (50 fmol) 3'-alkaline phosphatase-conjugated Label Probe oligo (Panomics) at  $50^\circ\text{C}$  for 30 min, and then washed three times. After the final wash, the alkaline phosphatase substrate dioxetane (Panomics) was added to the wells and incubated at room temperature for 5-10 min to detect luminescent signals using an Lmax microtiter plate luminometer (a molecular device). The reagent for the QuantiGene 2.0 DNA Assay was provided by Professor Zhang Lurong from Florida State University, United States.

In the same 96-well-plate, samples with a known amount of Human Genomic DNA were added as follows: 0, 50, 100, 200 and 400 ng/mL, respectively. A standard curve was mapped out by the computer. The standard Human Genomic DNA solution was obtained from Promega, Madison, United States.

**Quantitation of AFP:** The concentration of AFP in the serum samples from HCC patients was measured manually with the ARCHITECT AFP Reagent Kit (Abbott, Chicago, IL, United States) according to the calibrator dose response curve by the chemiluminescence method using an Abbott 2000 immunoassay instrument.

**Quantitation of AFU:** The activity of AFU in the serum samples from HCC patients was measured manually with a Shanghai Institute of Naval Medicine Biotechnology Center Kit (Shanghai, China) by a colorimetric method using a BA-88 semi-automatic biochemical analyzer.

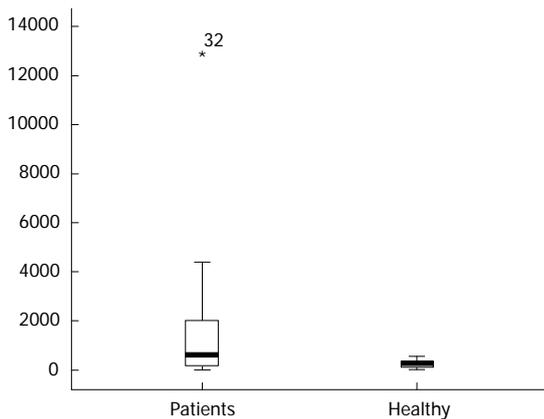


Figure 1 Circulating cell-free DNA levels in 39 hepatocellular carcinoma patients and 45 normal controls.

### Interpretation of the results

**Reference value of cfDNA:** The content of cfDNA in each sample was calculated according to the standard curve. A receiver operating characteristic (ROC) curve was drawn to select the Youden's index<sup>[5]</sup>, and the highest sensitivity and specificity (Youden's index = sensitivity + specificity-1) were selected as the cut-off values. A value greater than or equal to the cut-off value was regarded as positive, and smaller values were regarded as negative.

**Reference value of AFP:** According to the clinical diagnosis and staging criteria of HCC of the Chinese Anti-Cancer Association, a content of AFP greater than or equal to 400 ng/mL is considered positive, and a content smaller than this value is negative, excluding cases of pregnancy, embryonic tumors of the reproductive system, active liver disease and metastatic liver cancer.

**Reference value of AFU:** According to the reagent kit manual, the activity of serum AFU = (A determination tube-A control tube)/average A standard tube × 400, a value greater than 650 μmol/L per hour is considered positive, 550-650 μmol/L per hour is suspicious, and less than 550 μmol/L per hour is negative.

### Statistical analysis

Statistical analysis of the data was performed using the SPSS 13.0 software package. As the results of the concentrations of cfDNA, AFP and AFU in each group were normally distributed, they were presented as the median (range of detection). Measurement data were based on mean ± SD. Correlations between the three indices were evaluated with Pearson's correlation coefficient. The rates of interclass were determined using the  $\chi^2$  test. A *P* value less than 0.05 was considered statistically significant.

## RESULTS

### Results of quantitative detection of cfDNA

The content of serum cfDNA in the 39 HCC samples

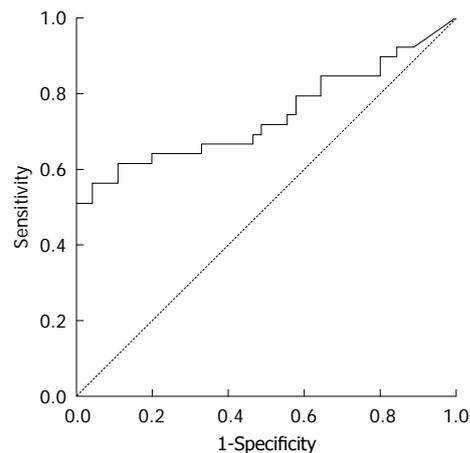


Figure 2 Receiver operating characteristic curve of serum circulating cell-free DNA in 39 hepatocellular carcinoma patients and 45 normal controls.

ranged from 12866.8 ng/mL to 0 ng/mL, with a median of 557.59 ng/mL compared with 569.2963 ng/mL, 0 ng/mL and 240.07 ng/mL in the 45 normal control samples, and a significant difference was observed between them (*P* < 0.05) (Figure 1). According to the ROC curve (Figure 2), the Youden index was maximal when the concentration of cfDNA was 509.9774 ng/mL, therefore 509.9774 ng/mL was defined as the positive value.

The ROC area under the curve (AUC) was  $0.742 \pm 0.058$ , therefore a sum score of the classifier at 509.9774 ng/mL was chosen as the optimal cut-off, as it had the highest Youden's index of 0.520. At this cut-off value, the sensitivity was 56.4%, the specificity was 95.6%, the accuracy was 77.4%, the positive predictive value was 91.7%, the negative predictive value was 71.7%, the positive likelihood ratio was 12.692 and the negative likelihood ratio was 0.048.

### Correlations between clinicopathological features and cfDNA expression

Correlations between clinicopathological features and serum cfDNA level were observed, suggesting that serum cfDNA level in HCC patients was correlated with intrahepatic and extrahepatic metastasis and gender (Table 1).

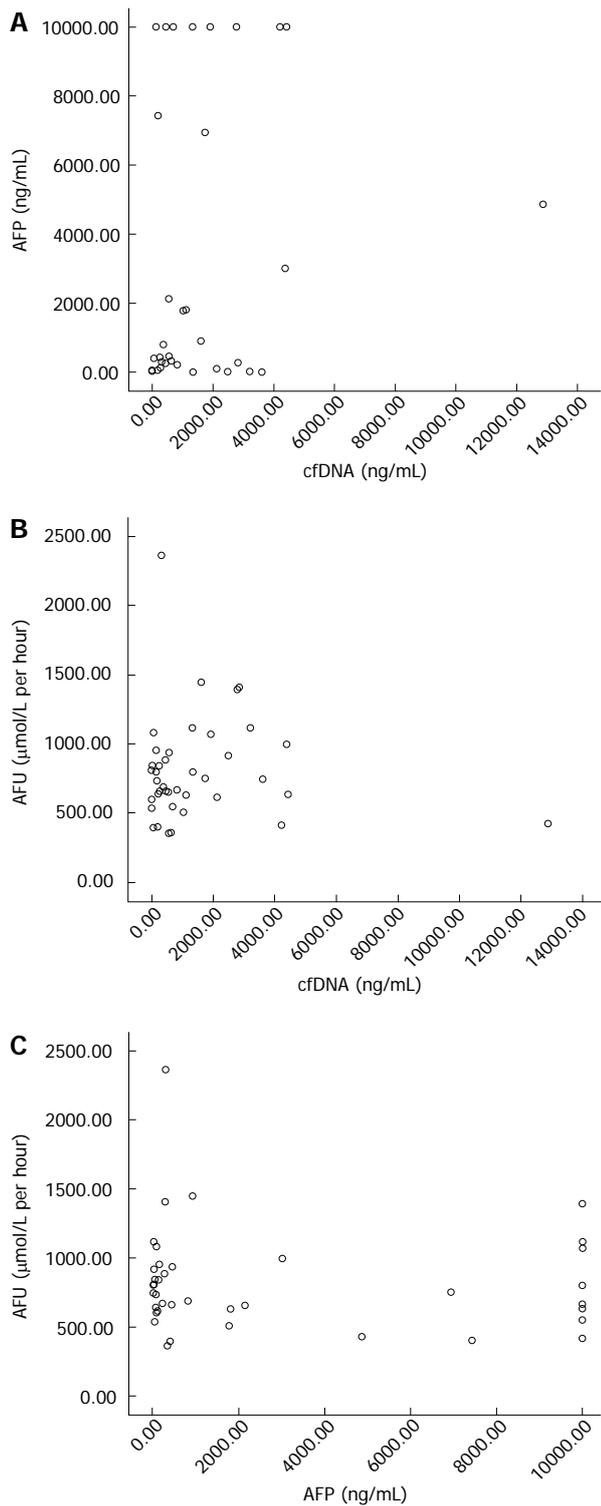
### Results of quantitative detection of AFP and AFU

The content of serum AFP in HCC samples ranged from > 10000 ng/mL to 3.92 ng/mL, with a median of 392.07 ng/mL, compared with 526.10 ng/mL, 1.31 ng/mL and 6.02 ng/mL in the normal control samples, and a significant difference was observed between them (*P* < 0.05).

The content of serum AFU in HCC samples ranged from 2366 ng/mL to 362 ng/mL, with a median of 744 ng/mL compared with 792 ng/mL, 121 ng/mL and 431 ng/mL in the normal control samples, and a significant difference was observed between them (*P* < 0.05).

### Correlation analysis between cfDNA, AFP and AFU

Scatter point diagrams were mapped out to analyze correla-



**Figure 3** Correlation coefficient. A:  $r = 0.243$  showing no significant correlation between circulating cell-free DNA and  $\alpha$ -fetoprotein; B:  $r = -0.0735$  showing no significant correlation between circulating cell-free DNA and  $\alpha$  L-fucosidase; C:  $r = -0.085$  showing no significant correlation between  $\alpha$ -fetoprotein and  $\alpha$  L-fucosidase.

tions between the three indices using SPSS13.0 (Figure 3).

**Detection of cfDNA in combination with AFP and AFU**

Compared with the diagnostic efficiency of cfDNA, AFP or AFU alone, combined detection improved the diag-

**Table 1** Relationship between expression of circulating cell-free DNA and clinicopathological features of 39 hepatocellular carcinoma patients

| Observation index                        | No. | No. of positive cfDNA (%) | P value  |
|--|-----|---------------------------|----------|
| Age (yr)                                 |     |                           |          |
| ≤ 60                                     | 26  | 16 (61.5)                 | 0.453255 |
| > 60                                     | 13  | 6 (46.2)                  |          |
| Sex                                      |     |                           |          |
| Male                                     | 32  | 19 (59.4)                 | 0.024449 |
| Female                                   | 7   | 3 (42.9)                  |          |
| HBsAg                                    |     |                           |          |
| Positive                                 | 31  | 16 (51.6)                 | 0.080857 |
| Negative                                 | 8   | 6 (75)                    |          |
| Intrahepatic and extrahepatic metastasis |     |                           |          |
| Positive                                 | 5   | 4 (80)                    | 0.000242 |
| Negative                                 | 34  | 18 (52.9)                 |          |
| Cirrhosis of liver                       |     |                           |          |
| Positive                                 | 20  | 10 (50)                   | 0.831179 |
| Negative                                 | 19  | 12 (63.2)                 |          |

cfDNA: Circulating cell-free DNA.

nostic efficiency of HCC to some extent (Table 2).

**DISCUSSION**

Circulating free cell DNA is the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases, including systemic lupus erythematosus (SLE), diabetes, cerebral stroke, rheumatoid arthritis, myocardial infarction, pulmonary embolism, preeclampsia, Whipple’s disease and malignant tumors<sup>[6-10]</sup>. Investigations have detected increased cfDNA in the plasma of patients with severe injuries, organ failure and multiple organ dysfunction syndromes. Elevated levels of cfDNA were also found in patients who had undergone organ transplantation<sup>[11]</sup>. Fetal cfDNA has been detected in maternal plasma and has been used for the prenatal diagnosis of fetal abnormalities<sup>[12]</sup>. Blunt traumatic injury and burn injury can also cause cfDNA release into the blood circulation<sup>[13]</sup>. Many studies have reported the detection of cfDNA in the serum of patients with various malignant and benign tumors. It is hypothesized that cfDNA may originate from lymphocytes and other nucleated cells in healthy individuals, however, the origin of cfDNA in malignant tumors is not fully understood<sup>[14]</sup>.

Previous studies have suggested the use of serum cfDNA in the diagnosis of HCC. One study showed that cfDNA level was significantly higher in HBV carriers than in normal controls<sup>[15]</sup>. Ren *et al*<sup>[16]</sup> suggested that cfDNA level correlated inversely with HCC prognosis, and was attributable to HBV infection in most cases, suggesting that cfDNA may be a predictive marker for the prognosis of HBV-related HCC. In HCV-related HCC patients, serum cfDNA levels were positively correlated with the increased expression of several inflammatory cytokine genes, suggesting that serum cfDNA is associated with local inflammation. cfDNA is a potential non-inva-

**Table 2** Diagnostic value of circulating cell-free DNA,  $\alpha$ -fetal protein and  $\alpha$  L-fucosidase for hepatocellular carcinoma

| Groups            | Sensitivity                   | Specificity                   | Accuracy        | Positive predictive value     | Negative predictive value     |
|-------------------|-------------------------------|-------------------------------|-----------------|-------------------------------|-------------------------------|
| cfDNA             | 56.4 <sup>ce</sup><br>(22/39) | 95.6 <sup>ce</sup><br>(43/45) | 77.4<br>(65/84) | 91.7 <sup>ce</sup><br>(22/24) | 71.7<br>(43/60)               |
| AFP               | 53.8 <sup>ce</sup><br>(21/39) | 91.1 <sup>ce</sup><br>(41/45) | 73.8<br>(62/84) | 84.0 <sup>e</sup><br>(21/25)  | 69.5 <sup>ce</sup><br>(41/59) |
| AFU               | 66.7 <sup>e</sup><br>(26/39)  | 75.6<br>(34/45)               | 71.4<br>(60/84) | 70.3<br>(26/37)               | 72.3<br>(34/47)               |
| cfDNA + AFP       | 71.8 <sup>e</sup><br>(28/39)  | 86.7 <sup>e</sup><br>(39/45)  | 79.8<br>(67/84) | 82.4<br>(28/34)               | 78<br>(39/50)                 |
| cfDNA + AFU       | 87.2<br>(34/39)               | 71.1<br>(32/45)               | 78.6<br>(66/84) | 72.3<br>(34/47)               | 86.5<br>(32/37)               |
| cfDNA + AFP + AFU | 89.7 <sup>a</sup><br>(35/39)  | 64.4 <sup>a</sup><br>(29/45)  | 76.2<br>(64/84) | 68.6<br>(35/51)               | 87.9<br>(29/33)               |

<sup>a</sup>*P* < 0.05 vs group circulating cell-free DNA (cfDNA) +  $\alpha$ -fetal protein (AFP); <sup>c</sup>*P* < 0.05 vs group cfDNA +  $\alpha$  L-fucosidase (AFU); <sup>e</sup>*P* < 0.05 vs group cfDNA + AFP + AFU.

sive cancer biomarker as it is easily isolated from the circulation<sup>[17]</sup>. Positive correlations were observed between cfDNA level, aspartate aminotransferase (AST) level and the number of leukocytes and neutrophils in HCV-related HCC patients, but not in HCV carriers<sup>[18]</sup>. The level of cfDNA in serum can function as a predictor of overall survival in distant organs after curative hepatectomy in patients with HCV-related HCC<sup>[19]</sup>. In our study, serum cfDNA level was significantly higher in HCC patients than in normal controls; patients with intrahepatic and extrahepatic metastasis had a significantly higher cfDNA level; and male patients had higher cfDNA levels as compared with female patients. Therefore, it is clear that serum cfDNA level is associated with intrahepatic and extrahepatic metastasis and gender in HCC patients, but is not closely associated with age, HbsAg or liver cirrhosis. The significance of serum cfDNA in the clinical diagnosis of HCC is as follows: First, serum cfDNA level is positively correlated with the degree of differentiation and metastasis and negatively correlated with the prognosis of HCC patients, suggesting that cfDNA may be useful in monitoring the pathogenic condition of HCC. Second, serum cfDNA level is relevant to the survival of HCC patients after hepatectomy, suggesting that cfDNA may be a useful marker for surgical or non-surgical treatment decision making.

Various techniques are available to quantify serum cfDNA, some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum, and these technologies have failed to reach expectations. The bDNA amplification-based Alu assay is a new technology to quantify serum cfDNA directly and does not require extraction of cfDNA from the serum. The Alu family is the largest of the short interspersed elements (SINEs). Each Alu sequence is approximately 300 bp with a high homology of 70%-98%<sup>[20,21]</sup>. The sequence AGCT at the 170 degree position can be recognized and cut by the restriction enzyme Alu. Recent studies<sup>[22,23]</sup> on the Alu sequence showed that it had a unique structure and physiological function, and was associated with a variety of malignant

tumors and other human diseases such as gene deletion or duplication of homologous recombination, gene rearrangement of chromosome translocation, and change in transcriptional level. The results of our study suggest that the bDNA technique may be a useful approach in the diagnosis of HCC, as amplification of the hybridization signal is needed instead of amplification of the target sequence. In addition, it is simple, economical, time saving and reproducible.

AFP is the most commonly used biomarker for HCC screening, and a continuous elevation in AFP is considered a risk factor for HCC. AFP has been widely used in HCC survey, diagnosis, evaluation of treatment effects and prediction of recurrence<sup>[24,25]</sup>. AFP can also be used to monitor treatment and prognosis. Vigilance is required when AFP is higher than 400  $\mu$ g/L or rises continuously. The survival period is very short in patients with an AFP value > 500  $\mu$ g/L and bilirubin > 2 mg/L. A rapid increase in AFP often suggests the occurrence of HCC metastasis. Postoperative elevation of AFP > 200  $\mu$ g/L often indicates incomplete resection of HCC tissue, or the occurrence of metastasis. AFP can also be used in other cancers, such as testicular cancer, teratoma, gastric carcinoma, and pancreatic carcinoma. However, not all HCC cases are diagnosed by AFP alone, as the false negative rate is approximately 30%. False positive AFP results are also seen in some patients with non malignant conditions such as viral hepatitis and cirrhosis. The level of AFP can also increase continuously in the blood or urine of pregnant women, but usually does not exceed 300  $\mu$ g/L.

AFU is a lysosomal acid hydrolase found extensively in human tissues, with higher activity in the liver and kidney. Some researchers<sup>[26]</sup> believe that increased serum AFU in HCC patients is associated with an increase in its synthesis, while others reported that hepatoma cells can produce a type of inhibitor to reduce substrate hydrolysis, resulting in substrate accumulation and a compensatory increase in AFU, which is an effective marker of HCC<sup>[27,28]</sup>. In the present study, the sensitivity of AFU, cfDNA and AFP was 66.7% 56.4% and 53.8%, respec-

tively; the specificity of AFU was only 75.6% compared with 95.6% for cfDNA and AFP, and 91.1% for AFP; the accuracy of cfDNA, AFP and AFU was 77.4%, 73.8% and 71.4%, respectively, with no significant differences between them. Although the sensitivity of AFU was the highest of the three, its specificity was very low. In contrast, the sensitivity of cfDNA and AFP was lower than that of AFU, and their specificity was relatively high. There were no significant correlations between cfDNA, AFP and AFU. The specificity of detection of cfDNA combined with AFP or AFU was lower. However, the sensitivity improved significantly, and the accuracy was similar. The sensitivity of combined detection with the three items was slightly improved, while the specificity was decreased. Taking into account the economic burden of HCC treatment, we believe that combined detection of any two of the three biomarkers is better than combined detection of all three biomarkers in the diagnosis of HCC. In our opinion, cfDNA in combination with AFP or AFU may effectively improve the clinical detection of HCC.

In conclusion, cfDNA for HCC detection has a high sensitivity and specificity, and the clinical significance is similar to AFP, and cfDNA is not correlated with AFP and AFU. The combined detection of cfDNA with one or two markers can significantly improve the diagnostic rate of HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide. Existing biomarkers for the diagnosis of HCC are  $\alpha$ -fetoprotein (AFP),  $\gamma$ -glutamyltranspeptidase isoenzymes II (GGT-II),  $\alpha$ -L-fucosidase (AFU) and acidic isoferritin, of which AFP remains the most specific marker for the diagnosis of HCC. However, not all HCC patients are diagnosed using AFP alone. Circulating free cell DNA (cfDNA) has been extensively studied over the past few decades, and has a high sensitivity and specificity in the detection of HCC.

### Research frontiers

cfDNA results from the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases. This research was designed to investigate the value of cfDNA in the diagnosis of HCC, determine correlations between cfDNA and clinicopathological features, and explore the value of combined detection of cfDNA, AFP and AFU in the clinical diagnosis of HCC.

### Innovations and breakthroughs

Various techniques are available to quantify serum cfDNA, some of these techniques require abstraction and purification as there is only a very small amount of cfDNA in the serum, and these technologies have failed to reach expectations. The bDNA amplification-based Alu assay is a new technology to quantify serum cfDNA directly and does not require the extraction of cfDNA from serum.

### Applications

The results of this study suggest that the quantitative analysis of cfDNA is sensitive and feasible, and the combined detection of cfDNA with AFP or AFU or both could improve the diagnostic sensitivity for HCC.

### Terminology

cfDNA: cfDNA results from the fragmentation of nucleic acids in body fluids, and has been described in the serum of healthy persons and patients with a variety of diseases. Alu: The Alu family of short (~300 bp) interspersed elements is one of the most successful mobile genetic elements, having increased to a copy number in excess of one million in primate genomes in the last 65 million years.

### Peer review

The study provides some new information. However, a brief mention regarding

the importance of studying circulating DNA and its pathophysiological relevance in cancer needs to be highlighted. The authors should be encouraged to present the observed levels of DNA in healthy controls and patients. This will facilitate the readers to understand the significance of the observed differences in both groups studied. The clinical translational potential of cfDNA as a diagnostic/prognostic biomarker for HCC is not addressed in detail, and needs to be elaborated.

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P- Reviewer Mishra PK

S- Editor Huang XZ L- Editor A E- Editor Lu YJ



## Protective effects of two *Lactobacillus plantarum* strains in hyperlipidemic mice

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Supported by Chinese Ministry of Education Doctor Degree grant, No. 20101333120011; Hebei Province Natural Science Fund, No. C2011203137 and No. 11965152D; Chinese Postdoctoral grant, No. 480013

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Received: December 7, 2012 Revised: February 3, 2013

Accepted: February 28, 2013

Published online: May 28, 2013

### Abstract

**AIM:** To investigate the effects of *Lactobacillus plantarum* (*L. plantarum*) CA16 and *L. plantarum* SC4 on hyperlipidemic mice.

**METHODS:** Male Kunming mice were fed a high-cholesterol diet for 28 d to construct hyperlipidemic models. Hyperlipidemic mice and normal mice were assigned to 3 groups which were separately treated with *L. plantarum* CA16, *L. plantarum* SC4, and physiological saline through oral gavage for 28 d. Total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) levels were measured by commercially available enzyme kits. FACS Calibur flow cytometry was used

to examine hepatic and renal nuclear factor-erythroid 2-related factor 2 (Nrf2) expression. The morphology of livers was checked by hematoxylin and eosin staining and optical microscope observation.

**RESULTS:** Compared with normal mice, hyperlipidemic mice possessed significantly higher TC ( $3.50 \pm 0.43$  vs  $2.89 \pm 0.36$ ,  $P < 0.01$ ), TG ( $1.76 \pm 0.07$  vs  $1.10 \pm 0.16$ ,  $P < 0.01$ ), and LDL-C ( $1.72 \pm 0.20$  vs  $0.82 \pm 0.10$ ,  $P < 0.01$ ) levels, resulting in an increase of atherogenic index (AI) ( $2.34 \pm 1.60$  vs  $0.93 \pm 0.55$ ,  $P < 0.05$ ) and LDL-C/HDL-C ratio ( $1.43 \pm 0.12$  vs  $0.51 \pm 0.16$ ,  $P < 0.05$ ). After treatment with *L. plantarum* CA16/*L. plantarum* SC4, TG ( $1.43 \pm 0.27/1.54 \pm 0.10$  vs  $1.76 \pm 0.07$ ,  $P < 0.01/P < 0.05$ ) and LDL-C ( $1.42 \pm 0.07/1.47 \pm 0.12$  vs  $1.72 \pm 0.20$ ,  $P < 0.01/P < 0.01$ ) in hyperlipidemic mice significantly decreased. In addition, TC, HDL-C, AI, and LDL-C/HDL-C ratio were all positively changed. Meanwhile, the treatment markedly alleviated hepatic steatosis and significantly stimulated Nrf2 expression ( $73.79 \pm 0.80/72.96 \pm 1.22$  vs  $54.94 \pm 1.84$ ,  $P < 0.01/P < 0.01$ ) in hepatocytes of hyperlipidemic mice.

**CONCLUSION:** *L. plantarum* CA16 and *L. plantarum* SC4 may protect against cardiovascular disease by lipid metabolism regulation and Nrf2-induced antioxidative defense in hyperlipidemic mice.

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**Key words:** *Lactobacillus plantarum*; Hypolipidemic; Nuclear factor erythroid 2-related factor 2; Metabolic syndrome; Hepatic steatosis; Cardiovascular disease

**Core tip:** Protective effects of *Lactobacillus plantarum* (*L. plantarum*) CA16 and *L. plantarum* SC4 strains on hyperlipidemic mice were found, including regulating lipid metabolism, alleviating hepatic steatosis and reducing cardiovascular disease (CVD) risk. The mechanism of intracorporal antioxidation of *Lactobacillus* strains may be related to stimulation of nuclear factor-

erythroid 2-related factor 2 (Nrf2) expression. Hypolipidemic effect and Nrf2-induced antioxidative defense may contribute to the reduction of CVD risk. We suggest that food fermented by the strains be used as part of the diet to relieve lipid metabolism related metabolic syndrome and to reduce the risk of CVD.

Wang LX, Liu K, Gao DW, Hao JK. Protective effects of two *Lactobacillus plantarum* strains in hyperlipidemic mice. *World J Gastroenterol* 2013; 19(20): 3150-3156 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3150.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3150>

## INTRODUCTION

Metabolic syndrome is a cluster of cardiovascular and type 2 diabetic risk factors<sup>[1]</sup>. The International Diabetes Federation defines metabolic syndrome as central obesity plus any two of raised triglycerides (TG), reduced high-density lipoprotein cholesterol (HDL-C), raised blood pressure, and raised fasting plasma glucose<sup>[2]</sup>. Disturbances of lipid homeostasis lead to metabolic syndrome and increase the risk of cardiovascular disease. A 1% increase in plasma cholesterol levels can increase the risk of coronary events up to 3%<sup>[3]</sup>. Elevated low-density lipoprotein cholesterol (LDL-C) accompanied with insufficient HDL-C is a risk predictor of atherosclerosis<sup>[4]</sup>. Hypercholesterolemia stimulates formation of free radicals, decreases activity of antioxidant enzymes, and eventually leads to elevated lipid peroxides including oxidized low density lipoprotein (Ox-LDL), which is an independent atherogenic factor<sup>[5]</sup>.

Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor that binds to antioxidant response elements (ARE) in the promoter regions of many antioxidative enzymes and phase II detoxifying enzymes<sup>[6]</sup>. Oxidative stress results in accumulation of Nrf2 in the cytoplasm and initiation of transcription of target genes after translocating into the nucleus<sup>[7]</sup>. Inhibition of LDL oxidation can protect against oxidative stress linked atherosclerosis. Belghitha found that bacterial levan was beneficial for antiarteriosclerosis hypolipidemic and antioxidant effects<sup>[8]</sup>. We suppose that both exogenous antioxidants and endogenous antioxidant enzymes can strengthen antioxidative defense. Therefore, antioxidant defense triggered by improving Nrf2 expression can be envisaged as a promising strategy in reducing the incidence of atherosclerosis.

Some strains of *Lactobacillus* have been studied as probiotics. They may protect against mutagens and carcinogen exposure, gastrointestinal disease, skin disorders, yeast infections, urinary tract infection, diabetes<sup>[9]</sup>, and immune dysfunction<sup>[10]</sup>. Hypolipidemic effects of LAB were shown in mice<sup>[11,12]</sup> and rabbits<sup>[13]</sup>. Antioxidative activity of LAB, a potential application valued in prevention of atherosclerosis, was also reported *in vitro*<sup>[14]</sup> and *in vivo*<sup>[15]</sup>.

Extracorporeal antioxidative activity of *Lactobacillus casei* KCTC 3260 may be caused by chelating metal ions<sup>[14]</sup>. However, an intracorporeal antioxidative mechanism of LAB has not been defined. In the present study, we investigated the change in Nrf2 expression in mice after the application of *Lactobacillus plantarum* (*L. plantarum*) CAI6 or *L. plantarum* SC4 to discuss the possible mechanism.

Probiotic strains may have acid or bile salt tolerance to colonize the gastrointestinal tract of the host. Several tests have been taken to screen strains tolerating low pH and high concentration of cholate<sup>[16]</sup>. Cells have been coated with sodium alginate to increase viscosity and stability of strains<sup>[17]</sup>.

The primary aim of this study was to investigate the effects of *L. plantarum* CAI6 and *L. plantarum* CS4 on alleviation of metabolic syndrome and prevention of cardiovascular disease (CVD) in high-cholesterol fed mice.

## MATERIALS AND METHODS

### Microbial cultures

*L. plantarum* CAI6 (GenBank accession number: KC470704) and *L. plantarum* SC4 (GenBank accession number: KC470705) were isolated from Chinese pickled cabbage and raw milk, respectively. The two strains could survive in MRS medium (pH 3.0) supplemented with 0.3% sodium taurocholate (date not shown). The strains were cultured in MRS broth and incubated at 37 °C for 24 h. Each activated culture was centrifuged and diluted with 0.9% saline water to obtain a preparation of  $2.0 \times 10^9$  cfu/mL. 0.1% (w/v) sodium alginate was added to make the strains have better acid resistance, bile resistance, and stability in digestive tract.

### Animals

Thirty-Six adult male Kunming mice with a weight of  $20 \pm 2$  g were obtained from the Academy of Military Medical Sciences (Beijing, China). The mice were housed in a controlled animal room at  $20 \pm 2$  °C and 60% relative humidity with 12 h light/dark photoperiod. Diet and water could be accessed freely. The care and handling of the animals were in compliance with the internationally accepted standard guidelines for use of animals, and was approved by Science and Technology Committee of Yanshan University on Animal Care and Use.

### Experimental protocol

Half of the mice were fed a high-cholesterol diet (75% basic diet, 10% lard, 10% soybean meal, 5% egg yolk) for a period of up to 28 d to construct a hyperlipidemic model (HM). For comparison, the other mice were maintained on standard laboratory diet for 28 d to develop a normal model (NM). Then the mice of HM and NM were randomly assigned to three groups ( $n = 6$ ), respectively.

Group I (NM): normal mice fed a standard laboratory diet and physiological saline (10 mL/kg); Group II (NM/CAI6): normal mice fed a standard laboratory diet and *L. plantarum* CAI6 (10 mL/kg); Group III (NM/SC4): normal mice fed a standard laboratory diet and *L. plan-*

**Table 1** Effects on body weight, diet intake, liver index, and serum lipid levels

| Group  | NM                        | NM/CAI6                   | NM/SC4                    | HM                        | HM/CAI6                   | HM/SC4                    |
|--|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| Body weight, food intake, and liver index of normal model and hyperlipidemic model mice after treatment for 30 d |                           |                           |                           |                           |                           |                           |
| IBW (g)  | 26.53 ± 2.96              | 25.47 ± 2.71              | 27.03 ± 2.09              | 33.17 ± 1.44 <sup>b</sup> | 31.32 ± 1.06 <sup>b</sup> | 30.78 ± 1.66 <sup>b</sup> |
| FBW (g)  | 33.43 ± 4.78 <sup>d</sup> | 28.50 ± 3.56 <sup>d</sup> | 32.30 ± 2.73 <sup>d</sup> | 39.05 ± 2.50 <sup>b</sup> | 36.68 ± 2.22              | 38.08 ± 1.87 <sup>a</sup> |
| TFI (g)  | 44.76 ± 6.94              | 47.07 ± 4.04              | 47.05 ± 2.51              | 52.29 ± 11.97             | 51.09 ± 6.34              | 53.32 ± 13.14             |
| <sup>1</sup> LI (%)  | 4.11 ± 0.30               | 4.08 ± 0.15               | 4.10 ± 0.49               | 4.65 ± 0.50               | 4.41 ± 0.58               | 4.54 ± 0.64               |
| Serum lipid levels in the different groups of mice   |                           |                           |                           |                           |                           |                           |
| TC (mmol/L)  | 2.89 ± 0.36 <sup>d</sup>  | 2.60 ± 0.24 <sup>c</sup>  | 2.85 ± 0.28 <sup>a</sup>  | 3.50 ± 0.43 <sup>b</sup>  | 3.16 ± 0.35               | 3.27 ± 0.36               |
| TG (mmol/L)  | 1.10 ± 0.16 <sup>d</sup>  | 1.06 ± 0.15 <sup>d</sup>  | 1.08 ± 0.13 <sup>d</sup>  | 1.76 ± 0.07 <sup>b</sup>  | 1.43 ± 0.27 <sup>d</sup>  | 1.54 ± 0.10 <sup>a</sup>  |
| HDL-C (mmol/L)   | 1.62 ± 0.52               | 1.76 ± 0.48 <sup>a</sup>  | 1.67 ± 0.14               | 1.20 ± 0.42               | 1.36 ± 0.55               | 1.31 ± 0.55               |
| LDL-C (mmol/L)   | 0.82 ± 0.10 <sup>d</sup>  | 0.75 ± 0.07               | 0.81 ± 0.09               | 1.72 ± 0.20 <sup>b</sup>  | 1.42 ± 0.07 <sup>d</sup>  | 1.47 ± 0.12 <sup>d</sup>  |
| <sup>2</sup> AI  | 0.93 ± 0.55 <sup>a</sup>  | 0.57 ± 0.41 <sup>d</sup>  | 0.71 ± 0.09 <sup>d</sup>  | 2.34 ± 1.60 <sup>a</sup>  | 1.65 ± 0.99               | 1.94 ± 1.47               |
| LDL-C/HDL-C  | 0.51 ± 0.16 <sup>a</sup>  | 0.43 ± 0.19 <sup>d</sup>  | 0.49 ± 0.04 <sup>d</sup>  | 1.43 ± 0.12 <sup>a</sup>  | 1.04 ± 0.16               | 1.12 ± 0.14               |

Data represent mean ± SD ( $n = 6$  for each group). <sup>1</sup>LI: Weight of liver (mg)/body weight (g), <sup>2</sup>AI = (TC-HDL-C)/HDL-C, <sup>a</sup> $P < 0.05$  vs NM group, <sup>b</sup> $P < 0.01$  vs NM group, <sup>c</sup> $P < 0.01$  vs HM group. IBW: Initial body weight; FBW: Final body weight; TFI: Total feed intake; LI: Liver index; TC: Total cholesterol; TG: Triglyceride; HDL-C: High-density lipoprotein cholesterol; LDL-C: Low-density lipoprotein cholesterol; AI: Atherogenic index; NM: Normal diet group; HM: High-cholesterol diet group; NM/CAI6: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CAI6 group; NM/SC4: Normal diet with *L. plantarum* SC4 group; HM/CAI6: High-cholesterol diet with *L. plantarum* CAI6 group; HM/SC4: High-cholesterol diet with *L. plantarum* SC4 group.

*tarum* SC4 (10 mL/kg); Group IV (HM): hyperlipidemic mice fed a high-cholesterol diet and physiological saline (10 mL/kg); Group V (HM/CAI6): hyperlipidemic mice fed a high-cholesterol diet and *L. plantarum* CAI6 (10 mL/kg); and Group VI (HM/SC4): hyperlipidemic mice fed a high-cholesterol diet and *L. plantarum* SC4 (10 mL/kg).

The mice were treated by intragastric administration for 28 d before being anesthetized with chloral hydrate and sacrificed. Food and water consumption and body weight were recorded daily. Blood samples were drawn from the ophthalmic venous plexus. After centrifugation (2000 rpm, 10 min, 4 °C), the serum samples were collected and stored at -20 °C. The liver and kidney were excised, rinsed in ice-cold physiological saline, weighed, and then stored at -20 °C.

### Serum lipids analysis

Serum TC, TG, HDL-C, and LDL-C levels were measured using commercially available enzyme kits (Beihuangtai clinical reagent Ltd, Beijing, CHN). The TC level was measured with an enzymatic method based on the conversion to a chromogen with maximum absorption at 500 nm by cholesterol ester hydrolase, cholesterol oxidase, and peroxidase<sup>[18]</sup>. The determination of TG was based on an enzymatic method coupled with lipase, glycerokinase, glycerol oxidase and peroxidase<sup>[19]</sup>. The HDL-C level was assayed by the same enzymatic method based on specific precipitation of VLDL-C and LDL-C in the presence of phosphotungstic acid. The LDL-C value was calculated by following formula:  $LDL-C = TC - HDL-C - TG/5$ <sup>[20]</sup>.

### Morphology of liver

Fresh livers of mice were fixed with 4% paraformaldehyde for 24 h, dehydrated gradually in a graded series of ethanol, clarified in xylene, and embedded in paraffin wax. Hematoxylin and eosin stained liver was observed by an optical microscope (X5Z-G, Chongqing Optical and Electrical Instrument Co., Ltd, CHN).

### Determination of Nrf2 expression in liver and kidney

Liver and kidney tissues suspensions were prepared in ice-cold 0.1 mol/L PBS. Antibodies were diluted in 0.1 mol/L PBS containing 0.1%NaN<sub>3</sub>. 10<sup>6</sup> cells/mL were incubated with primary anti-Nrf2 antibodies for 30 min on ice, mixed with 200 μL of 4 % paraformaldehyde, and incubated for 30 min at 4 °C. After washing twice with 0.1 mol/L PBS containing 0.1%NaN<sub>3</sub>, the supernatant was discarded by centrifugation. 3%BSA, 0.1%NaN<sub>3</sub> and 10% saponin (Sigma Co., United States) were added to cell pellets and blended for 15 min, then mixed with 500 μL PBS (pH 7.4). After removal of the supernatant, 0.5 μg of secondary FITC-conjugated rabbit anti-mouse antibody were added and incubated on ice for 60 min. Finally, the cells were resuspended in 1 mL of 0.1 mol/L PBS. The hepatocytes and nephrocytes were scanned using a FACSCalibur (Becton-Dickinson, United States) respectively, and fluorescence of Nrf2 positive cells were quantified. Nonspecific binding of secondary antibody was excluded by incubating the cells only with the FITC-labelled secondary antibody. For reproducibility, the experiment was repeated three times. The software BD CellQuest Pro (Becton Dickinson Biosciences, United States) was used and the data were calculated by fluorescence intensity formula [ $I = \text{Log}(x\text{-mode}) \times 340$ ].

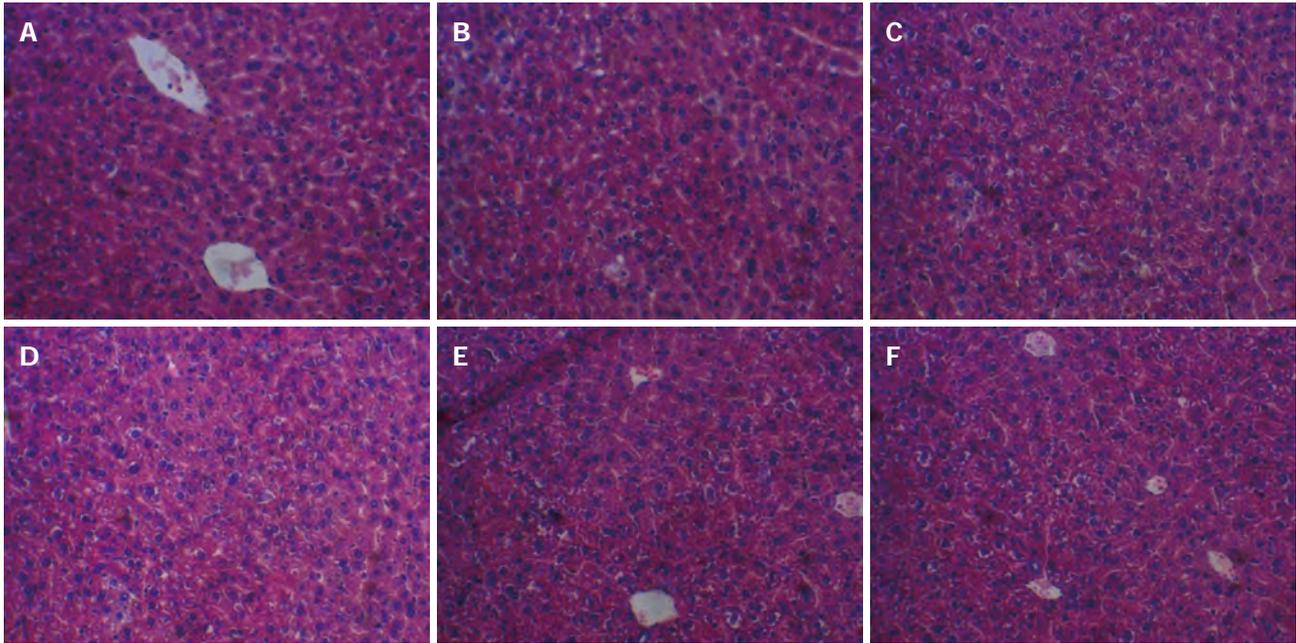
### Statistical analysis

All data were expressed as mean ± SD. Statistical analysis was performed using SPSS 13.0 software. Differences between the groups were analyzed by One-Way ANOVA followed by Duncan's multiple range tests. Statistical significance was considered at the  $P < 0.05$  level.

## RESULTS

### Effects on body weight, diet intake, and liver index

As shown in Table 1, the mice subjected to a high cholesterol diet had a significant increase in body weight (BW)



**Figure 1** Effects of *Lactobacillus plantarum* SC4 or *Lactobacillus plantarum* CAI6 on liver morphology in mice fed high-cholesterol diet. Tissues were stained with hematoxylin and eosin (20 × 10). A: High-cholesterol diet group; B: Normal diet group; C: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CAI6 group; D: Normal diet with *L. plantarum* SC4 group; E: High-cholesterol diet with *L. plantarum* CAI6 group; F: High-cholesterol diet with *L. plantarum* SC4 group.

compared with mice on a normal diet ( $P < 0.01$ ). In HM mice, oral administration of CAI6 or SC4 daily did not have any significant effect on BW, while CAI6-treated mice and SC4-treated mice had a significant ( $P < 0.01$  and  $P < 0.05$ , respectively) increase *vs* the NM group. In NM mice, CAI6 or SC4 had no significant effect on BW compared with NM mice receiving neither CAI6 or SC4. None of the groups showed a significant difference in total dietary intake or liver index. However, an increase in liver index in the HM group compared to the NM group was found, and CAI6 and SC4 decreased this.

#### Effect on plasma lipid profiles

Effect of the treatment on serum lipid levels was shown in Table 1. The TC, TG and LDL-C levels of mice in the HM group showed a significant increase ( $P < 0.01$ ) compared with the NM group. Meanwhile, HDL-C in the HM group decreased by 26% compared to the NM group. Moreover, a significantly increased ( $P < 0.05$ ) atherogenic index (AI) accompanied with a significantly reduced ( $P < 0.05$ ) LDL-C/HDL-C ratio was observed in the HM group. The HM mice orally administrated with strains (CAI6/SC4) showed different degrees of decline in serum TG (-19%,  $P < 0.01$ /-13%,  $P < 0.05$ ), TC (-10%/-7%), LDL-C (-17%,  $P < 0.01$ /-15%,  $P < 0.01$ ) levels, and an increase in HDL-C (+13%/+9%) level as compared to the HM group that did not receive CAI6 or SC4. As a result, those mice had a lower AI (-29%/-17%) and a higher LDL-C/HDL-C ratio (+23%/+11%) than the HM group receiving no treatment. As for the NM mice, the administration had no significant effect on serum TC, TG, HDL-C and LDL-C levels, but could reduce AI (-39%/-24%) and increase LDL-C/HDL-C ratio (+21%/+5%).

#### Hepatic morphology

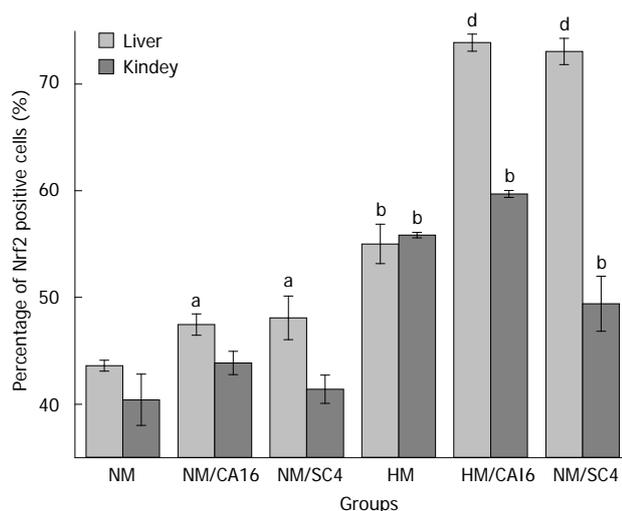
The livers of the HM group were larger compared with those in the NM group and became beige. The hepatic cells with clear cytoplasm, nucleus, nucleolus and central vein (A) in the NM, NM/CAI6 and NM/SC4 group, showed normal histology in Figure 1A-C<sup>[21]</sup>. The liver sections of the HM mice exhibited massive fatty changes and severe steatosis with cytoplasmic vacuoles confirmed by histopathological examination in Figure 1D. In addition, the size of lipid droplets in the HM/CAI6 and NM/SC4 groups were remarkably smaller than those of the HM group (Figure 1E, F), suggesting that the *L. plantarum* strains could reduce the build-up of lipid droplets and keep hepatocytes normal.

#### Effect on Nrf2 levels

Nrf2 expression in liver and kidney tissues was shown in Figure 2. Liver/kidney Nrf2 levels were significantly higher (+26%,  $P < 0.01$ /+38%,  $P < 0.01$ ) in the HM group than the NM group. Liver Nrf2 increased significantly in the HM/CAI6 (+34%,  $P < 0.01$ ) and HM/SC4 (+33%,  $P < 0.01$ ) groups respectively, compared with the HM group. A significant increase in liver Nrf2 level was also found in the NM/CAI6 (+9%,  $P < 0.05$ ) and NM/SC4 (+10%,  $P < 0.05$ ) groups as compared to the NM group. Contrary to the situation in the liver, the HM/CAI6 or HM/SC4 groups showed no significant increase in kidney Nrf2 level.

## DISCUSSION

The mice in the HM group possessed raised TG, reduced HDL-C, and elevated BW resulting in a high risk of developing metabolic syndrome. Accumulation of TG and



**Figure 2** Effect of *Lactobacillus plantarum* SC4 or *Lactobacillus plantarum* CA16 on liver and kidney nuclear factor-erythroid 2-related factor 2 expression in mice fed high-cholesterol diet. Results were expressed as mean  $\pm$  SD ( $n = 6$ ). <sup>a</sup> $P < 0.05$  vs normal diet (NM) group, <sup>b</sup> $P < 0.01$  vs NM group, <sup>d</sup> $P < 0.01$  vs high-cholesterol diet (HM) group. NM/CA16: Normal diet with *Lactobacillus plantarum* (*L. plantarum*) CA16 group; NM/SC4: Normal diet with *L. plantarum* SC4 group; HM/CA16: High-cholesterol diet with *L. plantarum* CA16 group; HM/SC4: High-cholesterol diet with *L. plantarum* SC4 group; NM: Normal diet group; HM: High-cholesterol diet group.

TC in the bloodstream has been proven related to atherosclerosis, and by extension, the risk of coronary heart disease and stroke. Elevated concentrations of oxidized LDL-C are associated with atherosclerotic plaque formation on the walls of arteries, but increased HDL-C levels may reduce the risk due to the ability of HDL transporting cholesterol back to the liver for excretion or to other tissues<sup>[22]</sup>. Therefore, the HM mice had an increased risk of CVD. The results showed that both AI, an indicator for severity of atherosclerosis<sup>[23]</sup>, and LDL-C/HDL-C, a valuable tool to evaluate coronary heart disease (CHD) risk<sup>[24]</sup>, were increased in HM mice, which is in agreement with the above view.

Serum TG, TC and LDL-C levels decreased in the HM/CA16 and HM/SC4 groups when compared with the HM group while serum HDL-C level increased revealing that *L. plantarum* CA16 and *L. plantarum* SC4 exerted a hypolipidemic effect and could relieve lipid related metabolic syndrome. AI and LDL-C/HDL-C ratios were also lower in the HM/CA16 and HM/SC4 groups, suggesting that *L. plantarum* CA16 and *L. plantarum* SC4 reduced the risk of CVD. Non-HDL-C, representing the total amount of cholesterol in the potentially atherogenic lipoproteins, was regarded as a risk predictor of cardiovascular outcomes on drug treatment<sup>[25]</sup>. After strains (CA16/SC4) treatment, non-HDL-C decreased by 22% (1.80 vs 2.30) and 15% (1.96 vs 2.30) respectively. Additionally, NM mice treated with strains (CA16/SC4) had positive changes in AI (-39%/-24%) and LDL-C/HDL-C ratio (-16%/-4%). So, we suppose that the strains have preventive and therapeutic effects for CVD.

The liver is the hub of fat synthesis and transporta-

tion. The liver can use fatty acids hydrolyzed from fat in food to synthesize cholesterol and TG. High fat intake of HM mice disequibrated lipid metabolism, resulting in accumulation of TG in liver and an increased increment of the liver index, and hepatic steatosis occurred<sup>[26]</sup>. The result of liver tectology proved that the *L. plantarum* strains had important potential in alleviating hepatic steatosis attributed to mediation of lipid metabolism and had protective effects on hepatic structure.

Oxidative stress is thought to be linked to atherogenesis<sup>[27]</sup>. Therefore, much attention has been paid to the antioxidant hypothesis in prevention and treatment of CVD<sup>[28,29]</sup>. In recent times, antioxidant activity of many hypolipidemic substances has been detected and the activities of SOD, CAT and GSH-Px are usually used as test standards<sup>[15,30]</sup>. To suffer ROS-mediated injury, cells initiate the Nrf2/ARE signaling pathway stimulated by Nrf2. Giovanni. E reported that the pathway plays an important role in vascular homeostasis and the defense of endothelial and smooth muscle cells against sustained oxidative stress associated with diseases such as atherosclerosis and preeclampsia<sup>[31]</sup>. Our results showed that liver and kidney Nrf2 expression was significantly ( $P < 0.01$ ) increased in the HM group compared with the NM group, revealing that a cholesterol-rich diet may bring about remarkable modifications in antioxidant defense mechanisms *in vivo*. Furthermore, oral administration of *L. plantarum* CA16 and *L. plantarum* SC4 both could significantly ( $P < 0.01$ ) increase liver Nrf2 levels compared with the HM group. So we envisage that spontaneous and strain-stimulated complex mechanisms conjointly promote Nrf2 expression in the liver. Accumulation of Nrf2 in the liver may initiate anti-oxidative stress and result in inhibition of Ox-LDL production, reducing the risk of atherosclerosis. Combining all the analyses above, the anti-CVD mechanism of the strains may be a regulation of lipid metabolism to alleviate metabolic syndrome and a stimulation of Nrf2 expression to initiate antioxidant defense. The actual mechanisms which account for strain-stimulated Nrf2 expression and the transcription and expression of antioxidase genes in Nrf2/ARE pathway need further study.

Dietary management has been shown to be an effective way to alleviate metabolic syndrome<sup>[32,33]</sup>. Some LAB-fermented probiotic products are commercially available<sup>[34]</sup>. Jeun *et al*<sup>[11]</sup> found that live *L. plantarum* was more effective than dead *L. plantarum* in reduction of plasma lipid levels and supposed that the active compounds may be derived from metabolic activity of live *L. plantarum*. Adding fermented foods seems like a good way to increase the number of live bacteria in a diet. LAB-fermented food, including both live LAB and active metabolin, seems the best form to apply LAB in our daily lives. The strains *L. plantarum* CA16 and *L. plantarum* SC4, which can be regarded as probiotics for their beneficial impacts, were isolated from milk and pickle, respectively. Therefore, *L. plantarum* CA16-fermented milk or *L. plantarum* SC4-fermented pickle may also exert those effects.

We suppose that the strains-fermented food may be used as a dietary approach to alleviate metabolic syndrome and prevent CVD.

## COMMENTS

### Background

Hyperlipidemia, an integral part of metabolic syndrome, is a major risk factor for cardiovascular diseases (CVD). Oxidation of low-density lipoprotein (LDL) cholesterol is linked to atherogenesis, and antioxidant drugs become a therapeutic approach. *Lactobacillus* regarded as probiotics have been proven to possess hypolipidemic and antioxidant effects. A probiotic diet, a kind of dietary management, for example fermented food, is a promising way to prevent and treat CVD.

### Research frontiers

CVD remains a major public health problem. The relationship between cardiovascular risk and serum lipid and related factors has been extensively studied to find valuable predictors of cardiovascular risk. Oxidative modification of LDL plays an important role in the genesis of arteriosclerosis. Based on the antioxidant hypothesis that suboptimal levels of principal antioxidant micronutrients are hitherto underrated risk factors for CVD, much research has focussed on the effects of antioxidant substances on CVD. Some *Lactobacillus* strains are probiotics, so novel *Lactobacillus* strains potentially used as probiotics have been continuously selected, and their applications have been investigated. Dietary approaches to alleviate metabolic syndrome have been shown in many studies.

### Innovations and breakthroughs

Recent research has highlighted the antioxidant hypothesis in prevention and treatment of CVD and demonstrated the antioxidant ability of *Lactobacillus* strains. However, the antioxidative mechanism of *Lactobacillus in vivo* has not been known. In the study, the authors investigated the effects of *Lactobacillus plantarum* (*L. plantarum*) CA16 and *L. plantarum* SC4 on nuclear factor-erythroid 2-related factor 2 (Nrf2) expression in hyperlipidemic mice. This is the first study to report the mechanism of intracorporal antioxidation of *Lactobacillus* strains may be the stimulation of Nrf2 expression. Furthermore, these studies suggest that *L. plantarum* CA16 and *L. plantarum* SC4 could reduce the risk of CVD by a hypolipidemic effect and Nrf2-induced antioxidative defense.

### Applications

As *L. plantarum* CA16 and *L. plantarum* SC4 have shown obviously protective effects on hyperlipidemic mice, food fermented by the strains may be used as dietary management to alleviate disorders of lipid metabolism related metabolic syndrome and to reduce the risk of CVD.

### Terminology

Metabolic syndrome is a combination of medical disorders, such as disturbances of glucose and lipid homeostasis, which increase the risk of developing CVD. Coronary heart disease, a type of CVD, is a narrowing or blockage of the coronary arteries, usually caused by atherosclerosis in a condition where an artery wall thickens as a result of the accumulation of fatty materials. Nrf2 is a transcription factor that regulates the expression of anti-oxidative stress enzyme genes. Stimulating the Nrf2 related signal pathway has been studied for treating diseases caused by oxidative stress.

### Peer review

This manuscript is worth publishing.

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P- Reviewer Verran DJ S- Editor Song XX  
L- Editor O'Neill M E- Editor Lu YJ



## Small undifferentiated intramucosal gastric cancer with lymph-node metastasis: Case report

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Received: December 25, 2012 Revised: March 25, 2013

Accepted: April 3, 2013

Published online: May 28, 2013

### Abstract

It has been reported recently that small undifferentiated intramucosal early gastric cancer (EGC) < 20 mm in size without any lymphovascular involvement or ulcerative findings had virtually no risk of lymph-node (LN) metastasis. Consequently, the indications for endoscopic resection were expanded to include such undifferentiated EGC lesions. We describe herein a case of a small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings that involved lymph-node metastasis. A 57-year-old female underwent pylorus preserving gastrectomy as standard treatment for an undifferentiated EGC 15 mm in size without any ulcerative finding. The surgical specimen revealed a signet-ring cell carcinoma with a moderately to poorly differentiated adenocarcinoma limited to the mucosa that was 15 mm in size with no lymphovascular involvement or ulcerative findings. This case involved LN

metastasis, however, and the lesion was diagnosed as pathological stage II A (T1N2M0) according to the Japanese Classification of Gastric Carcinoma.

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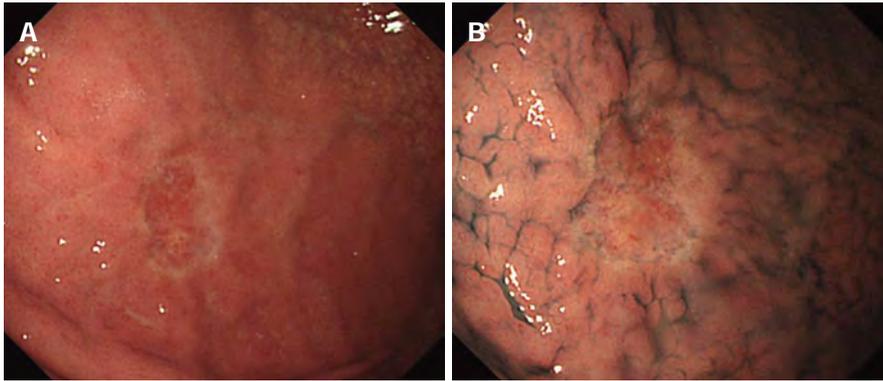
**Key words:** Early gastric cancer; Endoscopic submucosal dissection; Expanded indications; Lymph-node metastasis; Undifferentiated type

**Core tip:** Herein is described rare case of small undifferentiated intramucosal early gastric cancer (EGC) < 20 mm in size with lymph-node (LN) metastasis. It has been reported recently that small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings had virtually no risk of LN metastasis. Therefore, such small undifferentiated EGCs have become candidates for endoscopic resection. It was within this context we experienced the present case involving a small undifferentiated intramucosal EGC < 20 mm in size without lymphovascular involvement or ulcerative findings that evidenced LN metastasis.

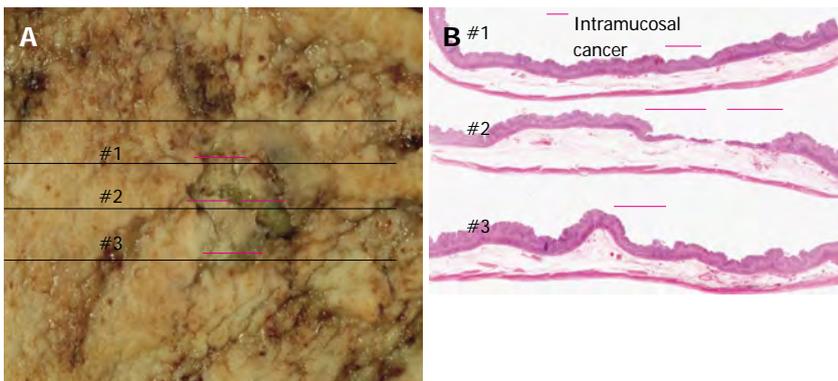
Odagaki T, Suzuki H, Oda I, Yoshinaga S, Nonaka S, Katai H, Taniguchi H, Kushima R, Saito Y. Small undifferentiated intramucosal gastric cancer with lymph-node metastasis: Case report. *World J Gastroenterol* 2013; 19(20): 3157-3160 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3157.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3157>

### INTRODUCTION

Endoscopic resection (ER) is generally indicated for early gastric cancer (EGC) with no risk of lymph-node (LN) metastasis. Hirasawa *et al*<sup>[1]</sup> reported there was virtually no risk of LN metastasis in undifferentiated EGCs < 20 mm



**Figure 1** Pre-treatment endoscopic examination. Esophagogastroduodenoscopy revealed pale depressed mucosal lesion on anterior wall of middle gastric body approximately 15 mm in size with no ulcerative finding and non-atrophic background mucosa. A: Conventional white light endoscopy; B: With indigo-carmine dye staining.



**Figure 2** Surgically resected specimen. A: Formalin-fixed specimen from pylorus preserving gastrectomy; B: Panoramic view of lesion with hematoxylin and eosin staining. Mucosal lesion 15 mm × 12 mm in size with no ulcer finding and pink lines corresponding to lesion depression (hematoxylin and eosin staining, × 1).

in size without lymphovascular involvement or ulcerative findings and such lesions could satisfy the indications for ER. We encountered, however, a case of a small undifferentiated intramucosal EGC meeting all of the above-mentioned criteria that actually involved LN metastasis and decided to report this seemingly rare occurrence.

## CASE REPORT

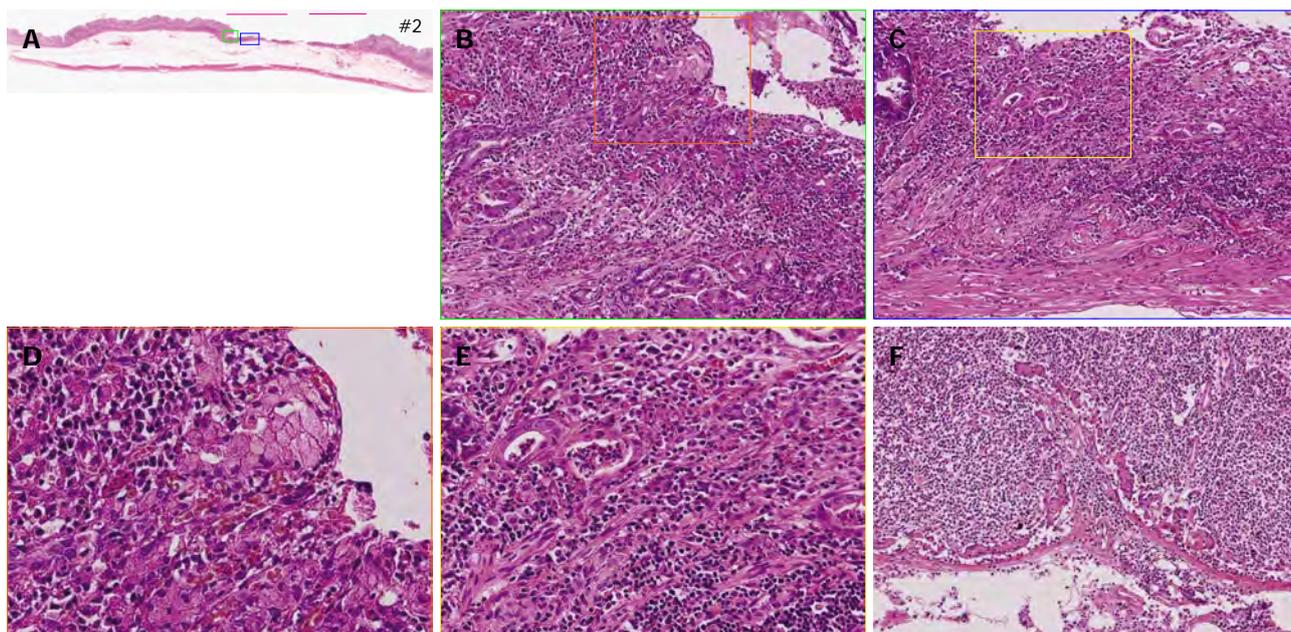
A 57-year-old female underwent an esophagogastroduodenoscopy (EGD) for a medical check-up in December 2008. The results indicated gastric cancer, and she was referred to our hospital for treatment. The EGD revealed a pale depressed mucosal lesion that was Type 0-IIc in accordance with the Japanese Classification of Gastric Carcinoma (JCGC)<sup>[2]</sup> located on the anterior wall of the middle gastric body (Figure 1). The size of the lesion was approximately 15 mm, and there was no ulcerative finding. The biopsy specimen revealed a signet-ring cell carcinoma. Computed tomography (CT) indicated no distinct regional LN or distant metastasis. Laboratory results were within normal limits and the serum carcinoembryonic antigen level was normal. We diagnosed this lesion as clinical (c) T1(M), cN0, cM0, cStage I A according to the JCGC; therefore, pylorus preserving gastrectomy was performed as the standard treatment. The surgical specimen revealed a signet-ring cell carcinoma with a moderately to poorly differentiated adenocarcinoma limited to the mucosa without lymphovascular involvement or ulcerative findings (Figures 2 and 3A-E). The lesion was 15 mm × 12 mm in size, and the resection margin was free of tumor cells. In addition, tumor cells were posi-

tive for mucin (MUC)5AC and negative for MUC6 with an MUC5AC/MUC6 double layer and Ki-67 localization both absent (Figure 4). Some portions of the resected regional LNs (lesser curvature LNs; LN station 3), however, included metastatic cancer cells (3/32) that were also identified as a signet-ring cell carcinoma (Figure 3F). This lesion was then diagnosed as pathological (p)T1(M), ly0, v0, pN2, pM0, pStage II A according to the JCGC. Four years after surgery, the patient was in good condition and disease free without any signs of tumor recurrence based on the latest follow-up EGD and CT examinations.

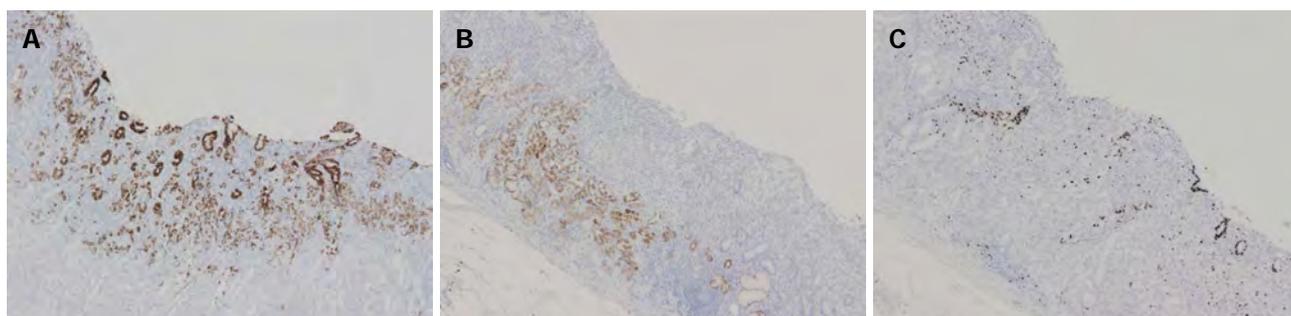
## DISCUSSION

ER is widely accepted as a minimally invasive treatment for intramucosal EGC with virtually no risk of LN metastasis<sup>[2-6]</sup>. An earlier report, however, demonstrated undifferentiated intramucosal EGC had a higher probability of LN metastasis (4.2%)<sup>[3]</sup>, and gastrectomy with regional lymphadenectomy has been considered essential treatment for such lesions. More recently, Hirasawa *et al*<sup>[1]</sup> reported small undifferentiated intramucosal EGCs < 20 mm in size without lymphovascular involvement or ulcerative findings had virtually no risk of LN metastasis (95%CI, up to 0.96%). Therefore, such small undifferentiated EGCs have become candidates for ER. It was within this context we experienced the present case involving a small undifferentiated intramucosal EGC < 20 mm in size without any lymphovascular involvement or ulcerative findings that evidenced LN metastasis.

Both Nasu *et al*<sup>[7]</sup> and Park *et al*<sup>[8]</sup> previously reported similar cases of a small undifferentiated intramucosal



**Figure 3 Histopathological findings.** A: Panoramic view with hematoxylin and eosin staining ( $\times 1$ ); B: Signet-ring cell carcinoma identified at lesion edge (green frame in A,  $\times 40$ ); C: Moderately to poorly differentiated adenocarcinoma limited to mucosa visible in lesion center. Low magnification view (blue frame in A,  $\times 40$ ); D: High magnification view of red frame in B ( $\times 200$ ); E: High magnification view of yellow frame in C ( $\times 200$ ); F: Image showing signet-ring cell carcinoma invasion of station 3 lymph node (N2) ( $\times 40$ ).



**Figure 4 Immunohistochemical staining.** Tumor cells positive for mucin (MUC)5AC (A,  $\times 10$ ) and negative for MUC6 with an MUC5AC/MUC6 double layer (B,  $\times 4$ ) and Ki-67 (C,  $\times 4$ ) localization both absent.

EGC  $< 20$  mm in size (13 and 17mm, respectively) without lymphovascular involvement or findings of ulceration that involved LN metastasis. In addition, Abe *et al*<sup>[9]</sup> reported three cases involving LN metastasis of small undifferentiated intramucosal EGCs  $< 20$  mm in size (10, 12 and 20 mm, respectively) without any ulcerative findings and concluded ER should not be indicated for undifferentiated EGCs  $> 10$  mm in size. There was no reference in those three cases, however, regarding the existence of lymphovascular involvement. Hirasawa *et al*<sup>[10]</sup> also reported a case with LN metastasis of a small undifferentiated intramucosal EGC that was 13 mm in size and without an ulcerative finding. According to their case report, routine histological examination of the endoscopic submucosal dissection specimen sectioned at intervals of 2 mm indicated an intramucosal tumor without lymphovascular involvement or ulceration. Hematoxylin and eosin staining of one of the 60 additional deep-cut sections of the original resected specimen, however, revealed lymphatic

involvement in the mucosa. The authors then suggested practical limitations in determining lymphovascular involvement through routine histological examinations may not always facilitate the detection of LN metastasis.

Based on the various published reports supportive of the findings in our particular case, the possibility of LN metastasis developing from a small undifferentiated intramucosal EGC  $< 20$  mm in size without ulceration exists even if there is no apparent lymphovascular involvement<sup>[7,8]</sup>. It is highly desirable, therefore, to clarify the predictive factors for the development of LN metastasis with such small undifferentiated intramucosal EGCs. Takizawa *et al*<sup>[11]</sup> evaluated the conditions in which LN metastasis was unlikely and ER of undifferentiated EGCs had a greater probability of being effective. Such conditions included intramucosal cancers without lymphovascular involvement or ulcerative findings and: (1) lesions  $< 10$  mm in size; (2) the presence of Ki-67 localization; (3) the presence of a double layer (MUC5AC/

MUC6); and/or (4) the presence of only a signet-ring cell carcinoma. The conditions in our case included a lesion > 10 mm in size, the absence of Ki-67 localization, the absence of a double layer (MUC5AC/MUC6), and the presence of not only a signet-ring cell carcinoma but also a moderately and poorly differentiated adenocarcinoma. In addition, more recently, Takizawa *et al.*<sup>[12]</sup> reported that LN metastasis was significantly more common in mixed predominantly undifferentiated (MU)-type intramucosal cancer than in pure undifferentiated (PU)-type intramucosal cancer and concluded MU-type tumors might have greater malignant potential than PU-type tumors. Actually, the present case was MU-type intramucosal cancer with lymph-node metastasis.

If it were possible to evaluate the pathology of all resected specimens in detail using deep-cut sections and immunohistochemical staining as indicated in the above-mentioned reports<sup>[10-12]</sup>, we might be able to predict those cases in which LN metastasis of small undifferentiated intramucosal EGCs would most likely occur, but it is not always feasible to perform such extensive pathological evaluations in clinical practice. We need to recognize the distinct possibility, therefore, of LN metastasis developing from small undifferentiated intramucosal EGCs < 20 mm in size without lymphovascular involvement or ulcerative findings because of the practical limitations related to such histological examinations<sup>[10]</sup>. It is, therefore, imperative that physicians thoroughly explain the possibility of LN metastasis to patients before performing ER for small undifferentiated intramucosal EGCs so as to obtain their informed consent and then conduct more careful follow-up surveillance examinations including CT scans, ultrasonography and/or EUS in addition to EGDs subsequent to ER treatment.

In order to satisfactorily demonstrate the validity of treating small undifferentiated intramucosal EGCs with ER, a large prospective study analyzing therapeutic outcomes will need to be conducted in the near future before the recently expanded indications for treating such lesions with ER should be accepted for general clinical use.

## ACKNOWLEDGMENTS

The authors wish to express their appreciation to Christopher Dix for his assistance in editing this manuscript.

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P- Reviewer Hoepfner J

S- Editor Zhai HH L- Editor A E- Editor Lu YJ



## Intraductal papillary neoplasm of the bile duct accompanying biliary mixed adenoneuroendocrine carcinoma

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Received: December 24, 2012 Revised: March 13, 2013

Accepted: April 3, 2013

Published online: May 28, 2013

per gastrointestinal endoscopy revealed mucus production from the papilla of Vater, characterized by its protruding and dilated orifice. Endoscopic ultrasonography visualized the polypoid tumor in the distal bile duct, but no invasive region was suggested by diagnostic imaging. Therefore, the initial diagnosis was IPNB. After endoscopic nasobiliary drainage, a pylorus-preserving pancreaticoduodenectomy was performed. Pathological examination of the resected bile duct revealed papillary proliferation of biliary-type cells with nuclear atypia, indicating pancreaticobiliary-type IPNB. In addition, solid portions comprised of tumor cells with characteristic salt-and-pepper nuclei were evident. Immunohistochemistry revealed expression of the neuroendocrine marker synaptophysin in this solid component, diagnosing it as a neuroendocrine tumor (NET). Furthermore, the MIB-1 proliferation index of NET was higher than that of IPNB, and microinvasion of the NET component was found, indicating neuroendocrine carcinoma (NET G3). This unique case of MANEC, comprising IPNB and NET, provides insight into the pathogenesis of biliary NET.

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**Key words:** Neuroendocrine tumor; Intraductal papillary neoplasm of bile duct; Intraductal papillary neoplasm of the bile duct; Bile duct

### Abstract

We present the first case of an intraductal papillary neoplasm of the bile duct (IPNB) accompanying a mixed adenoneuroendocrine carcinoma (MANEC). A 74-year-old woman presented with fever of unknown cause. Laboratory data revealed jaundice and liver injury. Contrast-enhanced computed tomography revealed a 20 mm polypoid tumor in the dilated distal bile duct, which exhibited early enhancement and papillary growth. Up-

**Core tip:** A 74-year-old woman presented with fever and jaundice. Computed tomography revealed a polypoid tumor in the dilated distal bile duct. Pylorus-preserving pancreaticoduodenectomy was performed. Pathological examination revealed the papillary proliferation of biliary-type cells with nuclear atypia in the dilated bile duct, indicating papillary neoplasm of the bile duct. A solid portion comprised of tumor cells with

characteristic salt-and-pepper nuclei was found. Immunohistochemistry revealed synaptophysin expression in the solid portion, diagnosing it as a neuroendocrine tumor (NET). This case provides insight into the pathogenesis of biliary NET.

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## INTRODUCTION

Intraductal papillary neoplasm of the bile duct (IPNB) is a new entity, defined as biliary neoplasms showing papillary or villous proliferation within the dilated lumens of intrahepatic and extrahepatic bile ducts by the 2010 World Health Organization (WHO) Classification of Tumors of the Digestive System<sup>[1]</sup>. IPNB encompasses several lesions, which were previously categorized as biliary papilloma, papillomatosis, papillary adenocarcinoma of the bile duct, and intraductal growth-type cholangiocarcinoma. The following three key pathologic features are characteristically evident in IPNB in varying combinations: (1) exophytic and papillary proliferation of neoplastic biliary epithelial cells, with delicate fibrovascular stalks within bile duct lumens; (2) mucin hypersecretion (macroscopic mucin is evident in some cases); and (3) variable dilatation or multilocular cystic changes of affected bile ducts.

Neuroendocrine tumors (NETs), including carcinoid tumors, are commonly found in several organs, including the pancreas and gastrointestinal tract. Most biliary NETs exist as a component of mixed adenoneuroendocrine carcinomas (MANECs)<sup>[2]</sup>. MANECs are found in hepatic hilar cholangiocarcinomas with hepatolithiasis, gallbladder cancers, and extrahepatic cholangiocarcinomas, and show a characteristic histology<sup>[3]</sup>. Moreover, since the NET components of biliary MANECs define prognosis, it is important to identify them and consider indications for adjunctive therapy, such as somatostatin analogues.

We encountered a patient with IPNB accompanying a NET in the extrahepatic bile ducts. This case is unique, has a different histology from most biliary MANECs, and provides insight into the histogenesis of NETs in biliary tumors.

## CASE REPORT

### Clinical course

A 74-year-old woman consulted a family physician for fever of unknown cause. Laboratory data revealed jaundice

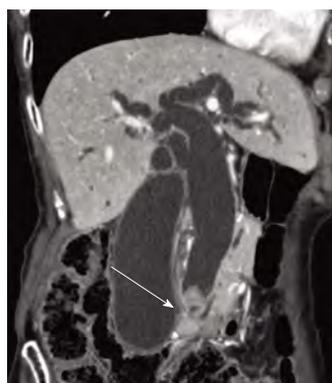
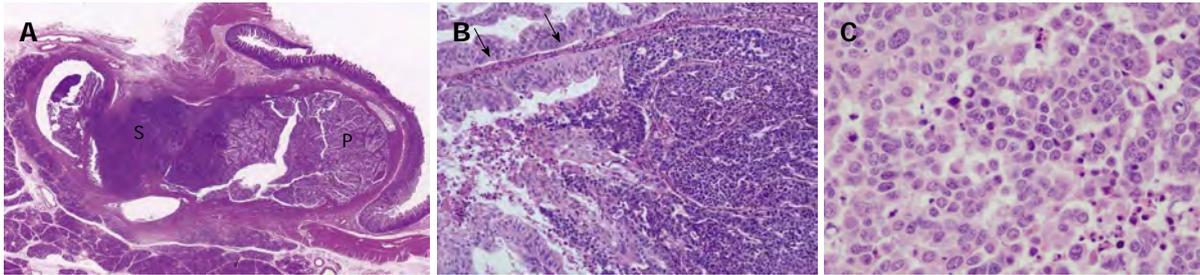


Figure 1 Computed tomography of the bile duct. Computed tomography revealed dilatation of the bile duct and an elevated lesion (arrow) at the bottom of the lower bile duct.

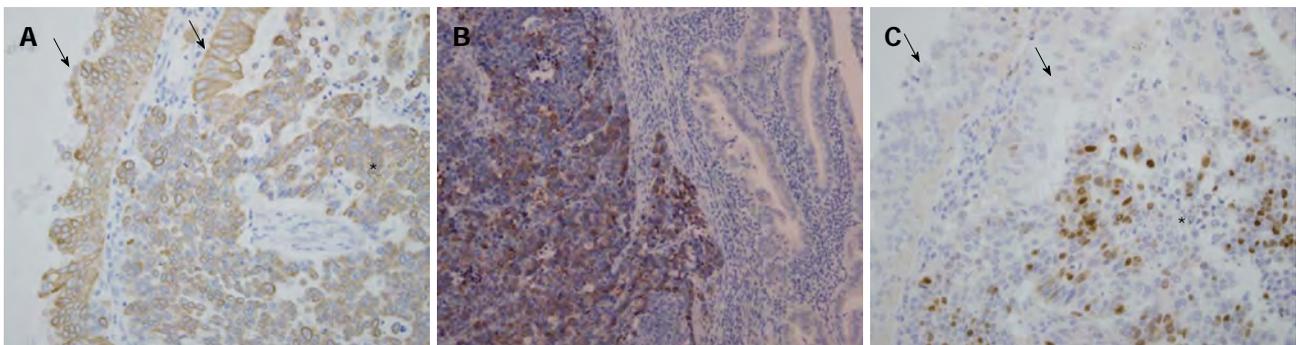
and liver injury. Computed tomography (CT) revealed an elevated lesion in the distal bile duct. She was admitted to our hospital for further examination and treatment. Enhanced CT revealed a 20 mm polypoid tumor, which exhibited early enhancement and papillary growth (Figure 1). Magnetic resonance image (MRI) and magnetic resonance cholangiopancreatography (MRCP) confirmed these findings. Mucus production was evident from the papilla of Vater, characterized by its protruding and dilated orifice. Endoscopic ultrasonography revealed a polypoid tumor in the distal bile duct. No invasive tumor regions were detected using these imaging techniques. Therefore, the initial diagnosis was IPNB. After endoscopic nasobiliary drainage (ENBD), a pylorus-preserving pancreaticoduodenectomy was performed. The postoperative course was uneventful, except for slight pneumonia.

### Pathology of the resected bile ducts

A whitish tumor occupying the dilated lumen of the distal bile duct was found, and the proximal portion of the bile duct was also dilated. As shown in Figure 2A, the tumor was comprised of two different areas: papillary and solid. The papillary proliferating area consisted of cholangiocyte-like columnar epithelial cells covering fine fibrovascular cores, and showed moderate-to high-grade intraepithelial neoplasia, indicating pancreaticobiliary-type IPNB (high-grade intraepithelial neoplasia) according to the WHO classification<sup>[1]</sup> (Figure 2B). Immunohistochemistry revealed expression of the biliary-type cytokeratin (CK): CK19 (Figure 3A) in these cells, but not CK20, confirming biliary-type IPNB. Conversely, the solid area lacking acinar/glandular structure (Figure 2A and B) was comprised of tumor cells with salt-and-pepper nuclei, a high nucleus-to-cytoplasm ratio, and increased nuclear chromatin (Figure 2C). Immunohistochemistry for neuroendocrine markers revealed synaptophysin expression (Figure 3B), but not chromogranin A and CD56 expression, indicating a NET component of MANEC. Furthermore, NET, as well as IPNB components, expressed CK19 (Figure 3A). The MIB-1 proliferation index of the NET component was significantly higher than that of the IPNB component (Figure 3C). Focal invasion of NET and IPNB components into the ductal wall was evident, but no pancreatic or lymph node metastasis was observed.



**Figure 2** Pathological findings from the resected bile duct (hematoxylin eosin staining). A: A semi macro cross-sectional image of the resected extrahepatic bile duct. In the dilated bile duct, the tumor was comprised of two distinct parts: papillary (P) and solid (S); B: The boundary between the papillary (right) and solid (left) areas. The papillary area consisted of a papillary proliferation of cholangiocyte-like columnar epithelial cells covering fine fibrovascular cores (arrows). In the solid area, a lacunar tumor was evident, but lacked distinct acinar/glandular structures. Magnification:  $\times 100$ ; C: The solid tumor at higher magnification. Tumor cells exhibited characteristic salt-and-pepper nuclei, a high nucleus-to-cytoplasm ratio, and increased nuclear chromatin. Magnification:  $\times 400$ .



**Figure 3** Immunohistochemistry for cytokeratin 19, synaptophysin, and Ki-67. A: Both the solid (asterisk) and papillary (arrows) components were positive for CK19. Magnification:  $\times 200$ ; B: Synaptophysin expression was evident in the solid component (left), indicating a neuroendocrine tumor, but not in the papillary component (right). Magnification:  $\times 100$ ; C: Although Ki-67-positive cells were scarce in the papillary component (arrows), many Ki-67-positive cells were identified in the solid component (asterisk). Magnification:  $\times 200$ .

## DISCUSSION

IPNB is characterized by a grossly visible, exophytic proliferation of neoplastic cholangiocytes with delicate fibrovascular cores. The WHO classification of digestive tumors<sup>[1]</sup> recognizes IPNB as a precancerous entity of cholangiocarcinoma. Surgical resection is the most optimal treatment for IPNB because mucin production by these tumors causes recurrent cholangitis and obstructive jaundice, even if these tumors are considered benign. Incomplete surgical resection often causes tumor recurrence; furthermore, recurrence in the remaining bile ducts can develop after apparently complete resection of even noninvasive tumors because of tumor multifocality<sup>[4,5]</sup>.

Radiographic imaging modalities, including CT, MRI, and MRCP, are recommended noninvasive techniques for detecting masses and evaluating the tumor extent. Although cholangiography and cholangioscopy carry risks of complications, these diagnostic techniques are useful for evaluating tumor extent, operative planning, and biliary intervention<sup>[6,7]</sup>. In this case, it was not difficult to evaluate the degree of tumor invasion using noninvasive radiographic techniques. However, pathological confirmation could not be obtained via biopsies or cytology. Peroral cholangioscopy and intraductal ultrasonography

may be necessary for pathological diagnosis. Unfortunately, we were unable to perform these invasive radiographies due to a lack of consent from the patient, who was suffering from pancreatitis after ENBD.

The degree of malignancy and histological atypia of IPNB ranges from borderline or low to moderate in the intraluminal papillary portions, to highly malignant and atypical in the invasive portions. IPNBs without invasive components are comprised of two subgroups: low-grade intraepithelial neoplasia and high-grade intraepithelial neoplasia. Moreover, the neoplastic cells of IPNB are cytologically classifiable into four phenotypes: pancreaticobiliary, intestinal, gastric (clear cell type), and oncocytic types<sup>[1]</sup>. This case was IPNB with associated pancreaticobiliary-type invasive carcinoma.

NETs in the digestive system, including in the gallbladder and extrahepatic bile ducts, are classified as: NET G1 [carcinoid; mitotic count:  $< 2$  per 10 high-power fields (HPF); and/or  $\leq 2\%$  Ki-67 index]; NET G2 (mitotic count: 2-20 per 10 HPF; and/or 3%-20% Ki-67 index); neuroendocrine carcinoma (large- or small-cell type); and MANEC, according to the 2010 WHO classification<sup>[2,8]</sup>. In biliary MANEC, the adenocarcinomatous component is located on the surface of the main tumor, and the majority of stromal invasion, including vascular invasion and lymph node metastasis, involves the NET

component<sup>[3]</sup>. Therefore, the NET component of biliary MANEC is considered to define prognosis<sup>[3]</sup>. In this case, contrary to typical biliary MANEC, the adenocarcinomatous component of MANEC was identified as IPNB. NET adjacent to the IPNB component resulted in both being intermingled in one mass (Figure 2B). Moreover, both NET and IPNB were immunophenotypically positive for CK19, suggesting that NET originated from the transformation of biliary tumor cells constituting IPNB. However, the NET component exhibited extensive proliferation and broad stromal invasion of the bile duct wall compared with IPNB, suggesting that the NET component defined the prognosis in this case.

In the pancreas, concomitant intraductal papillary mucinous neoplasm (IPMN; a pancreatic counterpart of IPNB) and pancreatic neuroendocrine tumor (pNET) are frequent, and the prevalence of pNET with IPMN was 2.8%-4.6% according to previous reports<sup>[9,10]</sup>. However, IPNB accompanying NET has not been previously reported. This case provides important insight into the histogenesis of biliary NET.

In summary, IPNB accompanying biliary MANEC in this patient provided important information for the elucidation of the histogenesis of NET in biliary tumors.

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**P- Reviewer** Folsch UR **S- Editor** Zhai HH  
**L- Editor** Rutherford A **E- Editor** Lu YJ



## Behçet's disease complicated by multiple aseptic abscesses of the liver and spleen

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Received: October 18, 2012 Revised: March 1, 2013

Accepted: March 8, 2013

Published online: May 28, 2013

### Abstract

Aseptic abscesses are an emergent entity and have been described in inflammatory bowel disease, especially in Crohn's disease, and in other diseases. However, aseptic abscesses associated with Behçet's disease are extremely rare. We report a Japanese male diagnosed with an incomplete type of Behçet's disease who developed multiple aseptic abscesses of the spleen and liver. In 2002, the spleen abscesses were accompanied by paroxysmal oral aphthous ulcers and erythema nodosum. As the patient's response to antibiotic treatment was inadequate, a splenectomy was performed. Severe inflammatory cell infiltration, largely of polymorphonuclear neutrophils, was observed without evidence of bacterial or fungal growth. Although the patient had no history of ocular symptoms or genital ulcers, a diagnosis of incomplete Behçet's disease was made according to the Japanese diagnostic criteria because of the presence of paroxysmal arthritis and epididymitis since 2002. In 2005, multiple liver abscesses developed with right hypochondrial pain and seemed to be attributed to Behçet's disease because the abscesses yielded negative results during a microbiologic investigation

and failed to go into remission under antibiotic therapy. Oral prednisone (15 mg/d) was started in May 2006, and the abscesses dramatically disappeared 4 wk after treatment. Although the patient had a relapse of the liver abscesses in association with the tapering of prednisone, the augmentation of prednisone dosage yielded a response. The abscesses of the liver and spleen were strongly suggested to be attributed to Behçet's disease. Clinician should be aware of the existence of aseptic abscesses as uncommon manifestations of Behçet's disease.

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**Key words:** Behçet's disease; Aseptic abscess; Spleen; Liver; Prednisone

**Core tip:** We report a Japanese male diagnosed with an incomplete type of Behçet's disease who developed multiple aseptic abscesses of the spleen and liver. Spleen abscesses developed with paroxysmal oral aphthous ulcers and erythema nodosum. A splenectomy was performed, and severe neutrophil infiltration was observed without evidence of bacterial or fungal growth. Multiple liver abscesses also developed with right hypochondrial pain and seemed to be attributed to Behçet's disease because the abscesses yielded negative results during a microbiologic investigation. Oral prednisone (15 mg/d) was started, and the abscesses dramatically disappeared. Clinicians should be aware of the existence of aseptic abscesses as uncommon manifestations of Behçet's disease.

Maeshima K, Ishii K, Inoue M, Himeno K, Seike M. Behçet's disease complicated by multiple aseptic abscesses of the liver and spleen. *World J Gastroenterol* 2013; 19(20): 3165-3168 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3165.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3165>

## INTRODUCTION

Behçet's disease (BD) is a systemic inflammatory disease commonly characterized by oral and genital ulcerations, with involvement of the skin and eye. The manifestations of BD are protean, and all symptoms and signs tend to recur either alone or in combination. Aseptic abscesses (AAs) are characterized by deep, sterile, round lesions consisting of neutrophil infiltration that do not respond to antibiotic therapy but improve with corticosteroid and immunosuppressive drugs. Clinical reports and case series concerning AAs in patients with inflammatory bowel disease, neutrophilic dermatoses, and other diseases have been published<sup>[1-5]</sup>. Although BD belongs to a group of neutrophilic dermatoses, AAs associated with BD are extremely rare. Here, we describe a patient with BD complicated by AAs of the liver and spleen, which were successfully treated with corticosteroid therapy.

## CASE REPORT

In February 2006, a 20-year-old Japanese male suffering from multiple liver abscesses associated with sporadic fever, right hypochondrial pain, and elevated inflammatory markers was admitted to our hospital. In 2002, at the age of 16, he developed oral aphthous ulcers, erythema nodosum, pericardial effusion, and multiple spleen abscesses (Figure 1A). As for the aphthous ulcers and erythema nodosum, resolution occurred spontaneously. Pericardiocentesis was performed. Effusive pericarditis was thought to be the cause of the pericardial fluid; however, it did not reaccumulate after pericardiocentesis. Because empirical antibiotic therapy had the least effect and symptoms such as fever and left hypochondrial pain persisted, an open splenectomy was performed in October 2002. Upon macroscopic examination, sections of the resected spleen showed multiple yellow nodular lesions (10-20 mm in diameter) (Figure 1B). Pathological examination confirmed the presence of severe inflammatory cell infiltration, largely of polymorphonuclear neutrophils, without evidence of bacterial or fungal growth. In addition to oral aphthosis and erythema nodosum, the primary symptoms of BD, paroxysmal arthritis and epididymitis have occurred since 2002. Although the patient had no history of ocular symptoms or genital ulcers, a diagnosis of incomplete BD was made according to the criteria of the BD Research Committee of Japan<sup>[6]</sup>. Spleen abscesses were suspected to be attributed to BD, but the association was difficult to prove. Paroxysmal oral aphthosis and arthritis persisted after splenectomy, and at the age of 19 in March 2005, multiple abscesses with an aggressive inflammatory response developed in the liver. Antibiotics were unable to demonstrate any benefit, and the liver abscesses seemed to be attributed to BD. Colchicine (with a 1.5 mg maximum daily dose) was started in July 2005. However, the patient had elevated inflammatory markers, fever, and right hypochondrial pain.

Upon physical examination, the patient did not present with fever, oral aphthosis, erythema nodosum, articular swelling, or abdominal tenderness at the time. The

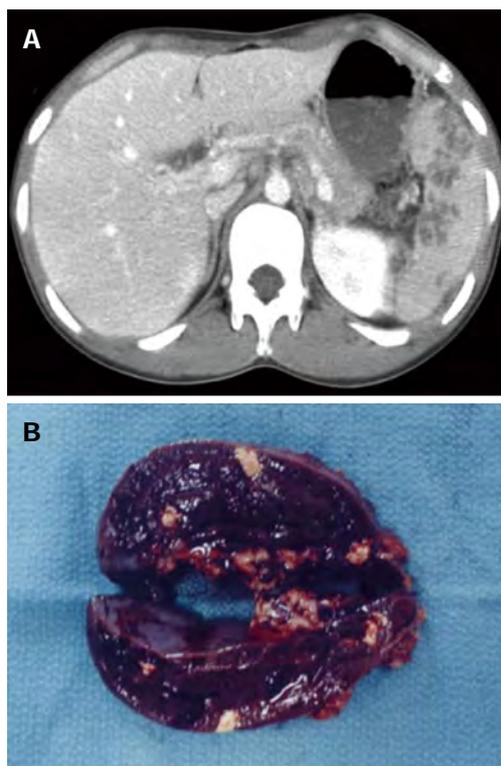
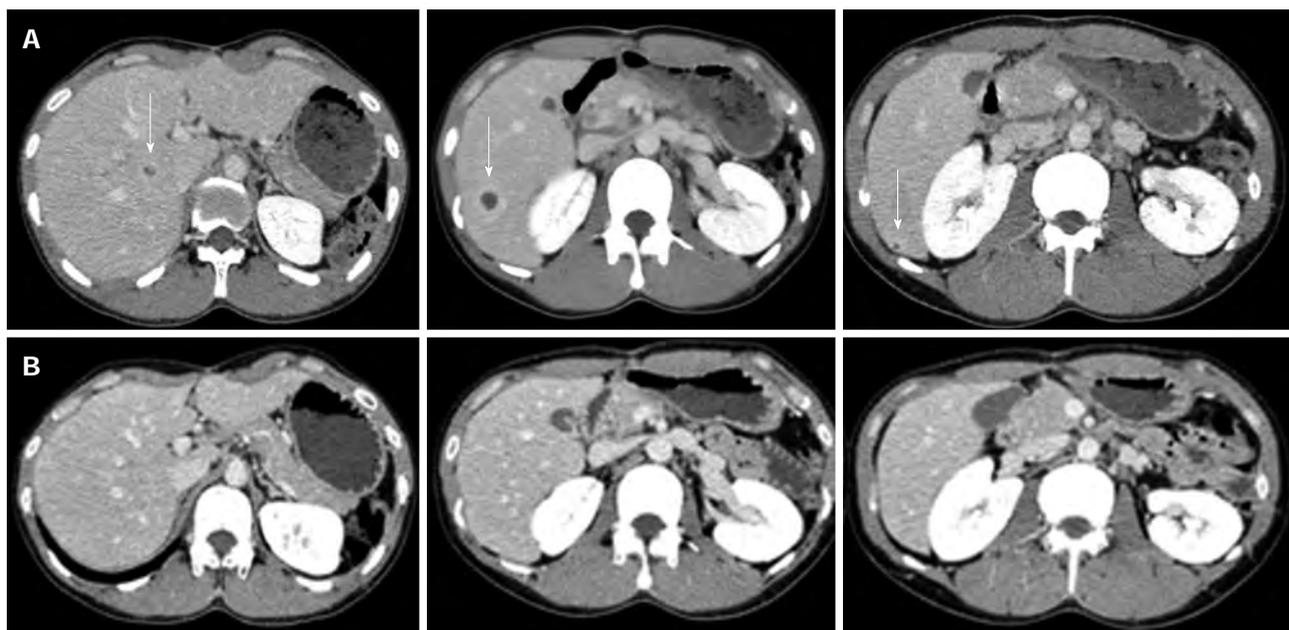


Figure 1 Contrast-enhanced abdominal computed tomography scan showing multiple spleen abscesses (A), and macroscopic findings of the cut surface of the resected spleen (B).

findings from the laboratory examinations are summarized as follows: white blood cell count, 13400/L (normal: 4000-9000/L) with 69% neutrophils; hemoglobin, 14.8 g/dL (normal: 12.0-16.0 g/dL); platelets,  $57.1 \times 10^4$ /L (normal:  $14.0-40.0 \times 10^4$ /L); aspartate aminotransferase, 21 U/L (normal: 10-40 U/L); alanine aminotransferase, 30 U/L (normal: 5-40 U/L); lactate dehydrogenase, 137 U/L (normal: 115-245 U/L); total bilirubin, 0.4 mg/dL (normal: 0.3-1.2 mg/dL); alkaline phosphatase, 583 U/L (normal: 115-359 U/L); gamma-glutamyl transferase, 123 U/L (normal:  $\leq 70$  U/L); and C-reactive protein (CRP), 11.0 mg/dL (normal:  $< 0.3$  mg/dL). The results of the kidney function tests were within the normal limits. Antinuclear antibodies (speckled staining pattern) showed a 40-fold positive result (normal:  $< 40$ -fold). Rheumatoid factor, antineutrophil cytoplasmic antibodies, and HLA-B51 antigen were negative (HLA-A24, B52, and B60 antigens were positive). Colonoscopy showed only a small aphthous ulcer in the terminal ileum; pathological examination showed non-specific chronic inflammation without any evidence of Crohn's disease (CD), such as noncaseating granuloma. Blood and urine cultures were negative. An abdominal ultrasound scan was performed, which showed multiple round lesions in the liver. Contrast-enhanced abdominal computed tomography scan demonstrated multiple areas of low-attenuation with ring enhancement in the liver (Figure 2A). Despite empirical antibiotic therapy, no clinical improvement was achieved. Ultrasound-guided percutaneous aspiration of the abscess yielded pus containing numerous neutrophils; no



**Figure 2** Contrast-enhanced abdominal computed tomography scan showing multiple liver abscesses (arrow) before (A) and 4 wk after corticosteroid therapy (B). Multiple areas of low-attenuation with ring enhancement were seen in the liver as indicated by the arrows (A). However, the areas vanished after treatment (B).

microbes were found in the culture. A fine needle biopsy of the liver lesions was performed, and a pathological examination revealed necrotic tissue containing inflammatory cells. Based on this evidence, these lesions were interpreted as aseptic liver abscesses associated with BD, and oral low-dose prednisone (15 mg/d), in addition to colchicine, was initiated in May 2006. A rapid improvement in the patient's symptoms occurred; the levels of CRP and liver enzymes reached normal range, and the liver abscesses disappeared within 4 wk of the initiation of corticosteroid therapy (Figure 2B). The dose of prednisone was gradually tapered; at 7.5 mg/d prednisone, however, the CRP level increased again, requiring a higher dose of prednisone. Therefore, the patient was maintained on low-dose prednisone therapy (10-12.5 mg/d) to restrain the inflammatory response. Elevated CRP was observed without obvious abnormality in August 2010, and the recurrence of AAs in the liver was detected by abdominal CT without abdominal symptoms in August 2011. After escalating the dose of prednisone to 20 mg/d, the liver abscesses vanished once again. The dose of prednisone was gradually tapered to 15 mg/d, and complete clearance of the liver lesions was achieved in February 2012.

## DISCUSSION

We present a patient with BD who developed AAs in the spleen and liver. AAs are an emergent entity. This condition has been described in inflammatory bowel disease (IBD), especially in CD, as well as in other diseases such as Sweet's syndrome and pyoderma gangrenosum<sup>[1-5]</sup>. Most patients with AAs have some underlying disease, and it has been proposed that AAs belong to the spectrum of

autoinflammatory multifactorial disorders<sup>[1]</sup>. The diagnosis of AAs is of exclusion on the basis of the following criteria suggested by André *et al*<sup>[1]</sup>: (1) deep abscess(es) upon radiologic examination, with neutrophilic features proven by surgical pathology or aspiration when performed; (2) negative blood cultures, negative serologic tests for bacteria, and, when surgical procedure or aspiration are performed, sterile standard, acid-fast bacillus and fungal cultures of pus; (3) failure of antibiotic therapy; and (4) rapid improvement by corticosteroids, sometimes in combination with immunosuppressive drugs. The main clinical manifestations of AAs are fever, abdominal pain, and weight loss. A rare case of BD complicated by multiple intrahepatic abscesses, which was dramatically resolved by antibiotic therapy, was reported<sup>[7]</sup>. There is also a case report of BD that was suspected to be complicated by sterile cerebral abscesses, although the clinical state of the patient gradually improved without immunosuppressive therapy<sup>[8]</sup>. In our patient, the liver and spleen abscesses were negative upon microbiologic investigation, and remission was not achieved under antibiotic therapy. Furthermore, the rapid resolution of the abscesses with corticosteroid therapy, even at the time of disease relapse in association with the tapering of prednisone, also favored a diagnosis of BD-associated AAs. Based on a PubMed search of the literature, our patient is the first unfulfilling case of sterile visceral abscesses associated with BD.

A differential diagnosis of CD *vs* BD can be difficult. Although ulcerative lesions at the ileocecal area are common in both diseases, we diagnosed the patient as having intestinal BD because of a lack of factors suggestive of CD: there was only one ulcerative lesion, its shape was not longitudinal, and histopathological examination showed no granulomatous lesions. It is also often difficult to dif-

ferentiate incomplete types of BD from Sweet's syndrome because some overlapping manifestations exist between BD and Sweet's disease. Unfortunately, a skin biopsy was not performed. However, we are convinced of the diagnosis of incomplete BD in this case because our patient had a chronic course with remissions and relapses, as well as a history of epididymitis and pericarditis, found in relatively rare manifestations of BD<sup>[9]</sup>.

Neutrophilic dermatoses are well-recognized cutaneous manifestations of systemic diseases such as IBD, and the wide spectrum of the disease includes Sweet's syndrome and pyoderma gangrenosum. These diseases may share common features such as sterile infiltration of polymorphonuclear leukocytes; BD is analogous to this condition<sup>[10]</sup>. Extracutaneous neutrophilic infiltrates are observed in all forms of neutrophilic dermatoses, but they predominate in Sweet's syndrome<sup>[11]</sup>. Sweet's syndrome associated with BD has been reported, and there may be similarities in the pathogenesis of BD and Sweet's disease<sup>[12]</sup>. Therefore, it seems reasonable that BD was complicated by AAs, although the mechanism remains unclear. Classically, Th1 immune response polarization has been known to be the main characteristic of BD immunopathogenesis<sup>[13]</sup>. The interleukin 17 (IL-17)-mediated (Th17) immune response may also contribute to immunological aberrations of BD<sup>[14]</sup>. IL-8-producing T cells were suggested to orchestrate neutrophil-rich pathologies of BD<sup>[15]</sup>. It was proposed that liver disease occurring in IBD is mediated by an aberrant homing of gut-derived T cells to the liver, with subsequent extensive lymphocyte infiltration of the liver<sup>[16]</sup>. Therefore, the mechanism by which AAs of the liver and spleen occurred in our case might be explained by an aberrant T cell-mediated immune response.

A patient with CD and associated AAs who underwent successful splenectomy was reported<sup>[17]</sup>. However, in our study, the patient developed AAs in the liver after splenectomy and finally needed immunosuppressive therapy. The risks of postoperative infection and thrombosis after splenectomy are now widely accepted. Accordingly, splenectomy as a treatment option might not be advisable in cases of AAs associated with BD.

In conclusion, we present a case of aseptic liver and spleen abscesses as the presenting picture of incomplete BD, in which complete remission was only obtained by corticosteroid therapy. The current case illustrates the possibility of the existence of AAs as uncommon manifestations of BD. Thus, physicians should be aware of this possibility to avoid a delay in diagnosis and unnecessarily aggressive therapies such as splenectomy.

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P- Reviewers Andre MFJ, Zippi M S- Editor Song XX  
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## Esophageal reconstruction with remnant stomach: A case report and review of literature

Song-Ping Xie, Guo-Hua Fan, Gan-Jun Kang, Qing Geng, Jie Huang, Bang-Chang Cheng

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Author contributions: Xie SP, Huang J and Cheng BC designed the report; Xie SP, Fan GH, Kang GJ and Geng Q managed the patients; Huang J performed surgical operation; Xie SP, Huang J and Cheng BC organized the report; Xie SP wrote paper.

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Received: January 20, 2013 Revised: March 27, 2013

Accepted: April 9, 2013

Published online: May 28, 2013

### Abstract

The number of patients developing esophageal cancer after gastrectomy has increased. However, gastric remnant is very rarely used for reconstruction in esophageal cancer surgery because of the risk of anastomotic leakage resulting from insufficient blood flow. We present a case of esophageal cancer using gastric remnant for esophageal substitution after distal gastrectomy in a 57-year-old man who presented with a 1-month history of mild dysphagia and a background history of alcohol abuse. Gastroscopy showed a 1.2 cm × 1.0 cm bulge tumor of the lower third esophagus with the upper margin located 39 cm from the dental arcade. Computed tomography of the chest showed lower third esophageal wall thickening. The patient underwent en bloc radical esophagectomy with a two-field lymph node dissection of the upper abdomen and mediastinum *via* a left-sided posterolateral thoracotomy through the seventh intercostal space. The upper end of the esophagus was resected 5 cm above the tumor. The gastric remnant was used for reconstruction of the esophago-gastrostomy and placed in the left thoracic cavity. The patient started a liquid diet on postopera-

tive day 8 and was discharged on the 10<sup>th</sup> postoperative day without complications. In this report, we demonstrate that the gastric remnant may be used for reconstruction in patients with esophageal cancer as a substitute organ after distal gastrectomy.

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**Key words:** Gastric remnant; Distal; Gastrectomy; Esophageal cancer; Substitution

**Core tip:** Gastric remnant is very rarely used for reconstruction in esophageal cancer surgery because of the risk of anastomotic leakage resulting from insufficient blood flow. We present a case of esophageal cancer using gastric remnant for esophageal substitution after distal gastrectomy in a 57-year-old man, who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis. The gastric remnant may be used for reconstruction in patients with esophageal cancer as a substitute organ after distal gastrectomy, with rapid recovery of bowel function and shorter hospital stay.

Xie SP, Fan GH, Kang GJ, Geng Q, Huang J, Cheng BC. Esophageal reconstruction with remnant stomach: A case report and review of literature. *World J Gastroenterol* 2013; 19(20): 3169-3172 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i20/3169.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i20.3169>

### INTRODUCTION

The number of patients developing esophageal cancer after gastrectomy has increased. Reconstruction is a challenge in esophageal cancer surgery when a previous subtotal gastric resection has been performed. In general, there has been a tendency to select the colon or jejunum

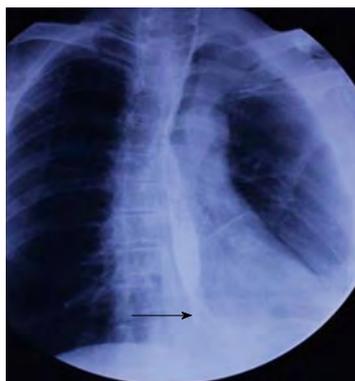


Figure 1 Barium esophagography. Esophagography shows a type 1 tumor, 1.5 cm in length, in the lower thoracic esophagus (arrow).



Figure 3 Upper gastrointestinal endoscopy shows a tumor 39 cm from the incisor, the anterior wall of the esophagus has a lip bulge about 1.2 cm × 1.0 cm.



Figure 2 Thoracic computed tomography shows a tumor 1 cm in diameter (arrow).

for substitution of the gastric tube, in order to avoid anastomotic leakage resulting from insufficient blood flow, which requires complicated operative procedures and leads to higher operative morbidity and mortality<sup>[1,2]</sup>. Herein, we report a patient with esophageal cancer and previous distal gastrectomy, who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis.

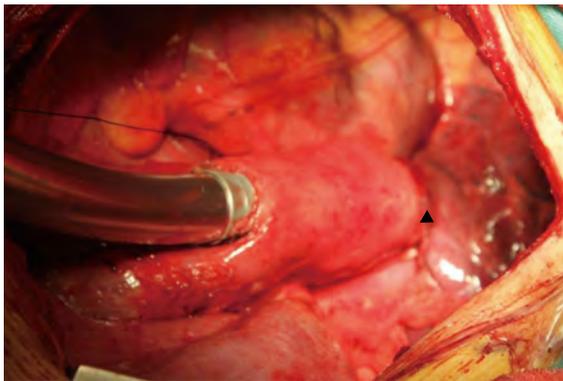
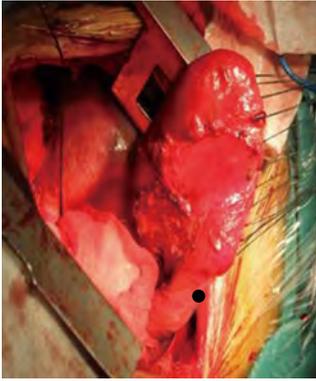
## CASE REPORT

A 57-year-old man had undergone distal gastrectomy with lymph node dissection 7 years previously for gastric cancer with a Billroth II anastomosis, pathologically diagnosed as highly differentiated adenocarcinoma, pT2N0M0; stage I b, according to the 7<sup>th</sup> edition of the UICC-TNM Classification of Malignant Tumors<sup>[3]</sup>. He was admitted to our hospital with a history of mild dysphagia with solids only for one month. His medical history was notable for alcohol abuse. Physical examination of the chest and abdomen revealed no abnormal findings. Barium esophagography revealed an approximately 1 cm arc filling defect in 1/3 of the lower esophagus with local mucosal damage and wall stiffness. The size of the gastric remnant was measured before surgery with contrast barium esophagography X-ray and was found to be 10 cm in length at the lesser curvature (Figure 1). Computed

tomography of the chest showed thickening of the lower third of the esophageal wall (Figure 2). Gastroscopy confirmed a 1.2 cm × 1.0 cm bulge tumor in the lower third of the esophagus with the upper margin located 39 cm from the dental arcade (Figure 3). The lesion did not stain with Lugol's solution. Pathological examination of the biopsy specimen revealed squamous cell carcinoma. Exclusion of cerebral, abdominal, skeletal, lymph node and other distal metastases (M0) was accomplished using [<sup>18</sup>F]-fluoro-2-deoxy-D-glucose positron emission tomography, and distant metastases to other organs were not found.

Prior to surgery, the complete colon was examined by endoscopy, and the bowel was prepared by mechanical cleansing. The patient underwent en bloc radical esophagectomy *via* a left-sided posterolateral thoracotomy through the 7<sup>th</sup> intercostal space, with a two-field lymph node dissection in the upper abdomen and mediastinum. The upper end of the esophagus was resected 5 cm above the tumor, and the left gastric artery and short gastric artery were then divided. The remnant stomach was freed up to the gastro-esophageal junction, and appeared rosy with adequate bloody supply probably due to the efferent jejunal flap with its wide gastrojejunal anastomosis (Figure 4). We decided to preserve the remnant stomach for reconstruction. The jejunum was freed from surrounding tissues. A gastric tube was extended far enough to reach the proximal esophagus. An esophagogastric anastomosis was performed mechanically under the level of the left inferior pulmonary vein using a circular stapler by an anterior gastrotomy to the high point of the fundus of the gastric remnant. The gastric remnant was placed in the left thoracic cavity. A micro-vascular anastomosis was not performed. Enteral nutrition therapy was *via* a jejunal stoma. The operative time was 266 min and the estimated blood loss was 435 mL.

Histologically, the tumor was diagnosed as a highly differentiated squamous cell carcinoma with tumor-free margins (Figure 5), and metastases in the abdomen and mediastinum lymph node were not found. The pathological stage was pT2N0M0; stage I b, according to the 7<sup>th</sup> edition of the UICC-TNM Classification of Malignant Tumors<sup>[3]</sup>.

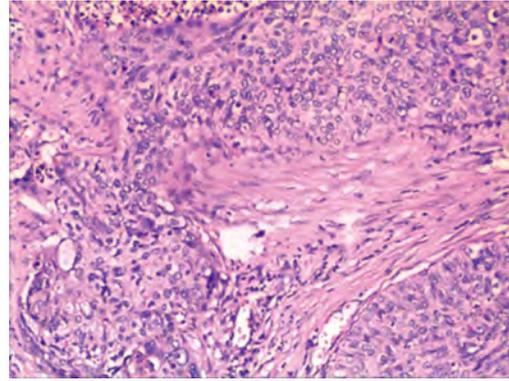


**Figure 4** Esophagus is anastomosed to the remnant stomach. During surgery, after cutting the left, right and short gastric vessels, the remnant stomach relies on the blood supply of the anastomotic stoma alone and is still rosy. Circle: Jejunum-stomach anastomotic stoma; Triangle: Esophagus-remnant stomach anastomotic stoma.

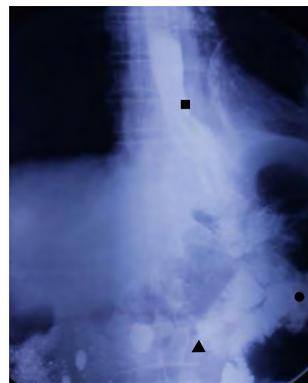
The postoperative course was uneventful, with no complications resulting from insufficient blood supply. The integrity of the anastomosis of the esophago-gastrostomy was confirmed by water soluble contrast radiography on postoperative day 7, with no signs of leakage or stricture (Figure 6). The patient started a liquid diet the following day and was discharged on the 10<sup>th</sup> postoperative day without complications. On follow-up at 6 mo after surgery, the patient was alive with no evidence of recurrence. He had improved food intake; mainly managing a solid diet with an average intake per meal of approximately 450 mL. Barium esophagography confirmed the absence of stricture, with efficient emptying of the remnant stomach.

## DISCUSSION

It was first reported in 1970 that a high incidence of gastrectomy (8.7%) was found in patients with esophageal cancer. Although surgical resection is the standard treatment for esophageal cancer after subtotal gastrectomy, the optimal management of this disease, including the surgical approach and the conduit for reconstruction, remains controversial. For patients with esophageal cancer and a history of gastrectomy, the esophagus is frequently reconstructed using colon interposition with a vascular pedicle<sup>[4]</sup>. The disadvantages of colon interposition include a long operative time for colon mobilization, highly



**Figure 5** The tumor was diagnosed as highly differentiated squamous cell carcinoma.



**Figure 6** Barium esophagography on the 7<sup>th</sup> postoperative day. Esophagography shows that barium passes smoothly through the anastomotic stoma, without leakage. Square: Anastomotic stoma; Triangle: Afferent loop of jejunum; Circle: Efferent loop of jejunum.

invasive procedures and additional anastomosis, which increase the incidence of postoperative complications<sup>[5]</sup>. Increased blood loss was observed when resolving adhesions in the upper abdominal cavity caused by gastrectomy. When using the remnant stomach, the procedure is easier, and the aims are to minimize surgical insults and to maximize the patient's quality of life. A reduction in the number of surgical maneuvers below the transverse colon and fewer bowel anastomoses represent real advantages<sup>[6]</sup>. In addition, use of the gastric remnant for reconstruction may offer substantial advantages to the elderly population in terms of fewer cardiac and pulmonary complications, rapid recovery of bowel function, shorter hospital stay, and a faster return to physical activities.

A remnant stomach can last for more than five years before the diagnosis of esophageal cancer. Establishment of the collateral circulation is important for healing of the anastomotic stoma. The blood supply of the remnant stomach may be maintained by the reconstituted microvascular supply from the widely anastomosed jejunal loop or vascular adaptation of the stomach and anastomotic site<sup>[6]</sup>, which may enhance perfusion of the gastric tube at the time of anastomosis and thus decrease anastomotic complications after esophagogastrectomy. Reavis demonstrated that gastrointestinal anastomoses were associated

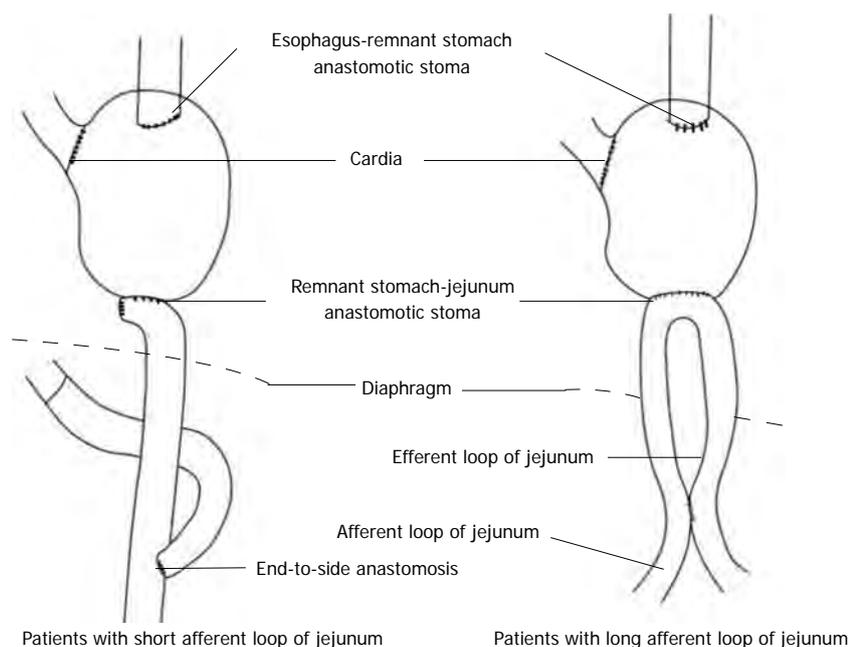


Figure 7 Scheme of the resection area of the gastric remnant and the esophagus (pre-operative), and reconstruction of the organs (post-operative).

with both vasodilation and angiogenesis and resulted in increased blood flow to the gastric fundus prior to esophago-gastric anastomosis in animals, which translated into a decrease in anastomotic dehiscence<sup>[7]</sup>. In our case, wide gastrojejunal anastomosis was seen in this operation. During surgery, 30 min after the left, right and short gastric vessels were cut, the remnant stomach was still rosy (Figure 5). We think that this wide anastomosis maintained the blood supply in the remnant stomach by reconstituting the intramural network. No complications occurred during the postoperative period, and barium esophagography on the 7<sup>th</sup> postoperative day showed that barium passed smoothly through the anastomotic stoma, without leakage. Preoperative bowel preparation should be conducted in such patients. If a poor blood supply in the remnant stomach is seen during surgery, then colon interposition should be used to replace esophageal surgery.

If the remnant stomach can not be lifted to the thorax to perform tension-free anastomosis with the esophagus during esophagectomy, the length of the afferent loop of the jejunum should be observed, if the afferent loop is short, it should be cut at the anastomotic stoma and an end-to-side anastomosis with the efferent loop of the distal jejunum should be performed; if the afferent loop is long, a side-to-side anastomosis should be performed 10-15 cm from the afferent and efferent loops, thus increasing the lifting height of the remnant stomach and jejunal loop (Figure 7).

We report a patient with esophageal cancer and previous distal gastrectomy who was successfully treated with esophagectomy and remnant stomach reconstruction without micro-vascular anastomosis. It is important to select an appropriate operative method for patients with esophageal cancer after distal gastrectomy, and its loca-

tion and stage must be determined. The remainder of the stomach can be used as an esophageal substitute depending on the curability of the esophageal cancer and the blood supply, which allows rapid recovery of bowel function and shorter hospital stay.

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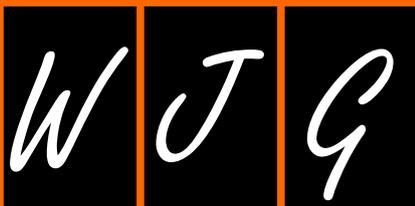
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# World Journal of *Gastroenterology*

*World J Gastroenterol* 2013 June 7; 19(21): 3173-3370



**EDITORIAL**

- 3173** Fortuitously discovered liver lesions  
*Dietrich CF, Sharma M, Gibson RN, Schreiber-Dietrich D, Jenssen C*

**REVIEW**

- 3189** E2F transcription factors and digestive system malignancies: How much do we know?  
*Xanthoulis A, Tiniakos DG*
- 3199** Sofosbuvir and ABT-450: Terminator of hepatitis C virus?  
*Zeng QL, Zhang JY, Zhang Z, Wang LF, Wang FS*

**ORIGINAL ARTICLE**

- 3207** Epidemiology, clinical-treatment patterns and outcome in 256 hepatocellular carcinoma cases  
*Fenoglio L, Serraino C, Castagna E, Cardellicchio A, Pomero F, Grosso M, Senore C*
- 3217** CT/<sup>99m</sup>Tc-GSA SPECT fusion images demonstrate functional differences between the liver lobes  
*Sumiyoshi T, Shima Y, Tokorodani R, Okabayashi T, Kozuki A, Hata Y, Noda Y, Murata Y, Nakamura T, Uka K*
- 3226** Mechanisms of trichostatin A inhibiting AGS proliferation and identification of lysine-acetylated proteins  
*Wang YG, Wang N, Li GM, Fang WL, Wei J, Ma JL, Wang T, Shi M*

**BRIEF ARTICLE**

- 3241** Survival after inflammatory bowel disease-associated colorectal cancer in the Colon Cancer Family Registry  
*Adams SV, Ahnen DJ, Baron JA, Campbell PT, Gallinger S, Grady WM, LeMarchand L, Lindor NM, Potter JD, Newcomb PA*
- 3249** Early dynamic transcriptomic changes during preoperative radiotherapy in patients with rectal cancer: A feasibility study  
*Supiot S, Gouraud W, Campion L, Jezéquel P, Buecher B, Charrier J, Heymann MF, Mahé MA, Rio E, Chérel M*
- 3255** Treatment of hepatitis C in compensated cirrhotic patients is equally effective before and after liver transplantation  
*Ponziani FR, Amicchiario EB, Siciliano M, D'Aversa F, Pompili M, Gasbarrini A*

- 3263** Stage and size using magnetic resonance imaging and endosonography in neoadjuvantly-treated rectal cancer  
*Swartling T, Kälebo P, Derwinger K, Gustavsson B, Kurlberg G*
- 3272** Low trypsinogen-1 expression in pediatric ulcerative colitis patients who undergo surgery  
*Piekkala M, Hagström J, Tanskanen M, Rintala R, Haglund C, Kolho KL*
- 3281** Possible ameliorative effect of breastfeeding and the uptake of human colostrum against coeliac disease in autistic rats  
*Selim ME, Al-Ayadhi LY*
- 3291** Gastric antisecretory and antiulcer activity of bovine hemoglobin  
*Al Asmari AK, Al Omani S, Elfaki I, Tariq M, Al Malki A, Al Asmary S*
- 3300** Anticancer effects of sweet potato protein on human colorectal cancer cells  
*Li PG, Mu TH, Deng L*
- 3309** Efficacy of adjuvant XELOX and FOLFOX6 chemotherapy after D2 dissection for gastric cancer  
*Wu Y, Wei ZW, He YL, Schwarz RE, Smith DD, Xia GK, Zhang CH*
- 3316** Differences in HER2 over-expression between proximal and distal gastric cancers in the Chinese population  
*Fan XS, Chen JY, Li CF, Zhang YF, Meng FQ, Wu HY, Feng AN, Huang Q*
- 3324** Effect of endogenous insulin-like growth factor and stem cell factor on diabetic colonic dysmotility  
*Wang Y, Xu XY, Tang YR, Yang WW, Yuan YF, Ning YJ, Yu YJ, Lin L*
- 3332** *PRSS1*\_p.Leu81Met mutation results in autoimmune pancreatitis  
*Gao F, Li YM, Hong GL, Xu ZF, Liu QC, He QL, Lin LQ, Weng SH*
- 3339** Gene expression profiles in peripheral blood mononuclear cells of ulcerative colitis patients  
*Miao YL, Xiao YL, Du Y, Duan LP*

## CASE REPORT

- 3347** Varicella zoster meningitis complicating combined anti-tumor necrosis factor and corticosteroid therapy in Crohn's disease  
*Ma C, Walters B, Fedorak RN*

- 3352** Endoscopic management of an esophagopericardial fistula after radiofrequency ablation for atrial fibrillation  
*Quénéhervé L, Musquer N, Léauté F, Coron E*
- 3354** Malignant solitary fibrous tumor involving the liver  
*Jakob M, Schneider M, Hoeller I, Laffer U, Kaderli R*
- 3358** Synchronous pancreatic solid pseudopapillary neoplasm and intraductal papillary mucinous neoplasm  
*Hirabayashi K, Zamboni G, Ito H, Ogawa M, Kawaguchi Y, Yamashita T, Nakagohri T, Nakamura N*
- 3364** Foreign body retained in liver long after gauze packing  
*Xu J, Wang H, Song ZW, Shen MD, Shi SH, Zhang W, Zhang M, Zheng SS*

- LETTERS TO THE EDITOR 3369** Is the American Association for the Study of Liver Diseases recommendation for hepatocellular carcinoma screening a cul-de-sac?  
*Brailon A*

**APPENDIX** I-VI Instructions to authors

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 25/F, Lucky Plaza,  
315-321 Lockhart Road, Wan Chai, Hong Kong, China

Fax: +852-65557188  
Telephone: +852-31779906  
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**PUBLICATION DATE**  
June 7, 2013

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## Fortuitously discovered liver lesions

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**Author contributions:** Dietrich CF and Jenssen C established the design and conception of the paper; Dietrich CF, Sharma M, Gibson RN, Schreiber-Dietrich D and Jenssen C analyzed the literature data; Dietrich CF provided the first draft of the manuscript, which was discussed and revised critically for intellectual content by Sharma M, Gibson RN, Schreiber-Dietrich D and Jenssen C; Dietrich CF, Gibson RN and Jenssen C provided figures; Dietrich CF, Sharma M, Gibson RN, Schreiber-Dietrich D and Jenssen C discussed the statement and conclusions, the comments of the reviewer, and approved the final version to be published.

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Received: December 25, 2012 Revised: March 22, 2013

Accepted: April 27, 2013

Published online: June 7, 2013

### Abstract

The fortuitously discovered liver lesion is a common problem. Consensus might be expected in terms of its work-up, and yet there is none. This stems in part from the fact that there is no preventive campaign involving the early detection of liver tumors other than for patients with known liver cirrhosis and oncological patients. The work-up (detection and differential diagnosis) of liver tumors comprises theoretical considerations, history, physical examination, laboratory tests, standard ultrasound, Doppler ultrasound techniques, contrast-

enhanced ultrasound (CEUS), computed tomography and magnetic resonance imaging, as well as image-guided biopsy. CEUS techniques have proved to be the most pertinent method; these techniques became part of the clinical routine about 10 years ago in Europe and Asia and are used for a variety of indications in daily clinical practice. CEUS is in many cases the first and also decisive technical intervention for detecting and characterizing liver tumors. This development is reflected in many CEUS guidelines, *e.g.*, in the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) guidelines 2004, 2008 and 2012 as well as the recently published World Federation for Ultrasound in Medicine and Biology-EFSUMB guidelines 2012. This article sets out considerations for making a structured work-up of incidental liver tumors feasible.

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**Key words:** Contrast-enhanced ultrasound; Hepatocellular carcinoma; Hemangioma; Focal nodular hyperplasia; Metastasis; Ultrasonography; Recommendations; Guidelines

**Core tip:** The presented paper is intended to discuss, comment and illustrate the recently published international guidelines on hepatic applications of contrast-enhanced ultrasound. Recommendations are based on a prospective multicenter study with more than 1000 histologically confirmed tumors and on national and international guidelines. The focus is on the important clinical work-up of the fortuitously discovered liver lesion. In contrast to most other published papers dealing with imaging methods, these recommendations also give advice for the clinician from a clinical point of view, including laboratory data. The described work-up includes different scenarios, *e.g.*, the asymptomatic (healthy) patient *vs* the oncological patient. Limitations of techniques and sources of error are also explained.

Dietrich CF, Sharma M, Gibson RN, Schreiber-Dietrich D, Jenssen C. Fortuitously discovered liver lesions. *World J Gastroenterol*

ol 2013; 19(21): 3173-3188 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3173.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3173>

## INTRODUCTORY CONSIDERATIONS

Guidelines for contrast-enhanced ultrasound were first published by the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) in 2004<sup>[1]</sup>. Further EFSUMB guidelines were published in 2008<sup>[2]</sup> and 2011<sup>[3]</sup>, and updated EFSUMB-World Federation for Ultrasound in Medicine and Biology liver guidelines have been concurrently published in the *European Journal of Ultrasound* (Ultraschall in der Medizin)<sup>[4]</sup> and *Ultrasound in Medicine and Biology*<sup>[5]</sup>. The presented paper is intended to discuss, comment and illustrate the recently published liver guidelines. The focus is on the important clinical work-up of the fortuitously discovered liver lesion<sup>[6]</sup>. The topic has been recently introduced by a German CME-article<sup>[6]</sup> which will be described in more detail.

### Frequency of incidental focal liver lesions

There are only few available data on the frequency of incidental focal liver lesions (FLL); this is true also for standard values in the abdomen<sup>[7-9]</sup>. In a forensic medicine autopsy series there were focal liver or gallbladder lesions ranging in size from 0.3 to 30 mm in 52% of 95 men (35-69 years old). The incidence rose with age<sup>[10]</sup>. Liver incidentalomas are found in 7.2%-33% of all patients investigated by computed tomography (CT) scan<sup>[11,12]</sup>. Liver tumors (5%) or focal fatty lesions (12%) were detected ultrasonically in 19% of a cohort of patients with Crohn's disease<sup>[13]</sup>. Reports on ultrasound screening at a population level in Asiatic countries suggest a prevalence for incidental focal liver lesions of 2.3%-6.2%<sup>[14,15]</sup>.

**Focal liver lesions in an asymptomatic and healthy population:** Fortuitously discovered liver tumors in asymptomatic and healthy persons without any previous history of malignant disease tend in most cases to be benign<sup>[13,16]</sup>. The core duty with regard to these patients is to document the benignity of the lesion or to exclude a malignant or inflammatory infiltration requiring treatment. In the case of a benign lesion further differential diagnostic characterization (diagnostic category) is desirable, although in most cases not absolutely necessary. In a well population of 64 patients referred for opinion about incidental solid liver lesions, Little *et al.*<sup>[17]</sup> found approximately 50% had hemangiomas, 7/64 had focal nodular hyperplasia (FNH) and 5/64 had adenomas. Approximately 25% had a neoplasm and 17% (11/64) had a malignant tumor. None of the lesions < 3 cm were malignant.

**Focal liver lesions in oncological patients:** Should there be signs of a malignant lesion, therapeutic options are generally dependent on knowing the correct diagnostic category. The procedure in patients with incidental

liver space-occupying lesions differs quite considerably from that which applies to patients with pathognomonic clinical symptoms, risk factors or a previous history of malignancy, who will also be briefly discussed in this review article in order to distinguish them. In patients with a tumor history, the probability that a focal liver lesion will be a metastasis is significantly higher. However, it must also be recalled that only 51%-88.8% of liver lesions detected by CT with a maximal size of 10-15 mm are actual metastases<sup>[18-20]</sup>. In this group at least 65% of single lesions < 15 mm are benign<sup>[19]</sup>.

In summary, focal liver space-occupying lesions are common opportunistic findings. A rational work-up strategy which is as noninvasive as possible must allow for the fact that the vast majority of these findings in asymptomatic persons are benign.

### What are the common focal liver lesions?

Common benign FLL include cysts, calcifications, FNH, nodular regenerative hyperplasia, biliary hamartoma (von Meyenburg complex), as well as regional hyper- and hyposteatoses on the one hand and true neoplasias on the other, *viz.* hemangioma and the significantly less common but prognostically important hepatocellular adenoma (HCA). Combined occurrence of FNH and hemangioma has been observed in one-third of cases<sup>[21]</sup>.

Inflammatory infiltrates, inflammatory pseudotumors<sup>[22]</sup>, abscesses and extramedullary hematopoietic lesions are more unusual<sup>[23]</sup>. Angiomyolipoma and other benign mesenchymal tumors, and benign (infantile) hemangioendothelioma, are true rarities<sup>[24,25]</sup>.

Common malignant FLL encompass primary [hepatocellular carcinoma (HCC) and cholangiocellular carcinoma (CCC)] and secondary neoplasias (metastases). Lymphomas can infiltrate the liver as primary or secondary tumors, but they and the extremely rare malignant mesenchymal neoplasias will not be further discussed here<sup>[26]</sup>. The prevalence of infective lesions such as hydatid disease is very region dependent but in most Western communities is uncommon<sup>[27]</sup>.

### Which imaging techniques are available?

The most commonly used imaging techniques are conventional B-mode ultrasound and color Doppler techniques. Compared to native ultrasound, contrast-enhanced (boosted echosignal) ultrasound (CEUS) improves the detection rate of FLL and makes it possible in nearly all cases to assess the malignant/benign status of hepatic space-occupying lesions in healthy liver parenchyma<sup>[28]</sup>. In large multicenter studies, CEUS revealed a diagnostic precision of over 90% in differentiating benign from malignant FLL<sup>[29,30]</sup>. Specific vascular patterns additionally allow, with a high level of reliability, FLL to be categorized into benign and malignant forms<sup>[31]</sup>. For assessment of the chest and abdomen in tumor patients (detection, staging), a CT scan is the method of choice, but it is considerably less useful than CEUS and magnetic resonance imaging (MRI) for the differential diagnosis of liver tumors<sup>[32,33]</sup>.

In the differential diagnosis of FLL, CEUS is better than the CT scan and at least equivalent to MRI<sup>[28,34-36]</sup>. A decisive advantage of CEUS in comparison to other cross-sectional methods is the real time representation of tumor vascularization along with much higher spatial and temporal resolution. Accordingly, FLL < 20 mm and even < 10 mm can be classified with a diagnostic reliability of over 80%<sup>[37]</sup>. Disadvantages of CEUS are the somewhat greater dependency on patient-specific factors, the lack of a complete field display and, with multiple lesions, the need to focus on a single lesion in the arterial phase. MRI, as well as contrast-enhanced imaging, is superior to CT for liver lesion detection and characterization. Commonly used MRI contrast agents fall into two main groups. The first group is extracellular gadolinium-based contrast agents, which provide similar contrast enhancement patterns to that seen with conventional CT contrast enhancement, although the signal enhancement is much stronger with MR. The second group is hepatobiliary agents, which have the properties of the first group but add another dimension to contrast enhancement as they are taken up by functioning hepatocytes and excreted into bile, resulting in further improvement to lesion detection and characterization<sup>[33]</sup>.

Elastographic imaging, especially transient elastography and acoustic radiation force impulse, has been shown to be of value to assess the severity of liver fibrosis in patients with chronic viral hepatitis to distinguish patients with mild fibrosis from those with significant fibrosis and liver cirrhosis. Strain elastography, shear wave elastography using acoustic radiation force impulse, and real time elastography are promising techniques for liver tumor characterization. Elastography has been studied recently to characterize focal liver lesions, and to differentiate between benign and malignant masses in a few studies, but so far cannot be recommended in daily routine<sup>[38-40]</sup>. The EFSUMB will soon be publishing guidelines and recommendations on the use of elastography and related methods.

#### **Which laboratory tests are available?**

Tumor markers are of low utility in the differential diagnosis of FLL. That also applies to alpha-fetoprotein, which is not raised in up to 40% of all cases of HCC, whereas nonspecific increases are, in contrast, common in active viral hepatitis and liver cirrhosis<sup>[41,42]</sup>. A rise in gamma-glutamyltransferase was observed in 76% of a group of 424 patients with FNH<sup>[21]</sup>. Laboratory test parameters are, however, very useful in the context of an FLL for diagnosing a hitherto undetected diffuse liver parenchymal pathology<sup>[42]</sup>.

#### **Appraisal of malignancy/benignity and prognosis**

The detection of a liver space-occupying lesion is followed by the question of whether it is malignant or benign. On the basis of the dual blood supply to the liver with arterial and its organ-specific portal venous sinusoidal circulation, analysis of the portal venous phase has shown that it is possible to classify malignant tumors reliably through use of ultrasound contrast media<sup>[29,31,35-37]</sup>.

The outcome is equally good with SonoVue<sup>®</sup> and Levovist<sup>®</sup><sup>[43-45]</sup>, and with Definity<sup>®</sup><sup>[46]</sup>.

A liver tumor can then be categorized (after excluding cysts and calcifications by B-mode imaging) as benign when enhancement or hypercontrast is demonstrated after 2 min, which is similar to normal liver parenchyma. In individual cases (particularly neuroendocrine tumors<sup>[47,48]</sup>) evaluation should also take place after > 2 min (up to 5 min)<sup>[27,49]</sup>.

**Limitations, source of error:** Erroneous diagnoses with contrast medium in ultrasound stem in particular from the inexperience of the investigator and incorrect instrument setting, as well as from concluding the investigation too early before the late phase.

The key source of error is bubble destruction due to an excessively high mechanical index or permanent sonication of the lesion. Premature destruction of the contrast medium can simulate faulty late-phase contrasting compared to non-sounded liver tissue. A FNH which is hyper-enhanced in the portal venous phase can therefore appear falsely hypo-enhanced due to the destroyed bubbles<sup>[50]</sup>.

Before every contrast medium ultrasound investigation, cysts and calcifications must be excluded by conventional ultrasound, since these structures do not exhibit contrast medium enhancement and could therefore be erroneously interpreted as a malignant infiltration.

The arterial phase is also of particular relevance for the correct appraisal of degenerative changes, *e.g.*, necrotic plaques and abscesses show no contrast onset either in the arterial phase or portal venous phase. It is of further significance, especially with benign liver tumors, that degenerative and shrinking processes can lead to changes in perfusion and thus in imaging correlates. Typical tumor features are therefore to be expected only with tumors of up to approximately 50 mm in diameter. Larger tumors, independent of their etiology, often show degenerative changes (calcifications, fibrosis, necroses, cystic areas and many other features).

Distinctive features have been reported with benign cholangiocellular adenoma<sup>[51]</sup>, benign cholangiofibroma<sup>[45]</sup>, rare bile duct tumors<sup>[52]</sup>, inflammatory pseudotumor<sup>[22]</sup>, sarcoma<sup>[26]</sup> and other rare tumor entities<sup>[27,49]</sup>. Allowance must also be made for artifacts<sup>[50]</sup>.

#### **What importance has histology?**

Due to the increasing quality of modern imaging techniques, particularly CEUS, the utility of ultrasound- or CT-guided biopsy for diagnostic categorization of focal liver lesions has fallen off considerably in the last few years<sup>[53]</sup>.

In the German Society for Ultrasound in Medicine (DEGUM) multicenter study, CEUS was evaluated as non-diagnostic in only 92 of 1349 cases of FLL (6.9%)<sup>[29,31,34-37,54]</sup>. Biopsy is therefore now only seldom required to establish whether a lesion is malignant or benign. Biopsy is still pertinent, however, in the differential diagnosis of malignant liver lesions, particularly before a planned palliative chemotherapy, *e.g.*, if several primary tumors are involved.

Because of a significant risk of puncture channel metastases, biopsy confirmation of HCC or metastases which are potentially resectable or likely to undergo liver transplantation should always be avoided if the imaging diagnosis appears to be clinically reliable<sup>[42]</sup>. Overall, imaging-guided biopsy of focal liver space-occupying lesions is a safe method with a clinically significant complication rate of approximately 0.5%<sup>[53]</sup>. As a rule, ultrasound-guided biopsy with a 20-18 gauge Trucut™ or aspiration cannula is the method of choice<sup>[53,55,56]</sup>. The diagnostic accuracy of ultrasound-guided biopsy is dependent on many factors and attains only about 90% even under optimal conditions<sup>[53,57,58]</sup>. There is a significant risk, especially with benign lesions, of overestimation (false-positive malignant diagnoses)<sup>[59]</sup>. Yield and sensitivity can be improved through CEUS-guided biopsy, in particular with small lesions which are not well demarcated on B-mode ultrasound, as well as tumors with necrotic components<sup>[60-62]</sup>. Highly promising new options for improving biopsy yield, particularly with lesions which are difficult to display ultrasonographically or are poorly accessible, include image fusion of radiological tomography procedures and CEUS, as well as three-dimensional electromagnetic or GPS-guided needle guidance<sup>[6,53,63,64]</sup>.

It is possible to characterize liver incidentalomas in over 90% of cases noninvasively. In terms of diagnostic reliability and cost-effectiveness, contrast-enhanced ultrasonography is the imaging method of choice. Magnetic resonance imaging is an equivalent diagnostic alternative.

#### ***What is the evidence base for the recommendations?***

Recommendations are based on national and international guidelines, on the prospective multicenter DEGUM study with more than 1000 histologically confirmed tumors<sup>[29]</sup>, as well as another prospective multicenter study from France<sup>[30]</sup>, and on published accounts of liver tumors, especially those histologically confirmed<sup>[26,45,65,66]</sup>. In the intervening period, there have also been two meta-analyses which validate the equivalence of CEUS, contrast-enhanced CT and contrast-enhanced MRI in the diagnosis of FLL<sup>[67,68]</sup>. The cost-effectiveness rules of the German Social Charter Sozialgesetzbuch V should also be observed in the work-up of liver tumors<sup>[69]</sup>; this applies to unnecessary secondary imaging as well as to biopsies which are not properly indicated.

## **INTRODUCTION: THE ASYMPTOMATIC PATIENT**

In an otherwise healthy patient, the accidental discovery of a focal liver lesion has an estimated probability > 95% of being benign, without the need for recourse to other imaging techniques<sup>[17]</sup>. Successive use of additional diagnostic procedures and measures is unable to improve the high general prediction rate for the malignant/benign nature of FLL, since the sensitivity, specificity and exactitude of all available procedures (with the exception of histology) are lower than the high clinical pretest prob-

ability. All diagnostic procedures are thus of service only to the individual affected patient in the context of recognition of prognostically relevant differential diagnoses. This diagnostic starting point of a fortuitously discovered focal liver lesion in the situation of a check-up in a completely healthy, asymptomatic person with a “blank-slate” medical history is, however, likely to be seldom encountered in such a straightforwardly exclusive way, in spite of a general trend towards “routine” and prophylactic investigations. More commonly, indications for investigation emerge which do not primarily suggest the existence of an FLL (*e.g.*, investigation of acute abdominal pains or during hospital admission for a cardiovascular condition), and in which the discovery of an FLL in spite of symptoms is still regarded as a major surprise. Moreover, the complete history is not always available in a patient referred for investigation. Accordingly, the first step following the discovery of a liver incidentaloma should always be to obtain a more detailed history. Further factors which should enter the initial evaluation are FLL number, size and (sonographic) morphology, as well as ultrasound criteria for the existence of a liver parenchymal pathology.

## **INTRODUCTION: THE SYMPTOMATIC PATIENT**

In a symptomatic patient, FLL is identified on the basis of the imaging process indication (symptomatology or previous history).

#### ***Patients with underlying malignant disease***

In a patient with underlying malignant disease and FLL, the probability of a malignant infiltration is thought to be about 50%, although this estimate is fettered by a high level of imprecision, especially since it is dependent on the underlying tumor entity. While a high percentage of very small solitary lesions are benign even in tumor patients, the probability of a metastasis increases depending on the following factors: size of the lesion(s), multifocal nature, presence of specific B-mode criteria (hypoechoic, halo) and, of course, existence of general symptoms, which are typical for a generalized tumor pathology. In the DEGUM multicenter study, a previous history of tumor in patients without liver cirrhosis increased the probability of malignancy for a given FLL by a factor of 1.8<sup>[35]</sup>. In the differential diagnosis of patients with hematological systemic diseases, it should be borne in mind that, along with infiltrations of the primary disease or extramedullary hematopoietic tissue foci, small hypoechoic liver lesions can correspond to multiple mycotic abscesses in both immune competent and suppressed patients. The indication for obtaining a histology specimen by biopsy for diagnostic confirmation is more often raised in this clinical situation, given that it has (diagnostic and prognostic) implications for treatment.

#### ***Patients with underlying inflammatory disease***

In patients with underlying inflammatory disease, confir-

mation or exclusion of an abscess is fundamental. CEUS has proved to be particularly helpful in this respect, since abscesses can be reliably identified due to their avascularity and immediately drained under ultrasonographic guidance. Extravascular administration of ultrasound contrast medium is of use in testing for successful drainage and, for instance, for reliably detecting (or excluding) a biliary communication<sup>[53]</sup>. Many other, but rarer, inflammatory infiltrations should be considered, *e.g.*, granuloma, inflammatory pseudotumor<sup>[22]</sup>, Bartonellosis<sup>[70]</sup>, *etc.*, which can ultimately only be reliably diagnosed histologically. In patients with rheumatological systemic diseases, nodular regenerative hyperplasias are a typical finding<sup>[71]</sup>. A study showed that patients with systemic lupus erythematosus are five-fold more likely to have liver hemangiomas than a control group<sup>[72]</sup>.

### **Patients with underlying chronic liver disease**

Existing chronic liver disease, particularly cirrhosis of the liver, raises the suspicion that every focal liver lesion is in fact a HCC, although benign FLL and other malignant liver tumors should of course be excluded. More than 80% of all detected FLL in a cirrhotic liver with a diameter  $\geq 20$  mm are HCC<sup>[42,73]</sup>. For FLL  $< 20$  mm the proportion of HCC is smaller, although it still lies well above 50%<sup>[42]</sup>. In the DEGUM multicenter study, 76.6% of FLL in hepatic cirrhosis patients were HCC, as opposed to a figure of only 6.1% in patients without hepatic cirrhosis<sup>[35]</sup>. Regeneration nodes (7.8%), metastases (4.3%), hemangioma (2.8%) and cholangiocellular carcinoma (2.5%) were considerably more infrequent than HCC<sup>[35]</sup>. Primary sclerosing cholangitis, but also chronic cholangitis in Caroli syndrome, fasciola hepatica fluke infestation ([www.efsumb.org](http://www.efsumb.org)) or hepaticolithiasis, as well as chronic hepatitis B and C and hepatic cirrhosis, are predisposing factors for the development of a CCC<sup>[74-77]</sup>.

**HCC:** HCC shows increased enhancement in the arterial phase compared to the surrounding liver parenchyma; however, less vascularized hepatocellular carcinomas are observed in up to 10% of patients<sup>[78]</sup>. Angioinvasion is typical of HCC and demonstration of a portal venous thrombosis indicative. Color duplex ultrasound is important and CEUS decisive for differentiating between banal portal venous thromboses and tumor thromboses. It is possible to demonstrate (arterial) blood flow signals in a tumor thrombus, but not in a purely coagulative thrombus. On use of echosignal boosters, the HCC typically shows an arterial hyperenhancement of the tumor compared to the circumferential liver tissue, while no contrast is yet apparent in the surrounding liver. A chaotic vessel pattern typically stands out in HCC as a sign of neovascularization<sup>[78]</sup>. Diagnostic criteria for HCC in patients with hepatic cirrhosis will not be more extensively dealt with in this article on the grounds of its specific terms of reference, and readers are directed to the current guidelines and the accompanying commentaries<sup>[21,79-83]</sup>. Only passing reference can also be made to the important and

much discussed differential diagnosis of cholangiocellular carcinomas in the cirrhotic liver<sup>[80,84]</sup>.

**Special case of HCC in the non-cirrhotic liver:** HCC is observed in up to 15%-20% of cases in the non-cirrhotic liver<sup>[85]</sup>. The majority of these HCC (“nearly all”) already appear hypervascularized in native color Doppler ultrasounds. In such cases it is possible that they may be confused with other hypervascularized liver tumors (FNH, HCA or hypervascularized metastasis). CEUS facilitates the correct procedure in nearly all these HCC (biopsy); all HCC cases observed by us showed hypoenhancement in the healthy liver parenchyma in the portal venous phase.

**HCC, intrahepatic cholangiocarcinoma, extrahepatic cholangiocarcinoma and guidelines:** Guidelines for clinical practice have become an integral part of the diagnosis and treatment of patients around the world. They provide an invaluable source of diagnostic algorithms, recommended treatments, safety information and training procedures. A discussion of currently published guidelines has been recently introduced<sup>[21,80-84,86]</sup> and will be summarized.

The American Association for the Study of Liver Diseases (AASLD) updated their 2005 Practice Guidelines<sup>[41]</sup> in July 2010, eliminating CEUS from the diagnostic algorithm for HCC, regardless of lesion size<sup>[42]</sup>. The reasons for this were two-fold: CEUS may cause false-positive HCC diagnosis in patients with cholangiocarcinoma, and CEUS is not available in the United States. The revised guidelines are not in agreement with the guidelines and recommendations of the EFSUMB, which includes CEUS in the characterization algorithm for FLL in non-cirrhotic patients as well as in cirrhotic patients with lesions  $> 2$  cm in size that emerge during a surveillance regimen<sup>[2]</sup>. The AASLD guidelines suggest only enhanced 4-phase multi-detector CT or dynamic contrast-enhanced MRI for the characterization of nodules  $> 1$  cm in size. The Asian Pacific Association for the Study of the Liver recommendations on hepatocellular carcinoma states that CEUS is as sensitive as dynamic CT or MRI in the diagnosis of HCC<sup>[87]</sup>. This dichotomy of clinical opinion presents difficulties for the physician attempting to optimize diagnostic potential for patients based upon imaging expertise on a case-by-case basis.

The rationale for the current AASLD guideline change was based on a single retrospective 21 patient case study from the Barcelona Hepatologist Group, which reported that intrahepatic cholangiocarcinoma (ICC) may occasionally (10 of 21 patients in mainly small nodules) display a vascular pattern similar to the vascular pattern considered indicative of hepatocellular carcinoma in CEUS, possibly leading to incorrect diagnoses<sup>[73]</sup>. There is no dispute that CEUS can, and perhaps should be, combined with other imaging modalities to make a definitive diagnosis in difficult cases. However, legitimate concerns have been raised over whether CEUS should be entirely removed from

important guidelines based upon a single, relatively small-scale study<sup>[88]</sup>. In contrast, recent studies from multiple investigators have produced extensive quantitative and qualitative data that suggest that CEUS is indeed valid as a primary diagnostic imaging modality in the characterization of HCC: These data are clearly supportive of the reinstatement of CEUS in the AASLD guidelines. The purpose of this review is to summarize these studies in order to validate the need for the continued use of CEUS as a pivotal diagnostic imaging tool in FLL diagnosis.

Importantly, ICC can also present variable enhancement patterns that relate to tumor size and cellular content. Chen *et al*<sup>[89,90]</sup> confirmed that whereas smaller ICC can present with homogeneous enhancement patterns similar to HCC, larger tumors may show diverse patterns due to compression-induced central hypovascularity and necrosis. Unfortunately, Vilana *et al*<sup>[73]</sup> did not address the developmental stage or cellular morphology of the ICC lesions evaluated by CEUS in their study. Even benign lesions can occasionally be misdiagnosed because of late-phase hypoenhancement; this can occur in lesions that contain scar tissue or pronounced fibrosis<sup>[54]</sup>. Knowledge of these pathomorphological enhancement and vascular patterns in FLL, and in HCC/ICC in particular, can be a critical factor in differential diagnosis. The use of a blood pool ultrasound contrast agent like SonoVue<sup>®</sup> is highly beneficial, in that it can aid demonstration of the hemodynamics and microvascular morphology of the liver and lesion in continuous real-time.

Several studies have suggested that CEUS is at least equal to CT and MRI for the diagnosis of FLL, including the differential diagnosis of HCC/ICC. Chen *et al*<sup>[90]</sup> compared enhancement patterns of pathologically proven ICC from 40 patients undergoing CEUS with SonoVue<sup>®</sup> with those generated in contrast-enhanced CT (CECT). They found that the correct diagnosis was made in 80% of the lesions at CEUS but in only 67.5% of the lesions at CECT. The arterial phase (AP) enhancement was consistent for both modalities, but the portal venous (PV) wash-out at CEUS was more pronounced than that at CECT. Visualization of the four enhancement patterns was equivalent in both modalities, indicating that it is pathophysiology, not the technology, which determines the enhancement pattern. Their conclusion was that CEUS is at least equivalent to CECT for the characterization of ICC.

Another study to assess the concordance of enhancement patterns at CEUS, CECT, and contrast-enhanced MRT (CEMRI) looked at 144 confirmed FLL including 49 HCCs<sup>[91]</sup>. Randomized image sets from each modality were evaluated by three blinded readers who answered identical questions regarding enhancement patterns and temporal changes in AP and PV enhancement. AP enhancement showed a mean concordance of > 76% across modalities. Concordance in the PV phase was lower at 61%. The majority of discordances were malignancies where sustained PV phase enhancement was seen at CECT and CEMRI but not at CEUS; included in this group were 18 HCCs, 6 metastases, and 3 ICCs. The

authors concluded that this result was due to the aforementioned tendency of CT and MRI contrast agents to diffuse into the interstitium from the leaky vessels of malignant lesions. Benign lesions showed sustained PV enhancement in all imaging modes. A further illustration of the importance of timing issues in comparing imaging modes for FLL was demonstrated in the examination of hemangiomas<sup>[65]</sup>; some smaller, rapidly enhancing hemangiomas showed trademark centripetal progression only with real-time CEUS.

The conclusions from numerous studies, including the DEGUM multicenter trial<sup>[34,36]</sup>, are that CEUS is an appropriate rapid first diagnostic procedure following initial identification of liver lesions by non-enhanced ultrasound in clinical routine, and that it is diagnostically non-inferior to CECT/CEMRI.

**Benign liver tumors in a cirrhotic liver:** Benign liver tumors should theoretically occur just as frequently in a cirrhotic liver as in patients with healthy livers<sup>[21,78,81,83]</sup>. Most studies report the opposite<sup>[35,36]</sup>. Before resection of hepatocellular carcinomas which may be susceptible to curative treatment, otherwise benign and in particular hypervascularized liver tumors (FNH, hepatocellular adenoma, shunt hemangioma, metastases especially of neuroendocrine tumors<sup>[47,48]</sup>) should be excluded in order to avoid incorrectly indicated operations.

#### ***Patients with otherwise predisposing diseases***

There are many kinds of patients with otherwise predisposing diseases. Examples here include hyperechoic adenomas in glycogen storage diseases, hemangiomas related to genetic syndromes with angiomatous malformations, focal biliary cirrhotic nodes in cystic fibrosis<sup>[92]</sup>. Readers are directed to specialist textbooks<sup>[27,49]</sup>.

The work-up of fortuitously discovered focal liver lesions is guided by contextual information from the clinical history, from which can be inferred the relative probability of malignant and clinically relevant benign lesions, the differential diagnostic spectrum and the utility of histological confirmation.

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## **HYPERECHOIC LIVER LESION**

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### ***Asymptomatic patient***

The most common hyperechoic (or better: more strongly echogenic) focal liver lesion is hemangioma, which is observed three times more often in women than in men (> 95%). Most hemangiomas (approximately 70%) are “typical” (Table 1) and can be correctly identified by conventional B-mode and color Doppler sonography; further measures are not necessary. In about 30% of patients there will be an atypical criterion, and in such cases contrast-enhanced imaging procedures can be used (CEUS, MRI)<sup>[65,91,93-100]</sup>. It should also be mentioned that hemangiomas in patients with fatty livers (hepatic steatosis) can appear to be isoechoic or hypoechoic compared to hyperechoic parenchyma. Lesions suspected of being

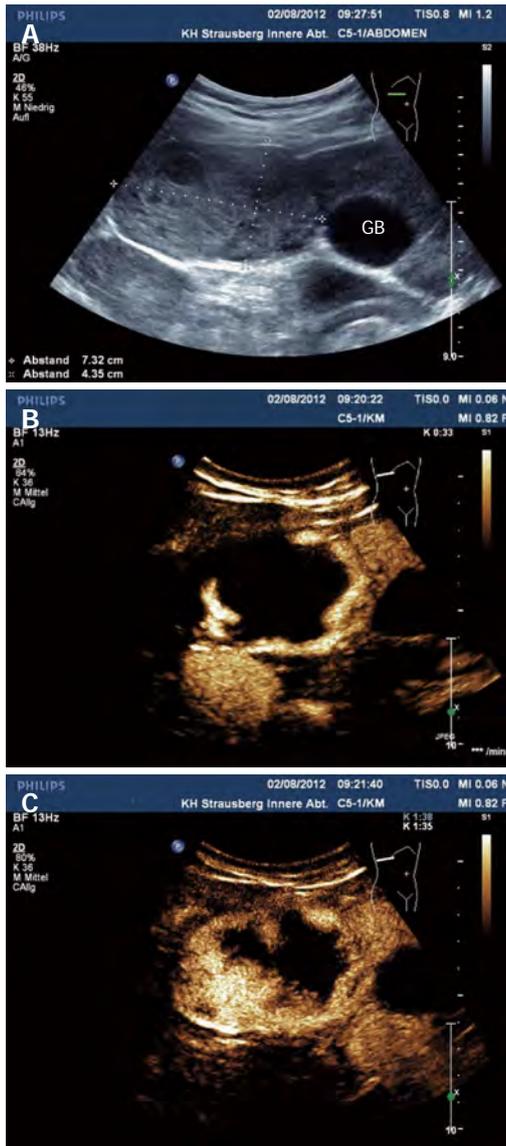


Figure 1 Hemangioma. Large hemangioma in B-mode (A), with typical peripheral nodular contrast enhancement (B) and centripetal fill-in (C). GB: Gallbladder.

hemangiomas which hypoattenuate in the portal venous phase after contrast medium administration should be biopsied and histologically confirmed<sup>[65]</sup>.

**Special features of hemangiomas:** Two signs typical of hemangiomas are peripheral nodular contrast medium enhancement and the iris-diaphragm phenomenon. The peripheral globular contrast medium enhancement is complemented by the centripetally progressive (partial) fill-in of the hemangioma (Figure 1).

Unfortunately, this typical contrast medium representation overlaps with the pattern in liver metastases arising from gastrointestinal carcinomas, and it is possible to confuse them<sup>[47]</sup>. Unlike metastases, which in the late phase progressively show less strongly echogenic contrast compared to normal liver tissue, hemangiomas are on a sustained basis more strongly enhanced than their surroundings.

Table 1 Typical criteria of hemangioma

#### B-mode criteria

|   |
|---|
| Less than 3 cm in diameter                        |
| Echo-rich structure                               |
| Homogeneous interior                              |
| Round or slightly oval shape                      |
| Smooth outline                                    |
| Absence of any halo sign                          |
| Possible detection of feeding and draining vessel |
| Absence of any signs of invasive growth           |
| Dorsal through-enhancement                        |

Reproduced with permission from Dietrich *et al*<sup>[65]</sup>.

The expression “high-flow hemangioma” (10% of hemangiomas), which is used in computer tomography, was marked by early onset and offset of contrast medium and can in our opinion be better described by the expression “shunt hemangioma”, since this hemangioma is characterized by arterial-portal venous shunts. Shunt hemangiomas are characteristically rather small (< 20 mm) and are typically found in areas of variable fatty degeneration compared to enveloping liver tissue. Normal liver tissue is predominantly (80%) perfused with lipid- and hormone-containing portal venous blood, whereas arterially perfused shunt hemangiomas show a lower lipid and insulin concentration than portal venous blood, which also leads to a lesser degree of fat deposition in surrounding hepatocytes. It is also known through angiographic and magnetic resonance tomographic studies that arteriovenous and/or portal venous shunts can lead to earlier contrast medium fill-in of the hemangioma<sup>[65]</sup> (Figure 2).

Liver hemangiomas can be solitary, but may also be multifocal and in very rare cases (in childhood) diffuse. An association with FNH is also not uncommon<sup>[24,101,102]</sup>. Readers are directed to the literature regarding special features of biopsy in hemangiomas<sup>[53,55,56]</sup> and rare hemangioma subtypes<sup>[65]</sup>. Ultrasound findings in 400 patients with hemangioma examined by CEUS have been recently published (Table 2)<sup>[81]</sup>.

#### Symptomatic patient

Very large hemangiomas can through extrusion, compression and very seldom hemorrhage themselves give rise to symptoms. Fundamentally, the criteria described for asymptomatic patients apply to symptomatic patients too. The indication for biopsy and histological confirmation may perhaps be more permissively made in daily routine if a multiplicity of confusing additional findings is present.

#### Differential diagnosis

In the differential diagnosis, consideration must be given to regional fat degeneration zones (which can often be unequivocally characterized based on their localization<sup>[103-106]</sup>), hyperechoic hepatocellular adenoma (particularly in storage diseases, which are extremely rare)<sup>[45,66,107]</sup> and neuroendocrine tumor metastases<sup>[47,48]</sup>, which in their early form

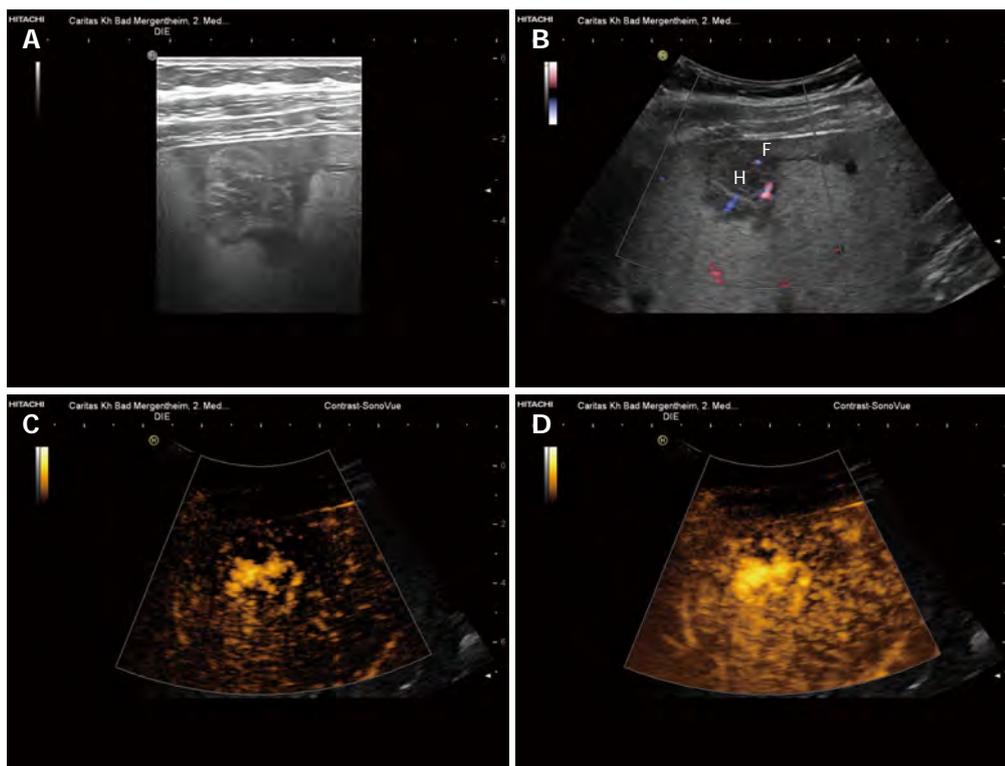


Figure 2 Shunt hemangioma. Shunt hemangiomas are typically small (often < 20 mm) with abundant arterio(porto)-venous shunts (functionally described as high flow hemangiomas). They are often surrounded by less fat-containing hypoechoic liver parenchyma (A, B) due to the dominant arterial blood flow in comparison to the reduced portal venous perfusion. Arterial contrast enhancement of the shunt-hemangioma is also shown (C, D). H: Hemangioma; F: Less fat-containing hypoechoic area. Reproduced with permission from Dietrich *et al*<sup>[65]</sup>.

can look remarkably like hemangiomas. Many rare causes of hyperechoic tumors have been described<sup>[49]</sup> and are only relevant insofar as indeterminate findings must be biopsied and histologically confirmed if a therapeutic decision is to emerge. Old abscesses and echinococcoses<sup>[53,108]</sup> and a miscellany of other disease syndromes should be borne in mind in the individual case.

### ISOECHOGENIC LIVER LESION

The most commonly isoechogenic liver lesions are focal nodular hyperplasia, hepatocellular adenoma, hepatocellular carcinoma and isoechogenic metastases. All these entities should be considered in symptomatic and asymptomatic patients equally, although the incidence profile is different. All isoechogenic lesions should be investigated using a contrast-enhanced imaging technique. Late-phase contrast medium hypoenhancement is a decisive indication for biopsy.

#### Asymptomatic patient

In asymptomatic patients, the differential diagnosis of FNH and HCA is the main objective. Both entities are far more commonly found in female patients. About 20% of FNHs occur multifocally, and co-association with hemangiomas is not uncommon<sup>[24,101,102,109]</sup>. It is important to exclude malignant differential diagnoses with a high degree of certainty, which is only possible with recourse

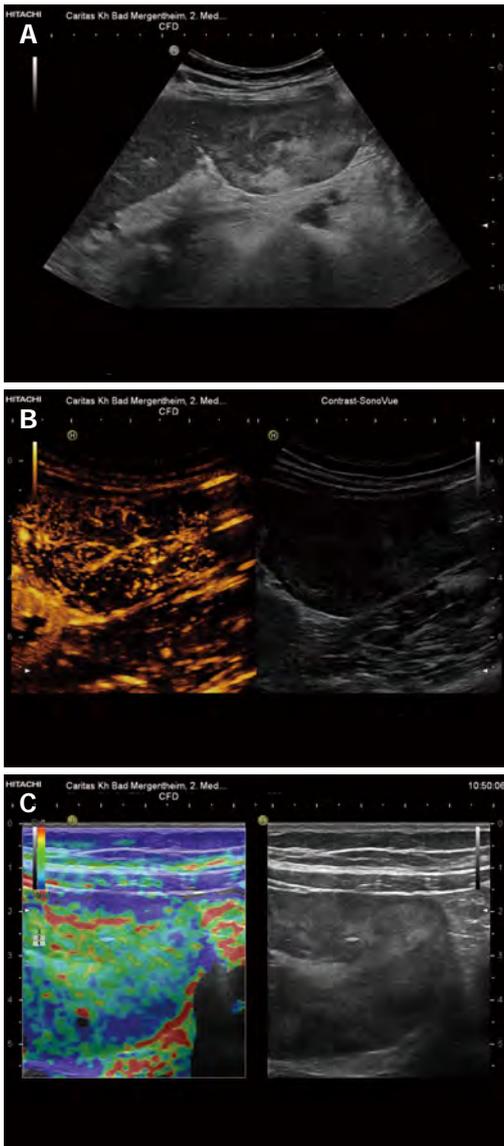
**Table 2** Ultrasound findings in 400 patients with hemangioma *n* (%)

| Characteristics   | <i>n</i> = 400 |
|---|----------------|
| Size, cm (mean ± SD)  | 2.6 ± 3.1      |
| B-mode, echogenicity  |                |
| Echo-rich   | 363 (91)       |
| Isoechoic or echopoor   | 37 (9)         |
| Vascularity assessed by colour Doppler imaging  |                |
| No intra-lesional vessels   | 379 (95)       |
| Intra-lesional vessels (hypervascular)  | 21 (5)         |
| Peripheral nodular enhancement  | 328 (82)       |
| Strong homogenous arterial enhancement  | 31 (8)         |
| No specific enhancement pattern or not determinable (e.g., due to size of the lesion, solitary fibrotic nodule) | 41 (10)        |
| Complete iris diaphragm phenomenon  | 320 (80)       |
| Incomplete iris diaphragm phenomenon  | 80 (20)        |

Reproduced with permission from Dietrich *et al*<sup>[65]</sup>.

to a contrast-enhanced technique.

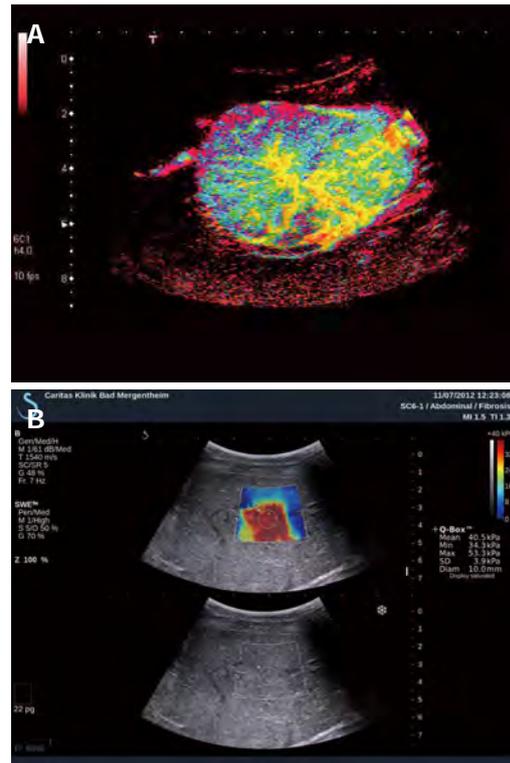
**Focal nodular hyperplasia and hepatocellular adenoma:** It has become possible to differentiate focal nodular hyperplasia from hepatocellular adenoma through echo-signal-boostered ultrasonography<sup>[66,81,110]</sup>. Unlike in FNH, portal veins and bile ducts are not present in hepatocellular adenoma. The two can therefore be differentiated through analysis of the portal venous phase<sup>[45,66]</sup>, which shows a typical hypoenhancement with HCA.



**Figure 3** Teleangiectatic focal nodular hyperplasia. Pedunculated liver tumor, histologically teleangiectatic focal nodular hyperplasia with signs of peliosis. A: B-mode imaging shows heterogenous echogenicity; B: Contrast-enhanced ultrasound reveals central arterial blood supply; C: Real-time elastography shows harder periphery and softer central portions of the lesion.

FNH typically exhibits arterial increased enhancement, which tends to be very marked in the first few seconds. Centrifugal (70%) or eccentric (30%) enhancement through one (or, in larger tumors, several) afferent correspondingly situated arteries is a diagnostic pointer. Further tumor enhancement takes place through a vessel architecture radiating out from this artery. FNH shows, in the portal venous and later phases, at least low-grade increased enhancement in about 95% of cases provided that bubble destruction does not lead to an error of interpretation<sup>[50]</sup> (Figures 3 and 4).

In the arterial phase, hepatocellular adenoma is hypervascularized, without however the characteristic vessel architecture described for FNH. Compared to the surrounding liver parenchyma, there is slightly poorer contrast medium enhancement in the portal venous and



**Figure 4** Focal nodular hyperplasia. A: In addition to conventional contrast-enhanced ultrasound (CEUS), parametric CEUS displays also the timeline of contrast enhancement (early enhancement in yellow, later enhancement in blue); B: Histologically proven. B-mode revealed isoechoic lesion, very difficult to identify. Shear wave elastography reveals very hard tissue of the lesion, shown in red in comparison to the surrounding soft liver parenchyma.

at least in the late phases (> 20 min), making it possible in most cases to differentiate it from FNH. Contrast medium hypoenhancement in the late phase is a decisive indication for liver biopsy. Ultrasound findings in 424 patients with FNH and 36 patients with HCA examined by CEUS are summarized in Table 3<sup>[21]</sup>.

**Possibilities of error:** Differentiating hepatocellular adenoma from highly differentiated hepatocellular carcinoma<sup>[111]</sup> is not possible by means of an imaging procedure, making it necessary to proceed to histological investigation of the tissue. It should be borne in mind here that error is inherent even in histological evaluation. Erroneous histological evaluations based on non-representative tissue samples and mixed forms of both tumors have been observed, with the result that in the individual case only resection and tissue preparation are unequivocal. Hepatocellular adenoma is frequently observed in patients with storage diseases<sup>[107]</sup>. Reliable tumor characterization of hepatocellular adenomas using imaging procedures is then only possible provided secondary regressive changes are not present, a phenomenon which is observed particularly with large (> 50 mm) adenomas.

**Symptomatic patient**

In symptomatic patients, the differential diagnosis of metastases and HCC is clinically of prime importance. Me-

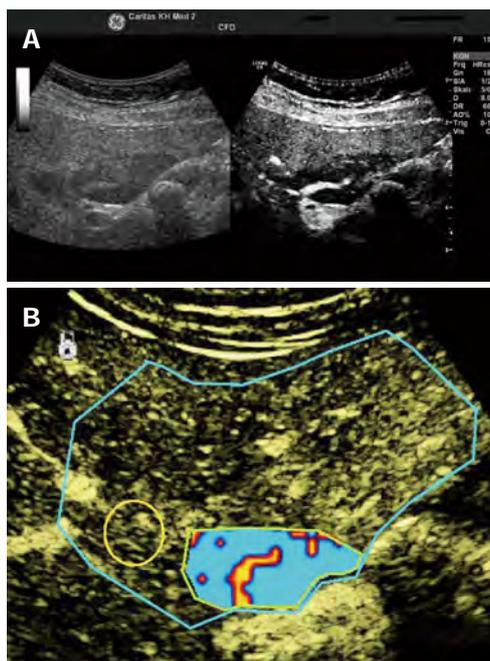


Figure 5 Focal fatty sparing. A: Focal fatty changes may simulate masses on conventional B-mode ultrasound; B: In the arterial, portal venous and late phases, focal fatty changes show similar enhancement patterns to that of the adjacent liver parenchyma. Contrast-enhanced ultrasound is helpful for the identification of the centrally located arteries. Typically centrally located arteries (and often also portal venous branches and hepatic veins) can be identified. Dynamic vascular pattern improves contrast imaging. Reproduced with permission from Cui *et al*<sup>[120]</sup>.

tastases can be identified with a high degree of reliability with CEUS in the portal venous and late phases due to their hypoenhancement. Biopsy and histological confirmation follow to determine the clinical course of action. Abscesses are diagnosed, contingent to severity, due to their avascularity. Hepatocellular adenomas should be included in the differential diagnosis in spite of their rarity, since they present in up to 30% of cases with nonspecific upper abdominal complaints, although they may also (if very rarely) manifest as acute abdominal pain (hemorrhage)<sup>[24,45,66]</sup>.

**Differential diagnoses**

It is important to note the variety of miscellaneous liver tumors. As a rule, where findings are unclear it is advisable to obtain histological confirmation or, if warranted by the clinical outcome, to proceed to surgery (*q.v.*, also “differential diagnoses of hypoechoic liver lesions”).

**HYPOECHOIC LIVER LESION**

The origin of hypoechoic lesions is considerably more varied and confusing. All hypoechogenic lesions (with the exception of those which can safely be categorized as regionally distinct fat degeneration zones) should be investigated using a contrast-enhanced imaging technique. Evaluation with CEUS in the portal venous and late phase is determinant in this context, and contrast

**Table 3** Ultrasound findings in 424 patients with focal nodular hyperplasia and 36 patients with hepatocellular adenoma

|   | Focal nodular hyperplasia (n = 424) | Hepatocellular adenoma (n = 36) |
|---|-------------------------------------|---------------------------------|
| Size of lesion, mm (range)              | 49 ± 24 (12-110)                    | 34 ± 22 (20-120)                |
| Conventional B-mode ultrasound          |                                     |                                 |
| Echo texture                            |                                     |                                 |
| Hypoechoic                              | 36 <sup>1</sup>                     | 8 <sup>1</sup>                  |
| Isoechoic                               | 382                                 | 20                              |
| Hyperechoic                             | 6                                   | 8                               |
| Central scar                            | 241                                 | 0                               |
| Colour/power Doppler imaging            |                                     |                                 |
| Hypervascular                           | 369                                 | 33                              |
| Radial vascular architecture            | 234                                 | 3                               |
| Contrast-enhanced ultrasound            |                                     |                                 |
| Arterial phase enhancement              | 424                                 | 36                              |
| (Early) Portal venous phase enhancement | 406                                 | 6                               |

<sup>1</sup>Depending on the technique and ultrasound machine used; all in a sonographically bright liver. Reproduced with permission from Dietrich *et al*<sup>[66]</sup>.

medium hypo-enhancement in the late phase is a decisive indication for liver biopsy.

**Asymptomatic patient**

In asymptomatic patients with fatty liver syndrome, the differential diagnosis of FNH and HCA is of prime importance. Malignant differential diagnoses must be excluded with a high degree of certainty, and this is only possible using a contrast-enhanced technique.

**Special features of focal fat sparing:** Regional areas of focal fat sparing are found in almost all patients with fatty liver<sup>[21,103-105]</sup> and are only seldom a diagnostic challenge. In terms of color duplex ultrasound, both focal fat deposition and focal sparing are in themselves inconspicuous; neither increased nor decreased blood perfusion is recognizable, since the liver tissue is in principle still normal tissue. The centrally situated afferent arterial and efferent venous vessels are typical, and can be recognized in many cases with the use of good instrument technology and a corresponding investigative technique (www.efsumb.org, case of the month). Even in the echosignal-boosted sequence, regionally distinct fat infiltration zones are apparent in contrast medium dynamics with centrally afferent artery and later as normal liver tissue.

In the arterial phase, afferent vessels can be displayed; in the portal venous phase they do not differ from their surroundings and can therefore be differentiated from true neoplastic entities. In this problem area CEUS is better than all other procedures (Figure 5).

**Symptomatic patient**

In symptomatic patients, the differential diagnosis of metastases (Figure 6) and HCC (Figure 7) is clinically of prime importance, the former being identifiable with a high degree of certainty using CEUS in the portal venous and late phase on account of their hypoenhancement.

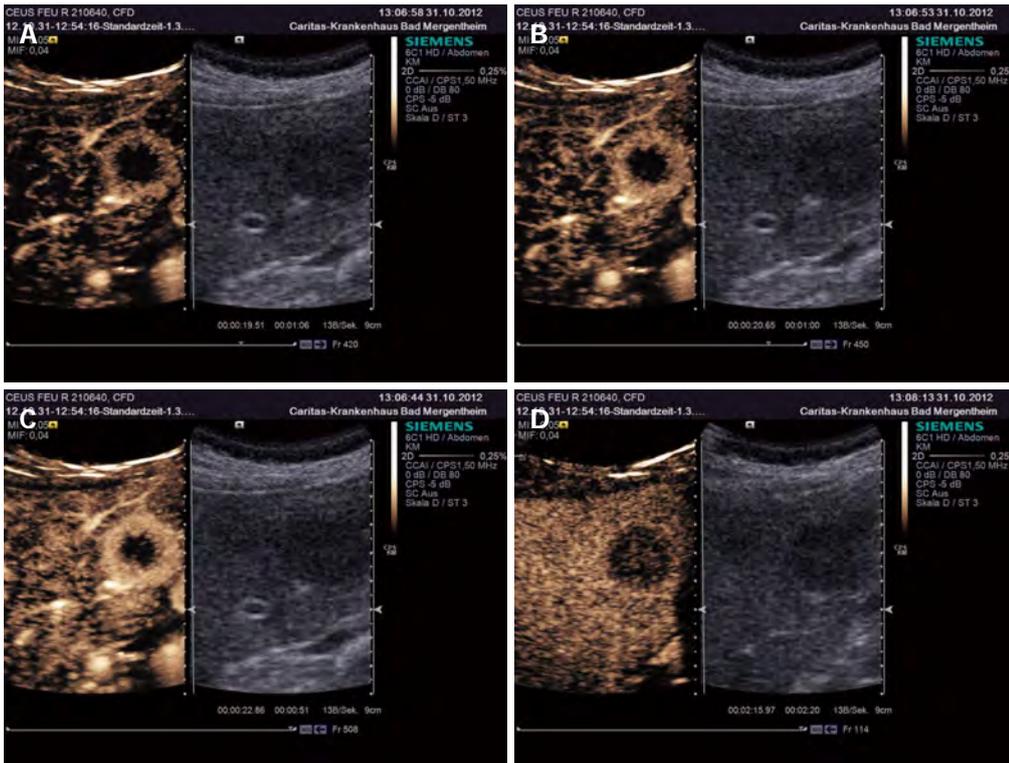


Figure 6 Liver metastasis. In contrast to the variably enhancing arterial phase (A-C), liver metastases are typically hypoechoic during the portal venous (sinusoidal) phase (D) which facilitates their reliable diagnosis.

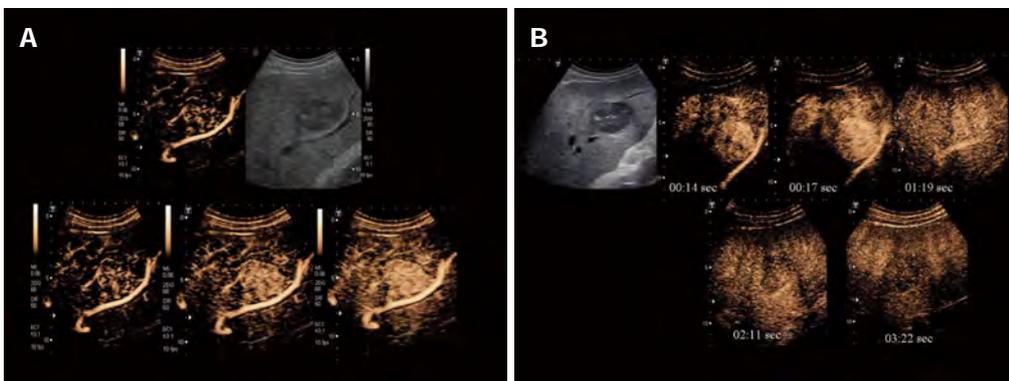


Figure 7 Hepatocellular carcinoma with arterial hyperenhancement (A) and hypoechoic enhancement in the portal venous phase (B).

Biopsy and histological confirmation follow to determine the clinical course of action. Inflammatory processes are diagnosed depending on the severity of the condition through their uneven enhancement (inflammation) or avascularity (abscess).

**Differential diagnoses**

Attention should be given to the wide range of miscellaneous liver tumors which are set out in the current literature and in textbooks<sup>[27,49]</sup>. As a rule, where findings are unclear it is advisable to obtain histological confirmation or, if warranted by the clinical outcome, to proceed to surgery. The main features of hepatic abscess have been published<sup>[2,112,113]</sup>. Typical enhancement patterns are summarized in Table 4. The evidence from the German

DEGUM study illustrates the key role that CEUS plays in diagnostic imaging of FLL; therefore, we discuss the study in more detail and refer to explanatory references<sup>[21,81]</sup>.

**Discussion of the DEGUM multicenter trial:** In total, 1349 patients (677 men and 672 women; mean age 59.8 years; range 12-91 years) were prospectively recruited at fourteen ultrasound centers<sup>[29]</sup>. In the majority of patients ( $n = 841$ ; 62.3%), the FLL was an incidental finding. Underlying liver cirrhosis was present in 234 patients (17.3%) while 364 patients (27.0%) were known to have an extrahepatic malignancy. Patients with liver lesions that could be diagnosed by B-mode ultrasound, such as cysts or distinctive hemangiomas, were not included in the study. These patients would not proceed for further examina-

**Table 4** Typical enhancement pattern for the characterization of focal liver lesions

| Lesion                                      | Arterial phase   | Portal venous and late phase                                   |
|---|--|--|
| Hemangioma (Figures 1 and 2)                | Circular vascular enhancement without central intratumoral vessels                 | Nodular enhancement and "fill-in" pattern                      |
| Focal nodular hyperplasia (Figures 3 and 4) | Radial centrifugal or "spoke wheel" pattern, then rapid, complete hyperenhancement | Iso- or hyperenhancement                                       |
| Focal fatty sparing (Figure 5)              | Central arterial blood supply  | Isoenhancement   |
| Metastasis (Figure 6)                       | Asymmetrical, chaotic enhancement configuration                                    | Hypoenhancement  |
| Hepatocellular carcinoma (Figure 7)         | Hyperenhancement and disorganized vascularity                                      | Iso- or hyperenhancement, depending upon tumor differentiation |

Reproduced with permission from reference Dietrich *et al*<sup>[81]</sup>.

tion in routine practice. Also excluded from the study were patients with malignant liver tumors that showed infiltration into the hepatic vasculature.

CEUS can be considered at least equal to, and in some ways superior to, CECT and CEMRI as a diagnostic tool. The reasons for differences between the modalities in lesion assessment may be method-related; the volume of microbubbles injected as a bolus (1.2-4.8 mL) administered over a period of a few seconds is more temporally sensitive than the larger volume of CT contrast agent (usually 100-150 mL) injected with a flow rate of 3-6 mL/min over 20-40 s. Diffusion of CECT contrast agent into the interstitium and the subsequent slow wash-out can result in erroneous diagnoses based upon PV enhancement patterns, whereas PV wash-out evident in CEUS may not be apparent in CECT. The pre-determined scan delay in CECT can miss the rapid initial wash-in and AP hypervascular response seen in CEUS. Extensive analyses have been carried out on the reasons for discordance between CEUS and CECT/CEMRI imaging in FLL, examining the effects of timing, diffusion, and fat content of the liver/lesion<sup>[114]</sup>.

Liver tumor characterization and other CEUS applications are described in more detail in currently published issues of *Ultraschall in der Medizin (European Journal of Ultrasound)*<sup>[21,80,81,83,84]</sup>.

Along with clinical context and medical history, echogenicity and ultrasound morphology set limits to the many-sided differential diagnostic spectrum of focal liver lesions. In determining whether a lesion is malignant or benign, the late phase of contrast-enhanced ultrasound is decisive, whereas diagnostic typing and characterization are particularly dependent on the evaluation of specific vascularization patterns in the arterial onset phase.

## ADDITIONAL CEUS IMAGING TECHNIQUES

Contrast-enhanced endoscopic ultrasound techniques<sup>[115,116]</sup>, real-time 3D reconstructions<sup>[86,117,118]</sup>, CEUS-guided cholangiodrainage<sup>[51]</sup> and other techniques have also been used to analyze liver tumor morphology, but the mentioned techniques are not part of this manuscript. EFSUMB is currently preparing recommendations and guidelines on the use of elastography (strain imaging methods), also dealing with liver pathology, and these will be published

soon (see also [www.efsumb.org](http://www.efsumb.org)).

## SUMMARY AND PERSPECTIVES

Computer tomography has only a limited pertinence in the characterization of liver tumors and is used essentially for staging. With CEUS, it is possible to determine whether almost all liver tumors are malignant or benign by analysis of the portal venous and late phases. Based on oncological considerations, this is followed by biopsy in patients with hypoenhancement in the portal venous and later phases, depending on whether knowledge of the histology will have a clinically applicable outcome. The predictive power of CEUS and other imaging procedures is limited in the cirrhotic liver. Attention must be paid to the special features of HCC and CCC in hepatic cirrhosis<sup>[80,84]</sup>. Dynamic ultrasound techniques<sup>[119-121]</sup> for the objectification of findings and treatment outcomes, as well as new indication ranges, are undergoing evaluation. Transient elastography for the evaluation of liver tumors is very promising<sup>[39,122-126]</sup> but has yet to establish itself as a routine method in this type of problem.

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E- Editor Li JY



## E2F transcription factors and digestive system malignancies: How much do we know?

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Supported by "Kapodistrias" Research Program, Special Accounts Research Fund 70/4/6549, National and Kapodistrian University of Athens, Greece

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Received: December 12, 2012 Revised: March 20, 2013

Accepted: March 28, 2013

Published online: June 7, 2013

### Abstract

E2F family of transcription factors regulates various cellular functions related to cell cycle and apoptosis. Its individual members have traditionally been classified into activators and repressors, based on *in vitro* studies. However their contribution in human cancer is more complicated and difficult to predict. We review current knowledge on the expression of E2Fs in digestive system malignancies and its clinical implications for patient prognosis and treatment. E2F1, the most extensively studied member and the only one with prognostic value, exhibits a tumor-suppressing activity in esophageal, gastric and colorectal adenocarcinoma, and in hepatocellular carcinoma (HCC), whereas in pancreatic ductal adenocarcinoma and esophageal squamous cell carcinoma may function as a tumor-promoter. In the latter malignancies, E2F1 immunohis-

tochemical expression has been correlated with higher tumor grade and worse patient survival, whereas in esophageal, gastric and colorectal adenocarcinomas is a marker of increased patient survival. E2F2 has only been studied in colorectal cancer, where its role is not considered significant. E2F4's role in colorectal, gastric and hepatic carcinogenesis is tumor-promoting. E2F8 is strongly upregulated in human HCC, thus possibly contributing to hepatocarcinogenesis. Adenoviral transfer of E2F as gene therapy to sensitize pancreatic cancer cells for chemotherapeutic agents has been used in experimental studies. Other therapeutic strategies are yet to be developed, but it appears that targeted approaches using E2F-agonists or antagonists should take into account the tissue-dependent function of each E2F member. Further understanding of E2Fs' contribution in cellular functions *in vivo* would help clarify their role in carcinogenesis.

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**Key words:** E2F; Colorectal cancer; Gastric cancer; Esophageal cancer; Pancreatic cancer; Hepatocellular carcinoma; Digestive system malignancies

**Core tip:** The E2F family of transcription factors has been in the focus of cancer research because its members regulate significant cellular functions related to cell cycle and apoptosis. E2Fs may act either as tumor-promoters or as tumor-suppressors, depending on the tissue. This review highlights the role of E2Fs in digestive system malignancies and their possible implication in diagnosis, treatment and prognosis.

Xanthoulis A, Tiniakos DG. E2F transcription factors and digestive system malignancies: How much do we know? *World J Gastroenterol* 2013; 19(21): 3189-3198 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3189.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3189>

## INTRODUCTION

The E2F family of transcription factors is involved in a vast number of cellular functions related to cell cycle and apoptosis. The study of E2F began in the mid 1980s when it was identified as a transcription activator of the adenoviral *E2* gene promoter<sup>[1,2]</sup>. The role of the prototype member, E2F1, in cancer was identified in the early 1990s through its ability to bind to and regulate the retinoblastoma protein pRb<sup>[3-7]</sup> with several members to follow either through their homology to E2F1 or through their association with pRb-related proteins (pocket proteins)<sup>[8-16]</sup>.

Early *in vitro* studies rose expectations that, through the traditional classification of the E2F family members into activators and repressors, accurate predictions about their contribution in human carcinogenesis could be possible, with oncogenic behavior expected for the former and tumor-suppressing function for the latter group. Nevertheless, *in vivo* studies have sunk the initial enthusiasm as their role appears to be by far more complicated<sup>[17]</sup>.

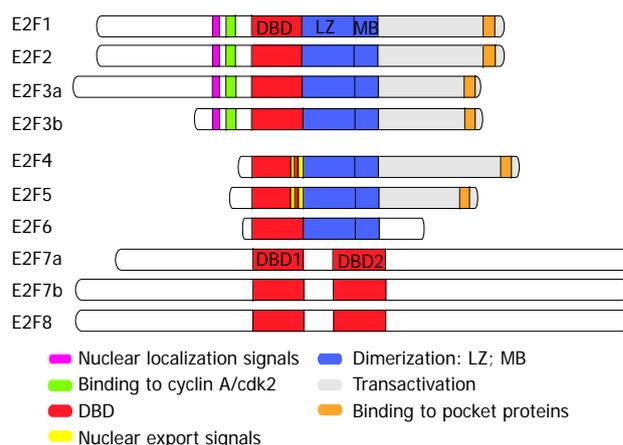
E2F1 is the most thoroughly investigated member of the E2F family in human malignancies. Other members have also been studied but in lesser extent. In non-small cell lung carcinoma, increased E2F1 and E2F3 expression has been associated with worse patient prognosis<sup>[18-21]</sup>. In breast cancer, enhanced E2F1 or E2F4 expression have been proposed as poor prognostic indicators, whereas increased E2F5 expression has been reported in certain histological subtypes<sup>[22-26]</sup>. In ovarian cancer, E2F1-5, E2F7 and E2F8 expression is reportedly increased. In addition, increased E2F4 and E2F7 expression has been related to better overall or disease-free survival, respectively, whereas that of E2F8 was linked to worse overall survival<sup>[27-30]</sup>. In prostate cancer, E2F2 and E2F3 expression increases, while E2F1 is absent<sup>[25,31]</sup>. In urothelial carcinomas of the bladder, E2F3 expression is enhanced, while that of E2F1 depends on the presence of invasion<sup>[25,32-37]</sup>. Increased E2F1 expression has also been observed in thyroid cancer, small cell lung carcinoma, glioblastoma and lymph node metastases from malignant melanoma<sup>[38-42]</sup>.

The aim of the current review is to summarize the collective knowledge on the role of various E2F family members in digestive system malignancies and to identify possible clinical implications for patients' diagnosis and prognosis and for future treatment strategy design.

## E2F STRUCTURE AND REGULATION

To date, eight members of the mammalian E2F family have been recognized and characterized<sup>[36,43]</sup>, though the most recently identified, namely E2F7<sup>[44,45]</sup> and E2F8<sup>[46,47]</sup>, bear little homology to their traditional counterparts (Figure 1). Two E2F3 proteins, E2F3a and E2F3b, have been identified, which are produced from the same gene through the use of alternate promoters<sup>[48,49]</sup>. E2F7 also has two isoforms, E2F7a and E2F7b, which are produced through by alternative splicing of the primary transcript<sup>[50]</sup>.

The greatest homology among the different members



**Figure 1 Mammalian E2F family of transcription factors.** In the context of digestive system malignancies, E2F1 may exhibit a tumor-suppressing role in colorectal, gastric and esophageal adenocarcinoma, as well as in hepatocellular carcinoma. On the contrary, in pancreatic ductal adenocarcinoma and esophageal squamous carcinoma a tumor-promoting role has been shown. A tumor-promoting role is demonstrated for E2F4 in colorectal, gastric and liver carcinogenesis. E2F8 may contribute to hepatocarcinogenesis, as it has been shown to be strongly upregulated in human hepatocellular carcinoma. DBD: DNA binding domain; LZ: Leucine zipper; MB: Marked box.

of this family is observed in the DNA binding domain. The older members E2F1-6 possess N-terminal DNA binding domain, followed by leucine zipper and marked box domains that mediate heterodimerization. With the exception of *E2F6*, the established members of the family possess a C-terminal transactivation domain containing the pocket protein binding region<sup>[36]</sup>. E2F1-3 have nuclear localization signals adjacent to their cyclin A-binding domain. This ensures their movement to the nucleus, thereby modulating E2F activity in a cell cycle-dependent manner<sup>[51-53]</sup>. E2F1-6 require heterodimerization with one of the dimerization partner (DP) proteins, DP1, DP2 or DP3 in order to form functional transcription factors which can bind DNA with high affinity<sup>[54-59]</sup>. E2F4 and E2F5 have bipartite nuclear export signals, which mediate their export to the cytoplasmic compartment and they rely on heterodimerization with one of DP proteins for their translocation to the cell nucleus<sup>[60,61]</sup>. The proteins DP2 and DP3 are thought to be alternatively spliced products of a single gene. The role of DP subunits is not completely elucidated and it appears that the function of E2F-DP heterodimer is dictated by the E2F subunit<sup>[56,57]</sup>.

The transcriptional activity of E2F1-5 is regulated through their binding with the pRb protein or the related pocket proteins p107 and p130<sup>[62-64]</sup>. E2F1-3 bind to pRb when the latter is found in its hypophosphorylated form<sup>[13]</sup>, E2F4 can bind to any of the pocket proteins, whereas E2F5 associates predominantly with p130<sup>[65,66]</sup>. Generally, when a pocket protein is hypophosphorylated can associate and block the transcriptional activity of E2F-DP heterodimers by masking the transcription activation domain of E2Fs, rendering them inactive (passive repression)<sup>[43]</sup>. Furthermore, the pocket protein/E2F-DP complex is guided to E2F binding sites where it can

recruit histone deacetylases that suppress transcription by remodelling the nucleosome (active repression)<sup>[67-70]</sup>. E2F6-8 are presumed to repress E2F-responsive genes independently of pocket proteins<sup>[10,11,50,71]</sup>.

## ROLE AND FUNCTION OF E2F TRANSCRIPTION FACTORS

E2F family members have been traditionally classified as activators and repressors. E2F1-3a are often referred to as activators because they transcriptionally activate certain target genes, for example cyclin E. This E2F subclass is expressed in a cell cycle-regulated manner exhibiting highest levels in the late G1 and S phase. In other words, they induce the entrance of quiescent cells in the S phase of cell cycle and overcome arrest mediated by the p16<sup>INK4a</sup> cyclin-dependent kinase inhibitor<sup>[43,72]</sup>. E2F3b-5 comprise the repressor subclass because their main function seems to be the repression of transcription of some E2F target genes when they associate with pocket proteins. This subgroup is expressed constitutively, but transcriptional repression by these factors takes place predominantly in cells which are in quiescence and early G1 phase<sup>[43]</sup>. E2F6-8 are also considered as repressors but they do so, as previously stated, by a manner independent of pocket protein family members<sup>[10,11,16,44-46,71]</sup>. The abovementioned classification, which is based on results of *in vitro* studies, is probably oversimplified and does not accurately reflect the dynamics of E2F-dependent transcriptional control<sup>[17,43]</sup>. The study of E2Fs' role *in vivo* has been challenging because of three main obstacles impending complete understanding of their functions. Firstly, there is a high degree of functional redundancy among activators and repressors. Secondly, there is a functional antagonism between E2F-mediated activation and repression in the regulation of normal cell proliferation. Thirdly, the members of this family have the ability to regulate each others expression, forming complex feedback loops to ensure a balance between activators and repressors in each phase of the cell cycle<sup>[17]</sup>.

## E2FS IN DIGESTIVE SYSTEM MALIGNANCIES

### Esophageal cancer

Amplification of E2F1 is often found in esophageal squamous cell carcinomas and the survival of patients with such aberration is significantly lower than that of patients without it<sup>[73]</sup>. Furthermore, positive E2F1 immunostaining correlates with histological grade and tumor stage with the overall survival being worse for patients with E2F1-positive tumors<sup>[74,75]</sup>. E2F1 expression is also shown to be positively associated with cell proliferation but not apoptosis<sup>[76]</sup>. Interestingly, the sequential transfer of the wild-type *p53* and *E2F1* genes into esophageal cancer cell lines induces tumor cell apoptosis *via* E2F1/ARF/MDM2/p53 pathway<sup>[77]</sup>. It is possible that reduced

apoptosis *in vivo* can be explained by a model suggested in non-small cell lung carcinoma, whereby tumors with deregulated E2F1/pRb network cannot promote p53-dependent apoptosis under conditions of *p53* mutation or MDM2 overexpression<sup>[19]</sup>. The situation is quite the opposite in esophageal adenocarcinomas arising in the background of Barrett esophagus. In such cases, E2F1 immunohistochemical expression exhibits a positive correlation with apoptosis and an inverse relationship with cell proliferation, implying that apoptosis is probably the tumor-suppressive mechanism activated by E2F1. The tumor-suppressing activity of E2F1 in adenocarcinomas of Barrett esophagus could be explained by the intestinal-type nature of the metaplastic mucosa and it may also be dictated by the embryological origin of the lower third of esophagus from the foregut<sup>[78]</sup>.

### Gastric adenocarcinoma

E2F1 is found to be overexpressed in about 40% of gastric adenocarcinomas, whereas gene amplification of E2F1 rarely occurs<sup>[79-81]</sup>. Experimental studies have provided evidence that adenovirus-mediated E2F1 overexpression in gastric carcinoma cells induces widespread apoptosis, probably through direct or indirect upregulation of phosphatase and tensin homolog (PTEN) expression, activation of caspase-3 and -9 and decrease in nuclear factor kappa-light-chain-enhancer of activated B cells expression *via* PI3K/PTEN/Akt signalling pathway, especially when combined with cyclin-dependent kinase inhibitors, such as roscovitine. This points to a tumor-suppressing role of E2F1 in this type of cancer<sup>[82-86]</sup>. Interestingly, at least one study has demonstrated the opposite effect, *i.e.*, downregulation of E2F1 significantly inhibited the infiltration and proliferation abilities of human gastric cancer cells. This inconsistency was attributed to the difference in activation of E2F1 at different points of the cell cycle to keep a dynamic balance<sup>[87]</sup>. E2F1 immunoreactivity was shown to independently predict favorable overall survival in gastric adenocarcinoma patients who received adjuvant chemoradiation therapy with 5-fluorouracil and leucovorin after gastrectomy and correlated with localized tumor, intestinal histological subtype and thymidylate synthase expression, supporting its role as a potential biological marker, predictive of clinical outcome in this particular setting<sup>[88]</sup>. Similar to colorectal adenocarcinomas, microsatellite unstable gastric adenocarcinomas frequently exhibit mutation of the adenosine-guanine-cytosine (AGC) repeat of the *E2F4* gene, that probably represents an early event in this context, allowing for additional gene anomalies to accumulate during tumor progression<sup>[89-94]</sup>.

### Colorectal adenocarcinoma

There are a number of studies that investigated the role of E2F family members, especially E2F1, in the context of colorectal cancer. E2F1 expression was indeed found increased in some early studies<sup>[81,95]</sup>. Later on, increased E2F1 expression was found directly related to increased

apoptotic levels and inversely related to cell proliferation, particularly when serial or semiserial sections were analyzed<sup>[25,96,97]</sup>, suggesting a tumor-suppressing role. E2F1 expression is higher in lung metastasis of colon adenocarcinoma and also correlates closely with the expression of thymidylate synthase in both the primary tumor and metastases, indicating worse response to 5-fluorouracil due to resistance<sup>[98,99]</sup>.

On the other hand, E2F4 has been directly associated with cell proliferation in colorectal cancer, suggesting a tumor-promoting role<sup>[97,100]</sup>. This finding is in agreement with experimental observations in cell line and animal models demonstrating increased nuclear expression of E2F4 in the replicating colon epithelium<sup>[101-104]</sup>. Of interest, the immunohistochemical expression of E2F1 was inversely correlated with that of E2F4 when studied in serial histological sections, suggesting a possible mechanistic interlink between the two family members that has yet to be identified<sup>[97]</sup>. An other interesting observation is that *E2F4* contains a stretch of 13 serine residues in its trans-activation domain encoded by a microsatellite trinucleotide AGC repeat within the *E2F4* gene. This repeat is often found mutated in various gastrointestinal tumors, including human colorectal cancer with microsatellite instability and it is believed to be one of the targets of DNA mismatch repair deficiency<sup>[93,105-107]</sup>.

E2F2 has only recently been investigated in colorectal adenocarcinoma. Its expression at the tissue level was found to be very low without any relationship to kinetic parameters, leading to the hypothesis that E2F2 expression does not contribute in colorectal carcinogenesis but rather reflects the functional redundancy between E2F members of the same subgroup<sup>[97]</sup>.

### Hepatocellular carcinoma

In a recent study, nuclear immunohistochemical expression for E2F1 was positively related to tumor apoptotic index in a series of human hepatocellular carcinomas, supporting a pro-apoptotic role of E2F1 in this type of cancer<sup>[108]</sup>. Interestingly, studies investigating HCC-cell lines or mouse models have provided evidence pointing towards a tumor-promoting role of E2F1 by demonstrating that its overexpression led to increased expression of upstream cell proliferation or anti-apoptotic genes<sup>[109-112]</sup>. Other investigators have demonstrated that, in a transgenic mouse model, endogenous c-myc was upregulated in the early stages of hepatocarcinogenesis, whereas p53 was overexpressed in the tumors, suggesting that both E2F1-mediated proliferation and apoptosis are operative but at different stages of hepatocarcinogenesis<sup>[113]</sup>.

E2F3 and E2F4 are also shown to be upregulated in HCC<sup>[114-116]</sup>. Of note, HCCs exhibiting microsatellite instability has shown deletions in AGC triplet repeats in the coding region of the *E2F4* gene in a similar manner as demonstrated for microsatellite unstable colorectal cancer<sup>[93,105-107,117]</sup>. These results suggest that both microsatellite instability and mutations of *E2F4* commonly occur in HCC and may play an important role in hepatocarcinogenesis<sup>[117]</sup>.

Lastly, it has been demonstrated that E2F8 is strongly upregulated in human HCC and it could thus contribute to oncogenesis and progression in this type of cancer. Mechanistic analyses indicated that E2F8 could bind to regulatory elements of cyclin D1, regulating its transcription and promoting accumulation of S-phase cells<sup>[118]</sup>.

### Pancreatic cancer

E2F1 may have a tumor-promoting role in pancreatic ductal adenocarcinoma. A direct correlation has been found between E2F1 immunohistochemical expression and cell proliferation index, as well as an inverse relationship between E2F1 immunopositivity and histological grade and disease-associated survival<sup>[119]</sup>. Interestingly, stable overexpression of E2F1 and decreased pRb expression resulting in the liberation of E2F in pancreatic cancer cell lines may be responsible for the demonstrated increase in chemotherapy-induced apoptosis<sup>[120]</sup>. Moreover, infection of pancreatic cancer cells lines by E2F1-expressing adenoviral vector has been shown to increase gemcitabine-induced apoptosis as well as etoposide- or roscovitine-induced apoptosis<sup>[121,122]</sup>.

## ISSUES IN E2F BIOLOGY

Deregulated expression of E2F family of transcription factors is a common phenomenon in human cancer. Little is known though about the magnitude and the nature of their contribution. The prevailing view is that E2F activators and repressors operate in a coordinated manner to achieve proper cell cycle progression and/or apoptosis and that disturbance of this well orchestrated interaction can contribute to carcinogenesis. According to the traditional classification of the different E2F members into activators and repressors, it would be expected that clear predictions about their function in cancer could be made. Unfortunately, this is not the case and this classical approach stemming from *in vitro* studies is openly challenged in practice<sup>[17,43]</sup>. To complicate things further, numerous E2F target genes have been identified reflecting the fact that E2Fs participate in cellular processes beyond the cell cycle<sup>[123-127]</sup>.

Regarding digestive system malignancies, E2F1 may exhibit a tumor-suppressing role in colorectal, gastric and esophageal adenocarcinoma, as well as in hepatocellular carcinoma, probably through pro-apoptotic activity<sup>[25,78,82-86,96,97,108]</sup>. On the contrary, a tumor-promoting role has been attributed to E2F1 in the context of pancreatic ductal adenocarcinoma and esophageal squamous carcinoma<sup>[73-76,119]</sup>. It is very interesting though that in experimental models involving adenoviral transfer of E2F1 in esophageal squamous and pancreatic cancer cell lines apoptosis was evoked<sup>[77,121,122]</sup>. The possible role, correlations and prognostic significance of E2F1 expression in digestive system malignancies is summarized in Table 1.

E2F4, a classical “repressor”, seems to play a tumor-promoting role in colorectal, gastric and liver carcinogenesis<sup>[90,92,93,97,117]</sup>. It is also well documented that AGC

**Table 1** Possible role, correlations and prognostic significance of increased E2F1 expression in digestive system malignancies

| Tumor                            | Role              | Clinicopathological relationships                                   | Prognostic significance | Ref.    |
|----------------------------------|-------------------|---|-------------------------|---------|
| Esophageal                       |                   |   |                         |         |
| Squamous cell carcinoma          | Tumor-promoting   | ↑ tumor stage, ↑ histological grade, ↓ overall survival             | Yes                     | [74,75] |
| Adenocarcinoma                   | Tumor-suppressing | ↑ survival  | Yes                     | [78]    |
| Gastric adenocarcinoma           | Tumor-suppressing | Localized disease, intestinal histological type, ↑ overall survival | Yes                     | [88]    |
| Colorectal adenocarcinoma        | Tumor-suppressing | ↑ survival  | Yes                     | [25,96] |
| Hepatocellular carcinoma         | Tumor-suppressing |   | No                      | [108]   |
| Pancreatic ductal adenocarcinoma | Tumor-promoting   | ↑ histological grade, ↓ disease-free survival                       | Yes                     | [119]   |

↑: Increased; ↓: Decreased.

repeats in the *E2F4* gene are commonly found mutated in various gastrointestinal malignant neoplasms exhibiting microsatellite instability and they are thought to represent one of the targets of DNA mismatch repair deficiency<sup>[89-94,105-107,117]</sup>. Of note, in colorectal cancer, an inverse relationship between the immunohistochemical expression of E2F1 and E2F4 has been demonstrated when examined in serial histological sections<sup>[97]</sup>. It would be interesting to investigate if such a relationship can be confirmed in other gastrointestinal cancers as well and, if so, whether there is a common “switch” connecting mechanistically these two opposing transcription factors.

## CONCLUSIONS AND PERSPECTIVES

E2F family of transcription factors serves key roles in cell cycle progression, apoptosis, cell differentiation and stress responses. Following their traditional classification into activators and repressors, it has been tempting to assign to them oncogenic or tumor-suppressing functions, thus predicting their role in carcinogenesis. A number of investigators have focused into showing a prognostic value of E2F expression in different kind of digestive tract malignancies<sup>[25,74-76,78,88,96,119]</sup>. Others have gone a step further and, based on their experimental work, suggest therapeutic strategies involving adenoviral transfer of *E2F* in a gene therapy context to sensitize cancer cells for conventional chemotherapeutic agents<sup>[82,121,122]</sup>.

However, clinical and experimental studies in mice openly challenge this traditional view, which does not appear to reflect the complexity of E2F function in tumorigenesis. It appears, though, that this function is exerted in a tissue-dependent manner. It is appealing to consider the development of treatment strategies involving E2F-antagonists, that would suppress cell proliferation, and E2F-agonists, that would promote apoptosis<sup>[128]</sup>. Nevertheless, targeted therapeutic approaches against E2F family members should take into account the tissue-dependent function of each member.

It is quite obvious that, although our knowledge on this intriguing family of transcription factors is seemingly increasing, little is known about their function *in vivo*. The introduction and use of modern molecular techniques and experimental models have identified numerous targets for these factors, helping us unravel the mystery of

their contribution in normal tissues. This can be the necessary step in order to clarify to which extent they exert pivotal roles in cancer development<sup>[17]</sup>.

## ACKNOWLEDGMENTS

The authors would like to thank Mrs. Maria Chatzopoulou for her kind contribution in creating Figure 1.

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**P- Reviewer** Lalli E **S- Editor** Huang XZ **L- Editor** A  
**E- Editor** Li JY



## Sofosbuvir and ABT-450: Terminator of hepatitis C virus?

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Supported by Grants from the National Key Basic Research Program of China, No. 2009CB522507, No. 2012CB519005; and Beijing Nova Program of China, No. Z12110702512071

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Received: February 20, 2013 Revised: March 31, 2013

Accepted: April 17, 2013

Published online: June 7, 2013

### Abstract

Combination therapy with peginterferon (pegIFN)- $\alpha$  and ribavirin (RBV) has been the standard of care (SOC) for chronic hepatitis C. Unfortunately, not all patients can achieve a sustained virologic response (SVR) with this regimen. SVR rates are approximately 80% in patients with hepatitis C virus (HCV) genotype 2, 3, 5 and 6 and 40%-50% in patients with genotype 1 and 4. Therefore, strategies to improve SVR rates have been an important issue for clinical physicians. Several direct acting antiviral agents (DAAs) have significantly higher SVR rates when combined with pegIFN- $\alpha$  and RBV than pegIFN- $\alpha$  and RBV alone. Treatments containing DAAs have several advantages over the previous SOC, including higher specificity and efficacy, shorter treatment durations, fewer side effects, and oral adminis-

tration. Based on these advantages, treatment with pegIFN- $\alpha$  and RBV plus telaprevir or boceprevir has become the current SOC for patients with genotype 1 HCV infection. However, many patients are either not eligible for therapy or decline treatment due to coexisting relative or absolute contraindications as well as an inability to tolerate the hematological side effects and adverse events caused by the new SOC. These factors have contributed to the advent of pegIFN- $\alpha$ -free regimens. The newest therapeutic regimens containing sofosbuvir and ABT-450 have shown promising results. In this review, we summarize the development of anti-HCV agents and the clinical efficacy of sofosbuvir and ABT-450-based therapies as well as the potential for future HCV studies.

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**Key words:** Sofosbuvir; ABT-450; Hepatitis C virus; Antiviral therapy; Sustained virologic response

**Core tip:** We are entering an era in which the development of antiviral agents and successful treatment of chronic hepatitis C is rapidly escalating. In this review, we have summarized the history of anti-hepatitis C virus (HCV) agents from interferon- $\alpha$  (IFN- $\alpha$ ) to the latest sofosbuvir- and ABT-450-based therapies. Although a new generation of direct acting anti-HCV agents has largely improved the sustained virologic response rates of patients, many unmet needs and questions remain, such as IFN-free regimens for difficult to treat patients, avoidance of cross-resistance, the role of interleukin-28B status as well as the management of some advanced and co-infected patients.

Zeng QL, Zhang JY, Zhang Z, Wang LF, Wang FS. Sofosbuvir and ABT-450: Terminator of hepatitis C virus? *World J Gastroenterol* 2013; 19(21): 3199-3206 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3199.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3199>

## INTRODUCTION

Chronic hepatitis C virus (HCV) infection is currently a major global health problem that affects 160 million people worldwide and is one of the main causes of chronic liver cirrhosis and hepatocellular carcinoma<sup>[1,2]</sup>. Compared with the former standard of care (SOC), the current SOC, consisting of peginterferon (pegIFN)- $\alpha$  and ribavirin (RBV) plus telaprevir or boceprevir, achieves higher rates of sustained virologic response (SVR), which is defined as undetectable HCV RNA in the serum for 24 wk after the end of treatment. SVR is associated with a better outcome for chronic hepatitis C (CHC) patients<sup>[3,4]</sup>. However, not all patients can achieve SVR<sup>[5,6]</sup>. In addition, triple therapy has many side effects and contraindications that result in a number of eligible patients refusing therapy<sup>[7,8]</sup>.

Based on these complications, pegIFN- $\alpha$ -free regimens could be an alternative for some patients. Several pegIFN- $\alpha$ -free regimens containing sofosbuvir and ABT-450 have shown high SVR rates with only 12 wk of treatment and mild adverse events. These regimens have the potential to be the newest SOC for CHC in the near future. Here, we summarize the clinical development history of anti-HCV agents as well as recent studies of the efficacy and adverse event profile of sofosbuvir and ABT-450 regimens. We also discuss the future focus for HCV studies.

## BRIEF DEVELOPMENT HISTORY OF ANTI-HCV AGENTS

### IFN era

Anti-HCV therapy is the backbone for the treatment of CHC. Since the late 1980s, IFN- $\alpha$  has gradually become the core of antiviral treatment<sup>[9-13]</sup>. However, IFN- $\alpha$  monotherapy achieved suboptimal efficacy until combined with RBV<sup>[14-22]</sup> (Figure 1). PegIFN- $\alpha$  has a higher plasma concentration and half-life than interferon, which results in an improved SVR rate as well as improved patient compliance as the pegylated form can be injected once weekly<sup>[23-25]</sup>. Higher SVR rates were achieved with combination therapy of pegIFN plus RBV than with pegIFN monotherapy, which has become the SOC during the past decade<sup>[26,27]</sup>. Meanwhile, the true meaning of SVR was unclear until a large cohort study demonstrated that patients who achieved SVR could be considered cured<sup>[28]</sup>. Unfortunately, not all patients, especially those infected with genotype 1 and 4 HCV could achieve SVR with the SOC. Furthermore, side effects, long-term treatment, contraindications and poor compliance all spurred the development of new agents with shorter treatment durations, fewer contraindications, oral administration, higher specificity, and fewer side effects.

### Era of direct acting antiviral agents

HCV is classified in the genus hepacivirus of the family flaviviridae. Once the virus is released into the cell, the

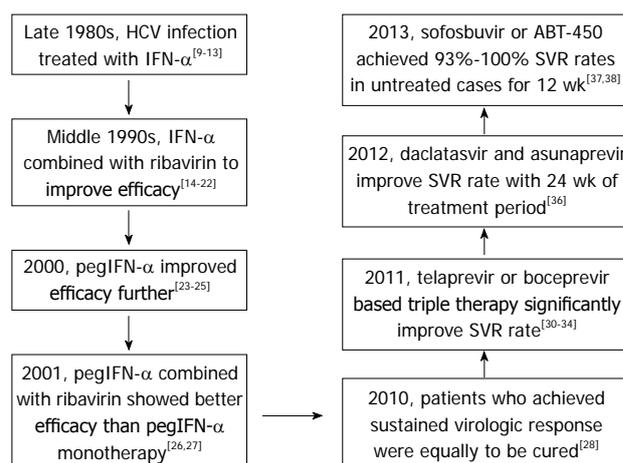


Figure 1 Brief development history of the anti-hepatitis C virus agents. HCV: Hepatitis C virus; IFN- $\alpha$ : Interferon- $\alpha$ ; SVR: Sustained virologic response; pegIFN- $\alpha$ : Peginterferon- $\alpha$ .

viral polyprotein is translated and cleaved by host proteases and the viral NS3-4A protease into ten mature proteins. Next, viral RNA is replicated into progeny RNA by the viral NS5B polymerase. Another viral protein, HCV NS5A, is indispensable for viral replication and assembly and could be a nonenzymatic target for therapeutics. With the structures of NS3/4A protease and NS5B polymerase solved and the rapid development of different cell culture models and biotechnology<sup>[29]</sup>, HCV research has been flourishing both on the bench and in industry. In particular, intensive efforts have focused on developing direct acting antiviral agents (DAAs) that can block the activity of viral enzymes.

The serine protease inhibitors telaprevir and boceprevir, approved by the United States Food and Drug Administration in 2011, were the first and currently only DAA to make it to the clinic. Telaprevir is a linear peptidomimetic HCV NS3/4A serine protease inhibitor, and boceprevir is a protease inhibitor that binds to the HCV NS3 active site. Treatment regimens consisting of telaprevir or boceprevir plus pegIFN- $\alpha$  and RBV had significantly higher SVR rates in genotype 1 patients and became the SOC thereafter<sup>[30-34]</sup>. However, this new SOC has more adverse events and a similar treatment duration when compared with the former SOC<sup>[35]</sup>, and monotherapy with telaprevir or boceprevir was shown to be impractical due to the rapid selection of resistant variants. In 2012, preliminary clinical data showed that combination therapy with the NS5A replication complex inhibitor daclatasvir and the NS3 protease inhibitor asunaprevir could also achieve high efficacy rates after 24 wk of treatment<sup>[36]</sup>, especially in prior null responders. These results showed the potential of a pegIFN-free regimen for 24 wk treatment duration. However, some patients in this study experienced viral breakthrough due to resistant variants, which ultimately resulted in treatment failure.

In January 2013, Gane *et al.*<sup>[37]</sup> and Poordad *et al.*<sup>[38]</sup> published clinical data on the efficacy of sofosbuvir- (also known as GS-7977) and ABT-450-based oral

**Table 1** Representative direct acting antiviral agent from 2011 to January, 2013

| DAA         | HCV genotype     | Course of DAA (wk) | Course of therapy (wk) | Dose                     | With or without pegIFN- $\alpha$ | Published year | Ref.    |
|-------------|------------------|--------------------|------------------------|--------------------------|----------------------------------|----------------|---------|
| Telaprevir  | 1a/1b/1c/unknown | 12/8               | 20/24/44/48            | 750 mg ( <i>tid</i> )    | With                             | 2011           | [30-32] |
| Boceprevir  | 1a/1b/unknown    | 24/32/44           | 28/36/48               | 800 mg ( <i>tid</i> )    | With                             | 2011           | [33-34] |
| Daclatasvir | 1a/1b            | 24                 | 24                     | 60 mg ( <i>qd</i> )      | With or without                  | 2012           | [36]    |
| Asunaprevir | 1a/1b            | 24                 | 24                     | 600 mg ( <i>bid</i> )    | With or without                  | 2012           | [36]    |
| Sofosbuvir  | 1/2/3            | 8/12               | 8/12                   | 400 mg ( <i>qd</i> )     | Without                          | 2013           | [37]    |
| ABT-450     | 1a/1b            | 12                 | 12                     | 250/150 mg ( <i>qd</i> ) | Without                          | 2013           | [38]    |

*qd*: Once daily; *bid*: Twice daily; *tid*: Thrice daily; DAA: Direct acting antiviral agent; HCV: Hepatitis C virus; pegIFN- $\alpha$ : Peginterferon- $\alpha$ .

**Table 2** Overview of 95 hepatitis C patients under sofosbuvir regimen

| Group | <i>n</i> | Genotype ( <i>n</i> ) | Status before treatment      | Therapeutic schedule                                | SVR rate |
|-------|----------|-----------------------|------------------------------|---|----------|
| 1     | 10       | 2/3 (4/6)             | Untreated                    | Sofosbuvir + RBV 12 wk                              | 100%     |
| 2     | 9        | 2/3 (3/6)             | Untreated                    | (Sofosbuvir + RBV 12 wk) + pegIFN $\alpha$ -2a 4 wk | 100%     |
| 3     | 10       | 2/3 (4/6)             | Untreated                    | (Sofosbuvir + RBV 12 wk) + pegIFN $\alpha$ -2a 8 wk | 100%     |
| 4     | 11       | 2/3 (4/7)             | Untreated                    | Sofosbuvir + RBV + pegIFN $\alpha$ -2a 12 wk        | 100%     |
| 5     | 10       | 2/3 (3/7)             | Untreated                    | Sofosbuvir 12 wk                                    | 60%      |
| 6     | 10       | 2/3 (0/10)            | Untreated                    | Sofosbuvir + RBV + pegIFN $\alpha$ -2a 8 wk         | 100%     |
| 7     | 10       | 1a/1b (9/1)           | No response to prior therapy | Sofosbuvir + RBV 12 wk                              | 10%      |
| 8     | 25       | 1a/1b (22/3)          | Untreated                    | Sofosbuvir + RBV 12 wk                              | 84%      |

The dose of sofosbuvir is 400 mg (*qd*), peginterferon- $\alpha$  (pegIFN- $\alpha$ )-2a is 180  $\mu$ g (*qw*), ribavirin (RBV) is 500 mg (*bid*) for patients with body weight < 75 kg, and RBV is 600 mg (*bid*) for patients with body weight > 75 kg. *qd*: Once daily; *bid*: Twice daily; *qw*: Once weekly; SVR: Sustained virologic response.

treatment regimens for 12 wk treatment duration. The results showed that 100% of genotype 2/3 patients and 93%-95% of genotype 1 patients achieved SVR. The sofosbuvir and ABT-450 studies were open-label clinical studies that assessed various combination and dosages of agents in previously treated and previously untreated patients. Sofosbuvir exhibits a higher barrier to resistance than ABT-450 because its target, the NS5B polymerase is highly conserved, and viral fitness is crippled if variants occur in the polymerase active site. Sofosbuvir also exhibits pan-genotypic antiviral activity compared to genotype-specific agents, such as ABT-450, asunaprevir, telaprevir and boceprevir. Although some mild adverse events were observed, these studies validated the feasibility of achieving extremely high SVR rates with a pegIFN-free regimen and a short treatment duration of 12 wk.

### General characteristics of representative DAAs

We summarize representative DAAs from 2011 to January 2013 in Table 1. There have been several treatment evolutions during this timeframe. First, most of these agents were designed for genotype 1 patients, with genotypes 2 and 3 only being considered recently. Second, the treatment period has gradually decreased to 12 wk. Third, dosing frequency has generally improved from three times daily to once daily, which will undoubtedly contribute to better patient compliance. Fourth, combination therapy has gradually shifted away from exclusive combinations with pegIFN- $\alpha$  to trials with and without pegIFN- $\alpha$  to finally pegIFN- $\alpha$ -free regimens. Finally, we found that at least two representative agents were developed from bench to bedside every year. Compared with other DAAs,

the sofosbuvir and ABT-450 regimens not only achieve extremely high SVR rates with 12 wk of treatment in treatment-naïve patients but are also efficacious in genotype 2 and 3 patients without the need for pegIFN- $\alpha$ .

## CLINICAL EFFICACY OF SOFOSBUVIR AND ABT-450

Sofosbuvir is a direct acting nucleotide polymerase inhibitor<sup>[39]</sup>, and ABT-450 is a potent macrocyclic HCV NS3 protease inhibitor. Both have been developed as oral therapies for the treatment of chronic HCV infection. Phosphorylated nucleotide analogues, such as sofosbuvir, are converted within the host hepatocyte to the active nucleoside triphosphate, which competes with natural nucleotides, thereby terminating RNA replication in the nascent viral genome. Sofosbuvir acts as a nonobligate chain terminator, targeting the highly conserved active site of the HCV-specific NS5B polymerase<sup>[38]</sup>. The mechanism of action of NS3 protease inhibitors has been thoroughly reviewed elsewhere<sup>[35]</sup>. As a potent inhibitor of CYP3A4 (the primary enzyme involved in first-pass metabolism of most protease inhibitors), ritonavir can increase the plasma concentration and half-life of ABT-450, decrease the emergence of resistance mutations in the NS3 gene, and permit once-daily dosing of ABT-450<sup>[38,40,41]</sup>.

### Clinical efficacy of sofosbuvir-based therapy

Gane *et al.*<sup>[37]</sup> published an open-label clinical trial of 95 previously untreated HCV genotype 1/2/3 patients and genotype 1 null responders (Table 2). The patients were

**Table 3 Overview of 50 hepatitis C patients under ABT-450 regimen**

| Group | n  | Genotype (n) | Status before treatment                 | Therapeutic schedule                      | Usage   | SVR rate <sup>2</sup> |
|-------|----|--------------|---|---|---|-----------------------|
| 1     | 19 | 1a/1b (17/2) | Untreated                               | ABT-450 + ritonavir + ABT-333 + RBV 12 wk | ABT-450, 250 mg ( <i>qd</i> )<br>Ritonavir, 100 mg ( <i>qd</i> )<br>ABT-333, 400 mg ( <i>bid</i> )<br>RBV, 1000 or 1200 mg/d <sup>1</sup> | 95%                   |
| 2     | 14 | 1a/1b (11/3) | Untreated                               | ABT-450 + ritonavir + ABT-333 + RBV 12 wk | ABT-450, 150 mg ( <i>qd</i> )<br>Ritonavir + ABT-333 + RBV Ditto  | 93%                   |
| 3     | 17 | 1a/1b (16/1) | No or partial response to prior therapy | ABT-450 + ritonavir + ABT-333 + RBV 12 wk | ABT-450, 150 mg ( <i>qd</i> )<br>Ritonavir + ABT-333 + RBV Ditto  | 47%                   |

<sup>1</sup>Body weight < 75 kg, 1000 mg/d, divided into doses of 400 and 600 mg, *bid*; Body weight ≥ 75 kg, 1200 mg/d, 600 mg, *bid*; <sup>2</sup>Sustained virologic response (SVR) in this study was defined as an hepatitis C virus RNA level of less than 25 IU/mL 12 wk after treatment. *qd*: Once daily; *bid*: Twice daily; RBV: Ribavirin.

**Table 4 Major adverse events during sofosbuvir and ABT-450 regimens**

| Adverse events                    | Sofosbuvir regimen | ABT-450 regimen <sup>1</sup> |
|-----------------------------------|--------------------|------------------------------|
| Headache                          | 32%-90%            | 14%-26%                      |
| Fatigue                           | 10%-70%            | 35%-47%                      |
| Insomnia                          | 10%-67%            | 0%-26%                       |
| Nausea                            | 0%-44%             | 21%-24%                      |
| Rash                              | 10%-60%            | 6%-21%                       |
| Anemia                            | 0%-44%             | No data                      |
| Dizziness                         | 4%-44%             | 5%-29%                       |
| Myalgia                           | 0%-40%             | No data                      |
| Diarrhea                          | 0%-30%             | No data                      |
| Vomiting                          | No data            | 0%-21%                       |
| Irritability                      | 0%-36%             | No data                      |
| Pruritus                          | 0%-33%             | 0%-21%                       |
| Decreased appetite                | 0%-50%             | No data                      |
| Upper respiratory tract infection | 0%-20%             | No data                      |
| Arthralgia                        | 0%-30%             | No data                      |
| Back pain                         | 0%-22%             | No data                      |
| Pyrexia                           | 0%-18%             | No data                      |

<sup>1</sup>Adverse events during ABT-450 regimen were only listed those that occurred in more than 20% of patients.

divided into 8 groups: sofosbuvir monotherapy and sofosbuvir plus RBV therapy with or without pegIFN- $\alpha$  for 8 or 12 wk. The results showed that 100% of previously untreated genotype 2/3 patients achieved SVR based on sofosbuvir plus RBV. For previously untreated genotype 1 patients, the SVR rate was 84%. Unfortunately, the SVR rate was only 10% for genotype 1 patients who had no response to previous therapy. These results indicate that genotype 2/3 untreated patients can be completely cured with 12 wk of sofosbuvir plus RBV therapy alone.

#### Clinical efficacy of ABT-450-based therapy

Poordad *et al.*<sup>[38]</sup> published an open-label, phase 2a clinical trial of 50 genotype 1 patients, including untreated patients and patients with no or partial response to prior therapy (Table 3). The patients were divided into 3 groups. Patients received a combination of ABT-333 (a nonnucleoside NS5B polymerase inhibitor), RBV and ritonavir plus two different dosages of ABT-450 for 12 wk. The results showed that 93% and 95% of previously untreated genotype 1 patients achieved SVR. Further-

more, 47% prior null and partial responders achieved SVR. These results indicate that almost all untreated patients with genotype 1 can achieve SVR with ABT-450-based regimens, and null and partial responders can achieve higher SVR rates than previously possible.

#### ADVERSE EVENTS OF SOFOSBUVIR AND ABT-450 REGIMENS

Both sofosbuvir and ABT-450 regimens have various adverse events (Table 4). The most frequent adverse events observed with sofosbuvir-based therapies were headache, fatigue, insomnia, nausea, rash, and anemia<sup>[37,42]</sup>. For ABT-450-based therapies, the most frequent adverse events were fatigue, nausea, headache, dizziness, insomnia, pruritus, rash, and vomiting. Some laboratory abnormalities were also observed during the treatment period, including anemia for sofosbuvir-based treatment and hyperbilirubinemia for ABT-450-based therapy. Some laboratory abnormalities were more common among patients receiving pegIFN- $\alpha$ -2a. Most adverse events and abnormalities were mild, and none led to treatment interruption.

#### INTERLEUKIN-28B POLYMORPHISM IN THE SOFOSBUVIR AND ABT-450 ERA

Genome-wide association studies have demonstrated that single nucleotide polymorphisms near the interleukin-28B (*IL-28B*) gene that encodes IFN- $\lambda$ 3 are closely associated with spontaneous and treatment-induced HCV clearance<sup>[43-46]</sup>. The rs12979860 CC genotype is associated with a two-fold greater SVR rate than the TT genotype in European-American individuals. Similar ratios have been observed in both African-American and Hispanic populations of genotype 1 chronic hepatitis C patients. The presence of the C-allele is always accompanied by higher SVR rates, indicating that this allele may favor the clearance of HCV. In studies of sofosbuvir and ABT-450, five groups achieved SVR rates of 100%, independent of IL-28B status (Table 5). Therefore, lower SVR rates may not be primarily due to *IL-28B* genotypes. Instead, these patients may have acquired resistant variants during treatment. However, it should be noted that the sample size

**Table 5 Interleukin-28B polymorphism in sofosbuvir and ABT-450 era**

| Group      | Status before treatment                 | IL-28B CC (n) | IL-28B CT (n) | IL-28B TT (n) | SVR rate |
|------------|---|---------------|---------------|---------------|----------|
| Sofosbuvir |   |               |               |               |          |
| 1          | Untreated                               | 5             | 4             | 1             | 100%     |
| 2          | Untreated                               | 4             | 4             | 1             | 100%     |
| 3          | Untreated                               | 4             | 4             | 2             | 100%     |
| 4          | Untreated                               | 4             | 5             | 2             | 100%     |
| 5          | Untreated                               | 2             | 6             | 2             | 60%      |
| 6          | Untreated                               | 3             | 6             | 1             | 100%     |
| 7          | No response to prior therapy            | 2             | 5             | 3             | 10%      |
| 8          | Untreated                               | 11            | 12            | 2             | 84%      |
| ABT-450    |   |               |               |               |          |
| 1          | Untreated                               | 10/9          | 7/7           | 2/2           | 95%      |
| 2          | Untreated                               | 5/4           | 7/7           | 2/2           | 93%      |
| 3          | No or partial response to prior therapy | 0/0           | 12/6          | 5/2           | 47%      |

IL-28B: Interleukin-28B; SVR: Sustained virologic response.

**Table 6 Outcome of representative direct acting antiviral agent-based therapy for genotype 1 null responders**

| Authors                              | n  | Therapeutic schedule  | SVR rate |
|--------------------------------------|----|---|----------|
| Zeuzem <i>et al</i> <sup>[32]</sup>  | 37 | (pegIFN- $\alpha$ 2a + RBV) 4 wk + (pegIFN- $\alpha$ 2a + RBV + telaprevir) 12 wk + (pegIFN- $\alpha$ 2a + RBV) 32 wk | 33%      |
| Bacon <i>et al</i> <sup>[33]</sup>   | 58 | (pegIFN- $\alpha$ 2b + RBV) 4 wk + (pegIFN- $\alpha$ 2b + RBV + boceprevir) 44 wk                                     | 52%      |
| Lok <i>et al</i> <sup>[36]</sup>     | 11 | Daclatasvir + asunaprevir 24 wk   | 36%      |
| Lok <i>et al</i> <sup>[36]</sup>     | 10 | Daclatasvir + asunaprevir + pegIFN- $\alpha$ 2a + RBV 24 wk   | 90%      |
| Gane <i>et al</i> <sup>[37]</sup>    | 10 | Sofosbuvir + RBV 12 wk  | 10%      |
| Poordad <i>et al</i> <sup>[38]</sup> | 7  | ABT-450 + ritonavir + ABT-333 + RBV 12wk  | 43%      |

pegIFN- $\alpha$ : Peginterferon- $\alpha$ ; SVR: Sustained virologic response; RBV: Ribavirin.

of these studies too small to reach a definitive conclusion on the role of IL-28B. Whether *IL-28B* genotype will be a predictive marker for treatment response with the new drug regimens requires further investigation with large sample sizes.

## PERSPECTIVES

### **Genotype 1 patients with no or partial response to prior therapy will be the focus of future studies**

Currently, the highest SVR rate observed in genotype 1 patients who have no response to prior therapy is 90%, which was achieved with daclatasvir and asunaprevir-based therapy (Table 6). SVR rates in this patient population treated with telaprevir and boceprevir regimens were only 33% and 52%, respectively, which was significantly inferior to the rates in treatment-naïve patients. Sofosbuvir plus RBV has achieved an excellent SVR rate in untreated genotype 1 patients compared with genotype 1 patients who had no response to previous treatment. While the ABT-450 regimen achieved high efficacy in genotype 1 patients with no or partial response to prior treatment, the SVR rate was still less than 50%. All of the above-mentioned results suggest that genotype 1 patients with no or partial response should be the focus of further investigation. Furthermore, genotype 1a patients have more opportunities to develop resistance to the DAAs combination from Poordad *et al*<sup>[38]</sup>. Genotype 1a accounts for 89%, 8 out of 9 patients with virologic failure

in group 3 who have been analyzed for the presence of resistance-associated variants. The genetic barrier to resistance of protease inhibitors is relatively lower for subtype 1a because this subtype only requires one nucleotide substitution to generate resistance, whereas the 1b virus requires two. Accordingly, genotype 1a patients may be more difficult to treat in new DAAs era.

### **DAA combinations with less cross-resistance may be the solution for genotype 1 patients with no or partial response to prior therapy**

Although it has not been investigated in a head-to-head study, genotype 1 patients with no or partial response to prior therapy have different SVR rates (10% vs 90%) with different DAA combinations (Table 6)<sup>[37,38]</sup>, indicating that different DAA combinations might be important to successfully treating these types of patients. Various combinations of DAAs are now under investigation<sup>[47,48]</sup>. However, why a particular combination may lead to an improved SVR rate in these patients remains unclear. It is possible that adding another DAA could complement the mechanisms of action of other agents in the regimen as well as decrease the appearance of cross-resistant variants. As Table 6 shows, 90% of genotype 1 patients with no response to prior therapy can achieve SVR<sup>[33]</sup>, suggesting that a potent DAA combined with pegIFN- $\alpha$  and RBV may be one choice for those patients who can endure the adverse events and long period of treatment.

### Combinations with less cross-resistance are the goal for the future

Various DAA combinations have now been investigated, such as asunaprevir plus daclatasvir; sofosbuvir plus RBV; sofosbuvir plus daclatasvir; faldaprevir plus BI207127; ABT-450/ritonavir plus ABT-333; ABT-450, ritonavir plus ABT-072; miracitabine, danoprevir plus ritonavir; and alisporivir plus RBV<sup>[47]</sup>. DAA combinations not only have the potential to increase antiviral efficacy but also to reduce the risk of viral breakthrough. When combining DAAs, it is important to consider combinations that have a low propensity for cross-resistance. The genetic barriers to resistance of DAAs appear to be an important factor during the development of resistance. When two agents with a low genetic barrier to resistance are combined, breakthrough occurs more quickly<sup>[49]</sup>. Adding on pegIFN- $\alpha$  or RBV<sup>[36]</sup> as well as nucleoside analogues with a higher genetic barrier might be better tactics for overcoming resistance<sup>[37,50]</sup>. For example, 90% of difficult-to-treat patients can achieve SVR with a combination of daclatasvir, asunaprevir, pegIFN- $\alpha$ 2a and RBV for 24 wk of treatment<sup>[36]</sup>. Furthermore, 84%-100% of patients can achieve SVR with a combination of sofosbuvir plus RBV for 12 wk of therapy<sup>[37]</sup>.

### PegIFN- $\alpha$ : To be with or not to be with

Whether or not to include pegIFN- $\alpha$  is a key issue in the DAA era. Several years ago, many hepatologists believed that HCV treatment would be IFN free. Now, however, the ability to do away with IFN is not so clear, especially in some difficult-to-treat patients. Although not compared in a head-to-head study, the SVR rate was relatively lower in refractory patients treated with a pegIFN-free regimen, as shown in Table 6. This information indicates that IFN-free regimens may be available for easy-to-treat patients in the near future, whereas IFN might be necessary for difficult-to-treat patients.

### Role of IL-28B in reducing treatment duration

In Tables 2 and 5, groups 4 and 6 differ with respect to treatment duration (12 and 8 wk, respectively). In group 4, 81% (9/11) of patients were genotype CC or CT. In group 6, 90% (9/10) of patients were genotype CC or CT. It will be interesting to investigate whether IL-28B polymorphisms could predict treatment duration in the DAA era, especially in patients with the potential to reduce the treatment course.

### Other aspects

In addition to above-mentioned situations, results from group 5 patients who received sofosbuvir monotherapy suggested the crucial role of RBV in maintaining an antiviral response (Table 2). However, the exact mechanism by which RBV contributes to SVR in the DAA era remains uncertain. Another area of important research in the future will likely be treatment of patients with cirrhosis, and hepatitis B virus and human immunodeficiency virus co-infected patients. Although the sofosbuvir- and ABT-450-

based therapies were well tolerated, the safety profile of other combination treatments remains to be seen.

## CONCLUSION

A series of clinical trials have demonstrated that we are currently experiencing a “watershed moment” for the treatment of hepatitis C<sup>[51,52]</sup>. Despite some unresolved questions, the recent achievements demonstrating DAAs as potent new HCV clearing agents with improved SVR rates appear to be encouraging, and it may be possible to cure nearly all HCV-infected patients in the near future. The progress of new anti-HCV agents might indicate that agents with specificity, sensitivity and a high barrier to resistance are the mainstay for conquering pathogen-related disease. Future studies may focus on the improvement of SVR rates in genotype 1 patients who have no or partial response to prior therapy as well as special patient populations, such as those with cirrhosis or co-infected patients. Furthermore, determining an optimal combination therapy with little cross-resistance and few adverse events as well as better understanding the status of IL-28B polymorphism and the potential mechanism of how RBV may synergize with DAAs are also areas of future study.

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**P- Reviewers** Estrabaud E, Quer J **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Li JY



## Epidemiology, clinical-treatment patterns and outcome in 256 hepatocellular carcinoma cases

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Received: November 28, 2012 Revised: January 30, 2013

Accepted: March 15, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To analyze the epidemiology, clinical characteristics, treatment patterns and outcome in hepatocellular carcinoma (HCC) patients.

**METHODS:** We analyzed clinical, pathological and therapeutic data from 256 consecutive patients, examined at S. Croce Hospital in Cuneo-Piedmont, with a diagnosis of HCC between 30<sup>th</sup> June 2000 and 1<sup>st</sup> July 2010. We analyzed the hospital imaging database and examined all medical records, including the diagnosis code for HCC (155.0 according to the ICD-9M classification system), both for inpatients and outpatients, and discovered 576 relevant clinical records. After the exclusion of reports relating to multiple admissions for the same patient, we identified 282 HCC patients. Moreover, from this HCC series, we excluded 26 patients: 1 patient because of an alternative final diagnosis, 8 patients be-

cause of a lack of complete clinical data in the medical record and 17 patients because they were admitted to different health care facilities, leaving 256 HCC patients. To highlight possible changes in HCC patterns over the ten-year period, we split the population into two five-year groups, according to the diagnosis period: 30<sup>th</sup> June 2000-30<sup>th</sup> June 2005 and 1<sup>st</sup> July 2005-1<sup>st</sup> July 2010. Patients underwent a 6-mo follow up.

**RESULTS:** Two hundred and fifty-six HCC patients were included (male/female 182/74; mean age 70 years), 133 in the first period and 123 in the second. Hepatitis C virus (HCV) infection was the most common HCC risk factor (54.1% in the first period, 50.4% in the second;  $P = 0.63$ ); in the first period, 21.8% of patients were alcoholics and 15.5% were alcoholics in the second period ( $P > 0.05$ ); the non-viral/non-alcoholic etiology rate was 3.7% in the first period and 20.3% in the second period ( $P < 0.001$ ). Child class A patients increased significantly in the second period ( $P < 0.001$ ). Adjusting for age, gender and etiology, there was a significant increase in HCC surveillance during the second period ( $P = 0.01$ ). Differences between the two periods were seen in tumor parameters: there was an increase in the number of unifocal HCC patients, from 53 to 69 ( $P = 0.01$ ), as well as an increase in the number of cases where the HCC was  $< 3$  cm [from 22 to 37 ( $P = 0.01$ )]. The combined incidence of stage Barcelona Clinic Liver Cancer 0 (very-early) and A (early) HCC was 46 (34.6%) between 2000-2005, increasing to 62 (50.4%) between 2005-2010 ( $P = 0.01$ ). Of the patients, 62.4% underwent specific treatment in the first group, which increased to 90.2% in the second group ( $P < 0.001$ ). Diagnosis period ( $P < 0.01$ ), Barcelona-Clinic Liver Cancer stage ( $P < 0.01$ ) and treatment *per se* ( $P < 0.05$ ) were predictors of better prognosis; surveillance was not related to survival ( $P = 0.20$ ).

**CONCLUSION:** This study showed that, between 2000-2005 and 2005-2010, the number of HCV-related

HCC decreased, non-viral/non alcoholic etiologies increased and of surveillance programs were more frequently applied.

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**Key words:** Hepatocellular carcinoma; Epidemiology; Clinical characteristics; Surveillance; Survival

**Core tip:** Hepatocellular carcinoma (HCC) is the 5<sup>th</sup> most common cancer and the 3<sup>rd</sup> leading cause of cancer mortality worldwide. In recent decades, the incidence of HCC has risen in Europe and the United States. This study showed that, between 2000-2005 and 2005-2010, the number of hepatitis C virus-related HCC decreased, non-viral/non alcoholic etiologies increased and of surveillance programs were more frequently applied.

Fenoglio L, Serraino C, Castagna E, Cardellicchio A, Pomero F, Grosso M, Senore C. Epidemiology, clinical-treatment patterns and outcome in 256 hepatocellular carcinoma cases. *World J Gastroenterol* 2013; 19(21): 3207-3216 Available from: URL: <http://www.wjnet.com/1007-9327/full/v19/i21/3207.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3207>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5<sup>th</sup> most common cancer and the 3<sup>rd</sup> leading cause of cancer mortality worldwide<sup>[1]</sup>. In recent decades, the incidence of HCC has risen in Europe and the United States; this trend may be explained to several factors, such as increased surveillance programs for HCC-high risk patients, improved management of chronic liver disease, and an epidemic of hepatitis C virus (HCV) infection in the late 1970s<sup>[2]</sup>. HCC related to HCV infection is a preventable disease whose incidence can be decreased by controlling HCV transmission; in Japan, the incidence of HCC began to decrease by 2000, mainly because of decreased HCV-related cases<sup>[3]</sup>. Considering the current provision of HCV prevention programs in Europe, can a similar decrease of HCV-related HCC be expected in the future?

HCC occurs two to four times more often among men than women, and within an established background of chronic liver disease (fibrosis or cirrhosis). Hepatitis B virus (HBV) and HCV infections are known independent risk factors for HCC, as well as alcohol consumption<sup>[4]</sup>. Nevertheless, 5%-30% of patients with HCC lack a defined, identifiable risk factor. Recently, it was suggested that non-alcoholic steatohepatitis (NASH) might account for a substantial portion of cryptogenic cirrhosis and HCC cases<sup>[5]</sup>. NASH is a more severe form of non-alcoholic fatty liver disease (NAFLD) that is associated with obesity, diabetes, dyslipidemia and insulin resistance<sup>[5]</sup>. International guidelines recommend surveillance for patients at high risk for HCC<sup>[6,7]</sup>, yet surveillance is not recommended in patients at risk for NASH.

Non-randomized trials and observational studies reported a survival benefit in small HCC recognized within surveillance programs, but these studies had unavoidable biases<sup>[8]</sup> and the evidence supporting surveillance efficacy in term of improving survival is limited. However, surveillance using 6-mo ultrasound (US) best identifies liver nodules less than 1 cm, and small HCC could benefit from more effective treatment<sup>[9]</sup>. Certain HCC treatments are potentially curative depending on cancer stage, liver function and performance status, as well as on resources and level of practitioner expertise<sup>[10]</sup>. The Barcelona Clinic Liver Cancer (BCLC) staging system is widely accepted in clinical practice and was adopted as the reference system by the American Association for the Study of Liver Disease (AASLD) in 2010<sup>[7,11]</sup>. The BCLC system includes data on patient's performance status, the number and size of nodules, cancer symptoms and liver function. The BCLC stratifies patients into separate prognostic categories and suggests treatment options according to the stage<sup>[11,12]</sup>. Patients with localized stage HCC could benefit from curative therapies, such as resection, percutaneous ablation or liver transplantation, whereas patients with intermediate or advanced stages may benefit from non-curative treatments, such as chemoembolization. Recent studies demonstrating that chemoembolization improves survival in well-selected patients with unresectable HCC<sup>[13]</sup> have led to a greater propensity for chemoembolization treatment, as well as a modification of indication to treatment, such as inclusion of more patients with well-compensated disease and advanced tumors<sup>[14]</sup>.

We created a database of consecutive HCC patients diagnosed in S. Croce General Hospital (Cuneo, Italy) from June 2000 to July 2010. Using this database, we designed the present study to assess clinical characteristics, treatment patterns and outcomes in patients with HCC.

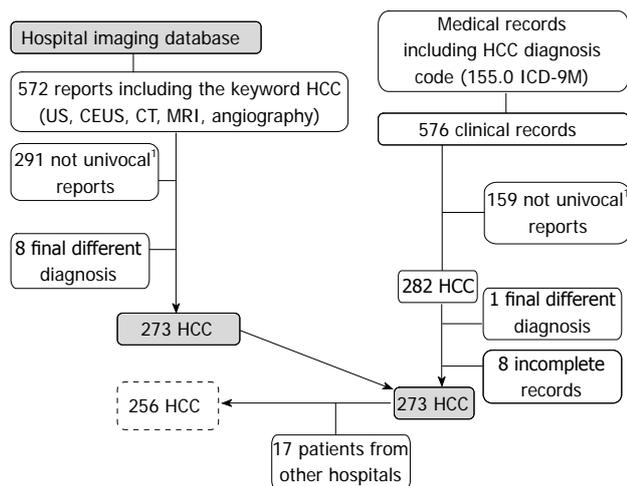
## MATERIALS AND METHODS

### Patients

Our population was a consecutive series of patients, examined at S. Croce Hospital in Cuneo, Piedmont, with a diagnosis of HCC between 30<sup>th</sup> June 2000 to 1<sup>st</sup> July 2010. All cases with initial HCC diagnosis were considered eligible; incident diagnosis as well as diagnosis was carried out during surveillance.

We analyzed the hospital imaging database and found 572 reports that included the keyword HCC. After exclusion of reports performed for the same patient and reports presenting a different final diagnosis (despite the presence of the keyword), we identified 273 actual HCC patients.

To better identify all the HCC patients referred to our hospital, we examined all medical records that included the diagnosis code for HCC (155.0 according to the ICD-9M classification system), both for inpatients and outpatients; we discovered 576 clinical records, and after exclusion of records presenting the same name (multiple admissions for the same patient), we identified 282 actual



**Figure 1** Selection of hepatocellular carcinoma patients. <sup>1</sup>Not-univocal: Reports/records repeated/belonging for/to patients already recruited. US: Ultrasound; CEUS: Contrast enhanced ultrasound; CT: Computed tomography; MRI: Magnetic resonance imaging; HCC: Hepatocellular carcinoma.

HCC patients. Nine patients were excluded from this series: one patient because of an alternative final diagnosis and eight patients because of lack of complete clinical data in the medical record (Figure 1).

To highlight possible changes in HCC patterns over the ten-year period, we split the population into two five-years groups, according to the diagnosis period: 30<sup>th</sup> June 2000-30<sup>th</sup> June 2005 and 1<sup>st</sup> July 2005-1<sup>st</sup> July 2010.

Patients were classified according to the cause of liver disease as follows: (1) HBV, if patients were hepatitis B surface antigen-positive; (2) HCV, if patients were serum antibody anti-HCV positive; (3) alcoholic, if the daily ethanol intake was > 60 g for women and > 80 g for men for more than 10 years; (4) multi-etiology, if there was a combination of these causative factors; and (5) others, when the cause was different from those cited above (as primary biliary cirrhosis, autoimmune hepatitis, hemochromatosis, *etc.*).

On admission, clinical (general conditions, presence of ascites, jaundice, *etc.*), biochemical (routine biochemical tests, liver function tests, alpha-fetoprotein, *etc.*) and imaging (US, computed tomography, magnetic resonance imaging, hepatic arterial angiography) parameters were assessed.

The HCC database included personal data, etiology of hepatic liver disease, biochemical tests, imaging features (number of nodules, size, presence of ascites, presence of hepatic thrombosis, and presence of metastasis), surveillance application (defined by two US examinations over the last 12 mo)<sup>[7]</sup> and Child Pugh score<sup>[15]</sup>.

We collected the following data regarding HCC treatment: type, number of procedures, number of admissions, days of hospitalization, treatment complications, presence of post-embolization syndrome (defined by the onset of fever, abdominal pain, moderate degree of ileus, moderate cholestasis and transaminase elevation, self-limited in less than 48 h)<sup>[7]</sup>.

### Diagnosis and severity of chronic liver disease

Diagnosis of cirrhosis was made by clinical (endoscopic and/or US signs of portal hypertension, and/or a nodular margin of the liver at US examination) and laboratory features; when possible and/or indicated, cirrhosis was confirmed by histology. The severity of liver dysfunction was scored according to the Child-Pugh classification<sup>[15]</sup>.

### Diagnosis and staging of HCC

Diagnoses of HCC were carried out according to internationally accepted criteria<sup>[6,7]</sup> and, if required, confirmed by cytology and/or histology.

HCC was classified as unifocal, paucifocal ( $\leq 3$  nodules), multifocal ( $> 3$  nodules), infiltrative and/or massive (infiltrating pattern of growth and/or a huge mass with a diameter  $> 10$  cm and an undefined boundary)<sup>[16]</sup>. The tumor size of expanding nodules was also measured (in cases of multinodular tumors, the largest one was measured).

HCC stage was scored according to the BCLC staging system<sup>[11]</sup>, recently validated by the AASLD<sup>[7]</sup>.

### Statistical analysis

Univariate associations were tested using the Pearson  $\chi^2$  test for proportions. Multivariate logistic regression models were fitted. All tests were two-sided with a significance level of  $P < 0.05$ .

Survival was calculated from the time of cancer diagnosis to death, with values censored at the date of the last follow up. The Kaplan-Meier method was used to estimate the cumulative probability of survival according to surveillance (yes *vs* no), BCLC stage (very-early and early *vs* others) and treatment (yes *vs* no). Difference between survival curves was assessed using the Log-Rank test; a  $P$  value  $< 0.05$  was considered significant.

A Cox proportional hazard model was fitted to test the role of prognostic factors associated with probability of death in the univariate analysis. All statistical analyses were performed using the SAS statistical package (SAS Institute, Cary, NC, United States).

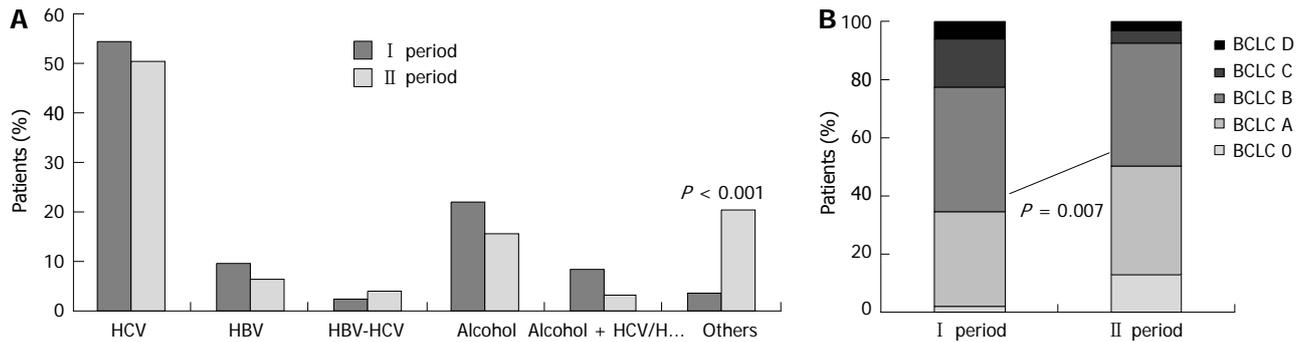
## RESULTS

Two hundred and seventy-three consecutive HCC patients was identified over the study period, but 17 patients were admitted to different health care facilities and were excluded because of difficulty in accessing to medical records and clinical data (Figure 1).

Ultimately, 256 HCC patients were included, 133 in the first group (2000-2005) and 123 in the second group (2005-2010).

### Clinicopathological features

The median age was 70-year-old (range 32-92 years), with no difference between the two groups. The male/female ratio was 182/74. Chronic liver disease was present in 252 cases (98.4%); cirrhosis in 234 (91.4%) and chronic hepatitis in 18 cases (7%). Four patients presented histologically normal livers.



**Figure 2** Different liver disease etiologies and distribution of Barcelona Clinic Liver Cancer stages in the first (I) period (2000-2005) and in the second (II) period (2005-2010). A: Different liver disease etiologies; B: Distribution of Barcelona Clinic Liver Cancer (BCLC) stages. HCV: Hepatitis C virus; HBV: Hepatitis B virus.

According to the Child-Pugh score, 159 cirrhotic patients (62.1%) were class A, 83 patients (32.4%) were class B and 14 patients (5.5%) were class C. Comparing the two groups, there were more class A patients in the second period (67 cases *vs* 92 cases,  $P < 0.001$ ). The distribution of different Child classes was not related to gender ( $P = 0.41$ ) or to age ( $P = 0.37$ ), but Child class B was more represented among alcoholic patients ( $P = 0.007$ ).

The most common cause of liver disease was HCV infection (134 cases, 52.3%), with a higher incidence in women than in men ( $P < 0.001$ ). The second most common HCC risk factor was alcoholic liver disease (48 cases, 18.7%), more frequently in men than in women ( $P = 0.01$ ). HBV infection was identified in 21 cases (8.2%), and an HBV-HCV infection was present in only eight patients (3.1%). Moreover, 15 patients presented a virus infection (6 patients with HBV infection and 9 patients with HCV infection) associated with alcohol consumption. Finally, in 30 cases, the cause of liver disease was different: 4 cases presented a diagnosis of primary biliary cirrhosis (1.6%), 2 cases of autoimmune hepatitis (0.8%), 3 cases of hereditary hemochromatosis (1.2%). Twenty-one cases (8.2%) presented only type 2 diabetes mellitus as potential risk factor for liver disease; however, none of these patients underwent a liver biopsy to confirm the presence of NAFLD-NASH. Comparing the two time periods by etiology (Figure 2A), we noticed a decreasing trend of HCV-related HCCs (72 cases *vs* 62 cases,  $P = 0.63$ ) in the second period, with a parallel increase of HCCs not related to viral infection nor to alcohol (5 cases *vs* 25 cases,  $P < 0.001$ ); in particular, there were significantly more patients with diabetes and HCC in the second period (3 cases *vs* 18 cases,  $P < 0.001$ ).

The diagnosis of HCC was made by histology and/or cytology in 55 patients and by imaging in 201 cases.

Alpha-fetoprotein (AFP) was  $> 100$  ng/dL in 59 cases (23%) and  $> 200$  ng/dL in 74 cases (28.9%), while in the remaining patients, the AFP value was not available.

Sixty-one cases (23.8%) were diagnosed in patients undergoing semiannual US surveillance, while the remaining 195 cases (76.2%) were diagnosed in patients occasionally submitted to US or during an US follow-up at an interval longer than 6 mo. Adjusting for age, gender, etiology and

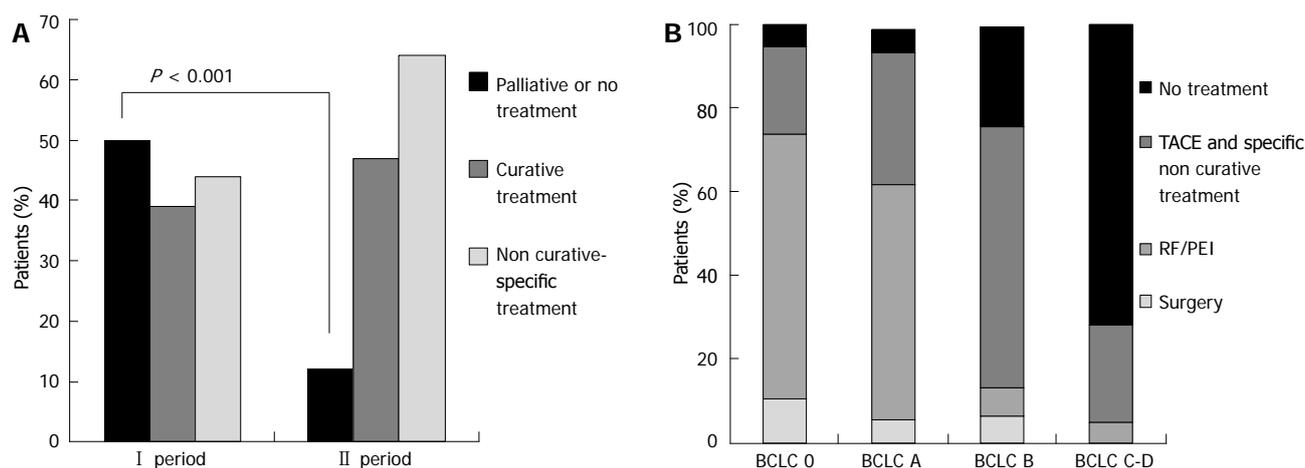
Child-Pugh class, the probability to undergo surveillance was inferior among alcoholic patients ( $P = 0.002$ ) and patients with “other causes” of liver disease ( $P < 0.001$ ). Comparing the two groups and after adjusting for age, gender, etiology, surveillance was more relevant in the second period than in the first ( $P = 0.01$ ).

At the time of diagnosis, most HCCs were unifocal (122 cases, 47.7%), 55 HCCs were paucifocal (21.4%), 62 HCCs were multifocal (24.3%) and 17 HCCs were massive (6.6%). The most common pattern of presentation of HCC over the whole study period was a single nodule more than 3 cm, but less than 5 cm, in diameter (128 cases, 50%). In the multivariate analysis, the probability of discovering a unifocal HCC and an HCC  $< 3$  cm in diameter was higher in patients undergoing surveillance ( $P = 0.001$  and  $P = 0.01$ , respectively) as well as in the second group ( $P = 0.02$  and  $P = 0.001$ , respectively). Portal thrombosis was detected in 29 patients (11.3%), more commonly in young patients ( $< 65$  years,  $P = 0.045$ ), in Child class B and C patients ( $P = 0.017$ ) and in multifocal HCCs ( $P = 0.02$ ). Extra-hepatic metastases were present in 19 patients (7.4%), whereas ascites were detected in 68 cases (26.6%).

Patients were grouped into five different stages according to the BCLC system (Figure 2B). Nineteen patients (7.4%) belonged to stage BCLC 0 (very-early), 89 (34.7%) to stage BCLC A (early), 109 (42.6%) to stage BCLC B (intermediate), 27 (10.6%) to stage BCLC C (advanced) and 12 (4.7%) to stage BCLC D (terminal). In the multivariate analysis, the probability of discovering a very early/early HCC (BCLC 0 or A) was higher in patients undergoing regular 6-mo surveillance ( $P = 0.003$ ) and in the second period ( $P = 0.007$ ).

### Treatment

Sixty-two patients (24.2%) received palliative care or no therapy. The untreated patient cohort presented with more advanced HCC (BCLC B  $P = 0.003$ ; BCLC C-D  $P < 0.001$ ) and a more elevated bilirubin value ( $P = 0.05$ ). There was a slight, but non-significant, trend to treat fewer women than men ( $P = 0.079$ ), and patients aged  $> 65$  years ( $P = 0.053$ ). Treatment distribution was not related to etiology.



**Figure 3** Treatment distribution in the two different periods and different Barcelona Clinic Liver Cancer stages. A: Treatment distribution comparing the two 5-year periods (2000-2005 vs 2005-2010); B: Distribution of treatments in different Barcelona Clinic Liver Cancer (BCLC) stages. TACE: Trans-arterial chemoembolization; RF/PEI: Radiofrequency/percutaneous ethanol injection.

Comparing the two 5-year periods, in the first period 50 patients (37.6%) did not receive any specific treatment for HCC, whereas in the second period, only 12 patients received no specific treatment (9.8%,  $P < 0.001$ ) (Figure 3A). This decrease was accounted for mainly by HCCs in the intermediate-advanced stage (BCLC B,  $P = 0.007$ ; BCLC C-D,  $P = 0.01$ ).

One hundred and ninety-four patients (75.8%) received HCC-specific treatment. During 2000-2005, 83 patients (62.4%) received a specific treatment, compared to 111 patients (90.2%) during 2005-2010. When adjusting for gender, age, etiology, BCLC stage and surveillance, the probability to receive specific treatment was higher in the later time period ( $P < 0.001$ ).

Among treated patients, 86 (44.3%) underwent curative procedures: surgical resection in 14 cases, percutaneous ablation in 70 cases [percutaneous ethanol injection in five cases, radiofrequency (RF) in 65 cases], and orthotopic liver transplantation (OLT) only in two cases. In three cases it was impossible to perform RF despite the initial indication because of the onset of pain during the procedure (two cases) or HCC location. These three patients underwent trans-arterial chemoembolization (TACE). In 2000-2005, 39 of 83 treated patients (47%) underwent a curative treatment; in the second period it was 47 of 111 treated patients (42.3%,  $P = 0.61$ ). Interestingly, over the entire study period, only 86 of 108 patients (79.6%) suitable for curative treatment according to BCLC stage effectively underwent it, whereas 15 patients (14%) underwent TACE and seven patients (6.5%) did not receive any specific treatment. In multivariate analysis, curative treatments were less probable in the intermediate-advanced stage (BCLC B,  $P = 0.01$ ; BCLC C-D,  $P = 0.007$ ), but there was no difference in curative treatment distribution according to gender, age, etiology and surveillance. In 2000-2005, 69.6% of potentially curable cases underwent a curative treatment, while 21.7% received only TACE and the remaining 8.7% did not receive any specific treatment. In 2005-2010, the

proportion of treated patients decreased to 61.3% ( $P = 0.04$ ), with 32.3% of potentially curable patients receiving TACE and 6.4% not receiving any treatment.

Over the entire study period, among treated patients, 108 (55.6%) underwent non-curative specific treatment: TACE (conventional or Drug-Eluting-Beds TACE) was performed in 90 cases, selective internal radiotherapy (SIRT) in four cases, chemotherapy and/or hormone-therapy in 14 cases. Interestingly, 44 patients who underwent TACE (48.8%) were in BCLC 0-A, *i.e.*, in curable stages (Figure 3B).

At multivariate analysis, TACE application was more common in patients aged  $< 65$  years ( $P = 0.04$ ), while it was less common in advanced-terminal stage (BCLC C-D  $P = 0.03$ ) and in patients with bilirubin  $> 2$  mg/dL ( $P = 0.02$ ); there was no difference in TACE application according to gender, etiology or surveillance.

Thirty-four patients received TACE (77.4% of a total of 44 patients receiving specific non-curative treatment; seven patients underwent chemotherapy and three patients underwent hormone-therapy) in the first period and 56 (87.5% of a total of 64 patients receiving specific non-curative treatment; four patients underwent chemotherapy and four patients underwent SIRT) in the second period ( $P = 0.25$ ). However, after adjusting for gender, age, etiology, BCLC stage and surveillance, the probability to receive TACE was higher in the second period ( $P = 0.01$ ). TACE application during the second period increased mainly for BCLC B ( $P = 0.001$ ), but also in BCLC 0-A ( $P = 0.04$ ).

Only 6 patients were treated with Sorafenib, all of them during the second period.

The prevalence of post-embolization syndrome was 20.4% (50 cases over 245 loco-regional procedures), with no significant differences between the two groups ( $P = 0.07$ ). Interestingly, in the first period, an antibiotic therapy (without any clinical and/or laboratory suspicion of infectious complication) was started in only six cases (31.6% of all cases), while in the second period, antibiot-

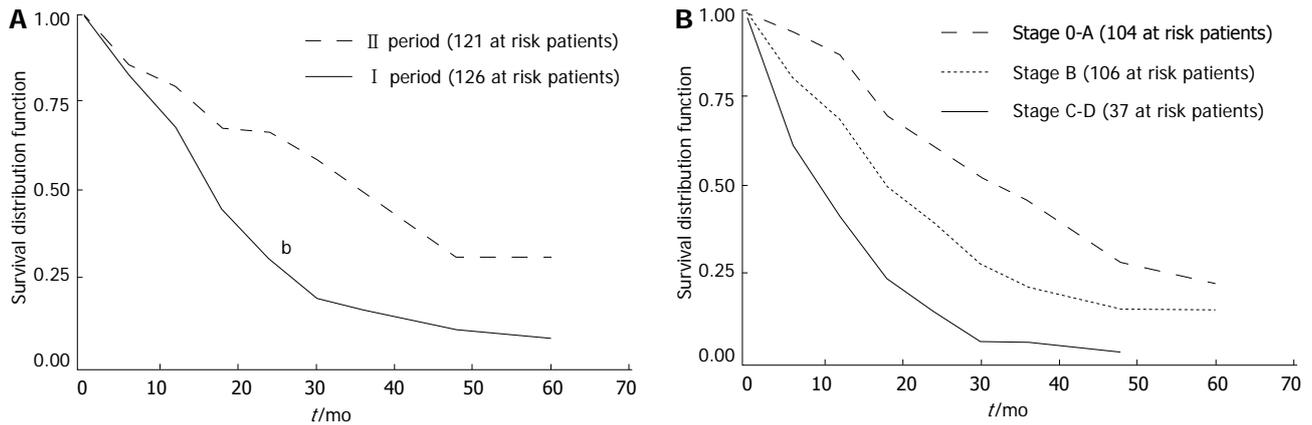


Figure 4 Survival in the two different periods, different Barcelona Clinic Liver Cancer stages. A: Survival in the two different periods, I = (2000-2005) vs II (2005-2010), <sup>b</sup>*P* < 0.01 vs II period; B: Survival in different Barcelona Clinic Liver Cancer stages.

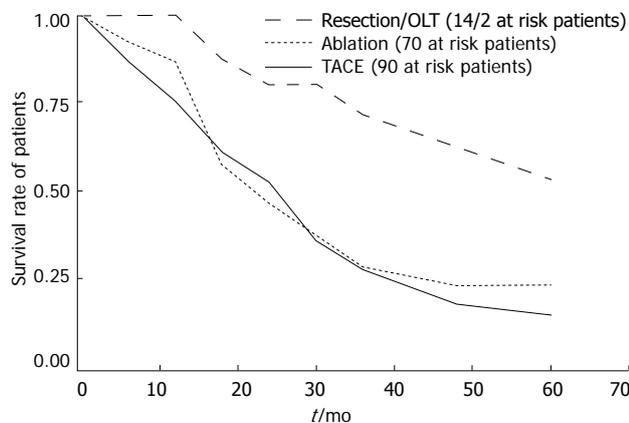


Figure 5 Survival according to treatment. OLT: Orthotopic liver transplantation; TACE: Trans-arterial chemoembolization.

ics treatment was administered to 14 patients (45.2% of all cases) (*P* = 0.5).

**Survival**

During follow up, nine patients (3.5%; seven patients from the first group and two from the second group) dropped out of the study. By December 31<sup>st</sup>, 2010, 172 patients (67.2%) had died and 75 patients (29.3%) were still alive.

The overall median survival from diagnosis was 22.4 mo (range 0-125 mo). Factors associated with better survival in HCC patients were diagnosis in the second period (*P* < 0.01) (Figure 4A), early BCLC stage (*P* < 0.01) (Figure 4B), and treatment (both curative treatments and TACE, *P* < 0.05) (Figure 5).

On the other hand, age (*P* = 0.17) and surveillance (*P* = 0.20) were not significantly associated with a better outcome (Table 1).

In the multivariate analysis (Table 2) only treatment (both curative treatments and TACE: HR = 0.37; 95%CI: 0.24-0.57) and BCLC stage (B *vs* A: HR = 1.50; 95%CI: 1.03-2.19; C-D *vs* A: HR = 1.75; 95%CI: 1.01-3.03) emerged as significant predictors of survival, while age,

gender and surveillance were not significantly associated with a better outcome; patients with a history of alcohol-related liver disease showed a trend toward an increased mortality, but this did not reach the level of statistical significance (alcohol *vs* viral infection HR = 1.43; 95%CI: 0.96-2.12).

Elevated bilirubin and alpha-fetoprotein levels were strongly associated with mortality in the univariate analysis (Table 1). However, they were also highly correlated with BCLC stage and the information was not available for about 20% of the patients. Therefore, they were not included in the multivariate model.

**DISCUSSION**

*Epidemiology*

This long-term retrospective study was carried out in a large series of consecutive HCCs recruited over ten years at Cuneo Hospital, which is the local referral center for the diagnosis and the treatment of HCC.

We believe that our findings closely represent the “real world” of HCC in Northern Italy. The number of cases identified reflects the current incidence of HCC in Italy, as reported in the latest epidemiological studies<sup>[17]</sup>. In Italy, HCC incidence is supposed to have started to decrease steadily by 2007<sup>[18]</sup>, as a consequence of reduced HCV infection related cirrhosis. We noticed this trend in our study, with an initial decrease of HCCs in more recent years (133 in the first period *vs* 123 in the second period), which may be related to a reduction in HCV-related HCCs. This phenomenon could be confirmed in the coming years through continuous monitoring of our population.

Our data confirmed that HCC occurs in a setting of chronic liver disease in 98% of cases<sup>[13]</sup>. The main risk factor for HCC was HCV infection, as reported in previous Italian series<sup>[19-21]</sup>, followed by alcohol, as in other developed countries<sup>[22]</sup>. Our data showed only a slight decrease in HCV-related HCC over the study period, although this figure probably would have been more relevant taking into account only the two-year extremes. Furthermore, in the second period there was a significant

**Table 1** Hepatocellular carcinoma mortality by patient's characteristics *n* (%)

|                   |                  | Yes        | No        | Total | <i>P</i> value |
|-------------------|------------------|------------|-----------|-------|----------------|
| Gender            | Men              | 120 (67.8) | 57 (32.2) | 177   | 0.24           |
|                   | Women            | 52 (74.3)  | 18 (25.7) | 70    |                |
| Age (yr)          | ≤ 65             | 39 (61.9)  | 24 (38.1) | 63    | 0.17           |
|                   | > 65             | 133 (72.3) | 51 (27.7) | 184   |                |
| Calendar period   | 2000-2005        | 116 (92.1) | 10 (7.9)  | 126   | < 0.01         |
|                   | 2006-2010        | 56 (46.3)  | 65 (53.7) | 121   |                |
| Etiology          | Viral infection  | 108 (69.7) | 47 (30.3) | 155   | < 0.01         |
|                   | Alcohol          | 38 (80.9)  | 9 (19.1)  | 47    |                |
|                   | Other            | 25 (56.8)  | 19 (43.2) | 44    |                |
| BCLC stage        | A                | 59 (56.7)  | 45 (43.3) | 104   | < 0.01         |
|                   | B                | 79 (74.5)  | 27 (25.5) | 106   |                |
|                   | C-D              | 34 (91.9)  | 3 (8.1)   | 37    |                |
| Treatment         | No               | 60 (93.8)  | 4 (6.3)   | 64    | 0.04           |
|                   | Yes              | 112 (61.2) | 71 (38.8) | 183   |                |
| Surveillance      | No               | 134 (72.0) | 52 (28.0) | 186   | 0.20           |
|                   | Yes              | 38 (62.3)  | 23 (37.7) | 61    |                |
| Alpha-fetoprotein | Normal           | 104 (59.8) | 70 (40.2) | 174   | < 0.01         |
|                   | Elevated (> 15)  | 43 (91.5)  | 4 (8.5)   | 47    |                |
| Bilirubin         | Normal           | 48 (57.8)  | 35 (42.2) | 83    | < 0.01         |
|                   | Elevated (> 2.1) | 98 (77.8)  | 28 (22.2) | 126   |                |

BCLC: Barcelona Clinic Liver Cancer.

increase in HCCs unrelated to either viral infection or to alcohol. These cases were mainly patients with type 2 diabetes mellitus. This figure might reflect an increased attention to HCC in this specific population as a consequence of recent studies that reported diabetes and obesity as possible independent risk factors for HCC through the development of NAFLD and NASH<sup>[5,23]</sup>. To date, there are no evidence-based recommendations as to whether this group should be screened for HCC, as in other HCC high-risk groups<sup>[7]</sup>. Nonetheless, future data may change this recommendation, and there is increasing interest in this issue in the international literature.

### Clinical features and treatment

Surveillance programs in HCC high-risk populations have led to the early detection of small tumors eligible for curative therapies<sup>[19,24-26]</sup>, which may lead to improved outcomes<sup>[13]</sup>. Surveillance with US every 6 mo for detection of early HCC is therefore applied extensively in clinical practice and is recommended by the guidelines for HCC management<sup>[6,7]</sup>.

However, in our study, less than one-third of HCCs (23.8%) were discovered under surveillance, in agreement with other Italian studies<sup>[27-29]</sup>. On the other hand, surveillance has increased significantly in recent years, probably as a result of increased dissemination of international evidence demonstrating the benefit of surveillance in HCC risk groups. Moreover, in our population, surveillance was inferior in alcoholic patients and in patients with "non-viral/non-alcoholic" cirrhosis. This may be related to poor compliance to strict follow up in the former group, and to a current lack of specific recommendations in the later.

The prognosis in HCC patients is determined by tumor stage, underlying liver function reserve and general

**Table 2** Predictors of hepatocellular carcinoma mortality

|                 |                 | Multivariate analysis-Cox proportional hazards model |           |
|-----------------|-----------------|--|-----------|
|                 |                 | HR   | 95%CI     |
| Gender          | Men             | 1  |           |
|                 | Women           | 1.22   | 0.86-1.72 |
| Age (yr)        | ≤ 65            | 1  |           |
|                 | > 65            | 1.20   | 0.82-1.75 |
| Calendar period | 2000-2005       | 1  |           |
|                 | 2006-2010       | 0.67   | 0.46-0.95 |
| Etiology        | Viral infection | 1  |           |
|                 | Alcohol         | 1.43   | 0.96-2.12 |
|                 | Other           | 0.95   | 0.59-1.53 |
| BCLC stage      | A               | 1  |           |
|                 | B               | 1.50   | 1.03-2.19 |
|                 | C-D             | 1.75   | 1.01-3.03 |
| Treatment       | No              | 1  |           |
|                 | Yes             | 0.37   | 0.24-0.57 |
| Surveillance    | No              | 1  |           |
|                 | Yes             | 1.10   | 0.74-1.65 |

BCLC: Barcelona Clinic Liver Cancer.

health status<sup>[6,7,9]</sup>. The BCLC staging system<sup>[11]</sup> takes into account all these factors, and, to date, it is recommended as the reference staging system for HCC<sup>[7,12]</sup>.

As far as tumor stage is concerned, over the entire study period, the most common HCC presentation pattern was a single nodule with a diameter between 3 and 5 cm; notably, in the 2005-2010 period, there was a significant increase in unifocal HCC and in small HCC (< 3 cm of diameter), as a result of the increased use of surveillance programs. In a previous Italian study<sup>[30]</sup>, multifocal HCCs were more common among multi-etiology cirrhotic patients, while unifocal HCCs were more common among HCV-related cirrhosis and among younger patients (< 65 years). In our study, the morphological pattern at the time of diagnosis was not associated with etiology or age, but multifocal HCC was moderately more prevalent among Child B and C class patients than among Child A class patients. With regard to liver function, the majority of our population was in Child class A, in keeping with other Italian studies<sup>[19,31]</sup>. Moreover, there was an increase in the number of Child class A patients during the second period, which could also be related to the increased use of surveillance.

Finally, as far as HCC staging is concerned, almost half of the population was in BCLC stage B (intermediate stage) at the time of diagnosis. As expected, very-early/early HCCs (BCLC stage 0 and A) were more common in patients who underwent surveillance and, consequently, were present in the 2005-2010 cohort. To date, this is the first Italian study to apply the BCLC staging system; other Italian groups have used the CLIP score<sup>[19-21]</sup>. Given the complexity of HCC management in clinical practice, we strongly emphasize the need to use a common staging system that can easily define patient groups for different therapies and that can stratify them into separate prognosis categories<sup>[7]</sup>. The BCLC staging system meets all these characteristics. We stress that the use of guidelines is very important for providing

patients with the best treatment options.

In our population, the proportion of HCC patients who underwent specific treatment grew significantly during the last period; however, this growth was not associated with an increased use of curative treatments, as we could expect given the higher number of BCLC 0-A patients in the second period, but may be caused by the increased use of non-curative treatments, such as TACE, in intermediate stage HCCs (BCLC B) in recent years. In fact, in our population, potentially curative therapies were underutilized, even among very early/early HCCs. Similar findings have been obtained in previous studies<sup>[21,30,32]</sup>. This may have two possible explanations: firstly, the lack of application of BCLC staging system leading to an incorrect allocation of early HCCs to the proper therapeutic approach; and, secondly, the BCLC staging system does not take into account patients' age, leading to the possibility that, in elderly patients, non-curative treatments were preferred over curative therapies, given the slow HCC growth and the survival benefit provided by non-curative therapies<sup>[33]</sup>.

On the other hand, there was a proportion of patients in our population with intermediate-advanced HCC who underwent curative treatments, namely "overtreated patients", as reported in another Italian series<sup>[30]</sup>.

Among curative therapies, the most common choice was percutaneous ablation with radiofrequency. This switch from resection to the less invasive and less expensive percutaneous ablation therapy is a current practice supported by the good results achieved in terms of survival. In addition, this preference may be related to the relative high median age of our population (70 years, range 32-92 years); indeed, ablation permits a shorter hospital admission with less severe post-operative complications.

Overall, a large proportion of our population received TACE over the ten-year study period. The use of TACE increased significantly during the last years, mainly in intermediate stage HCCs, as a result of current evidence that TACE improves survival in patients who cannot benefit from curative treatment and who do not have severe impairment in liver function, vascular invasion and extra-hepatic diffusion<sup>[13,34,35]</sup>. However, our data shows an increased use of TACE in very-early/early HCC stages, and this trend may be explained by the older median age of this patients' group during the second period, leading to a preference of TACE over curative therapies.

The prevalence of post-embolization syndrome was similar to other series, but in the last years we noticed an increment in antibiotic consumption, despite the absence of signs of infection; this finding underlines the widespread and increasingly frequent inappropriate use of antibiotic treatment in clinical practice.

Liver transplant had a very limited role in the therapy of HCC in our population. Despite a relatively large number of potentially OLT-eligible patients (38 patients, 63% Child-A), only five subjects were submitted to OLT evaluation and two (1%) were definitively transplanted.

This low rate of OLT is hard to explain considering that in Piedmont, three liver transplant centers were available at that time. Consequently, at least in our region, OLT should be regarded as a virtual rather than a real therapeutic option.

Notably, almost one-third of HCC patients did not receive any specific treatment; obviously, untreated patients presented more commonly an intermediate-advanced HCC, and there was a non-significant trend in treating fewer patients aged more than 65 years and women. Nonetheless, the rate of untreated patients decreased during the last period, as a result of the parallel increase in treating intermediate-advanced HCCs with non-curative therapies.

### Survival

As expected, the prognosis of our patients was dictated by liver function, BCLC stage, and diagnosis period.

As far as surveillance is concerned, it was effective in detecting HCCs at an early stage, where curative treatment may be more effective. This fact explains the previously reported survival benefit for patients in surveillance programs<sup>[36]</sup>. Therefore, the effect of surveillance on survival was usually not significant in multivariate analysis after adjusting for these factors in most studies<sup>[37-39]</sup>. In addition, at multivariate analysis, surveillance has not proved to be an independent prognostic factor.

Chen *et al*<sup>[40]</sup> reported that HCC surveillance resulted in early diagnosis of liver cancer, but not in mortality reduction, because therapy was ineffective. We observed a similar scenario, with an increased rate of HCCs diagnosed at an early stage in the last period, but without a parallel increase in application of curative therapies. Therefore, the surveillance program must be accompanied with appropriate treatment options for patients with newly diagnosed HCC, to improve their survival and justify the expense.

With regard to the therapeutic impact on survival, there was an obvious survival benefit in patients that underwent surgery (OLT plus resection) over the TACE group. Ablation was the most extensively applied curative therapy, but there was only a small survival benefit over the TACE group. This fact may be explained by an inappropriate selection of patients for loco-regional therapy (ablation or TACE). Moreover, as reported by Tseng *et al*<sup>[20]</sup>, it is possible that in early HCC the two therapies did not differ in terms of survival, but only in terms of recurrence.

Therapy "*per se*" resulted as an independent favorable prognostic variable in our patients, suggesting that the amenability to treatment identifies a subset of patients with expected better survival.

In conclusion, our cohort study (being in between a population-based and a referral center-based investigation) offers a picture close to what actually occurs in clinical practice. As reported in previous series<sup>[19-21,27,28,30]</sup>, in the real clinical practice, the approach to HCC is far from being adequate, and this tumor remains an undertreated or inappropriately treated complication, despite positive

changes that have occurred during recent years. We stress that this state of affairs should be a stimulus for further implementation of surveillance and improved employment of the various therapeutic opportunities available.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the 5<sup>th</sup> most common cancer and the 3<sup>rd</sup> leading cause of cancer mortality worldwide. In recent decades, a rising incidence has been reported in Europe and United States. Hepatitis B virus and hepatitis C virus infections are known independent risk factors for HCC, as well as alcohol consumption.

### Research frontiers

Approaches to HCC treatment are potentially curative depending on cancer stage, liver function and performance status, as well as on resources and the level of practitioner expertise. The Barcelona Clinic Liver Cancer (BCLC) staging system has come to be widely accepted in clinical practice as the reference system to stratify patients into separate prognostic categories and to suggest the treatment options according to the stage.

### Innovations and breakthroughs

Data showed a significant increase in HCCs related to neither viral infection nor alcohol; these cases were mainly patients with type 2 diabetes mellitus. This figure might reflect an increased attention in research HCC in this specific population, as a consequence of recent evidence reporting diabetes and obesity as possible independent risk factors for HCC through the development of non-alcoholic steatohepatitis and non-alcoholic fatty liver disease.

### Applications

The study identified the increased use of surveillance in the latest years, probably as a result of increasing diffusion and knowledge of international evidence about related benefits. However, at multivariate analysis, surveillance has not proved to be an independent prognostic factor. In this population study, potentially curative therapies were underutilized, and this should be a stimulus to a more adequate employment of different therapeutic opportunities.

### Peer review

The authors present an interesting series of patients with HCC treated over 10 years in a single institution. Changes in epidemiology and a lack of strict adherence to the BCLC treatment recommendations follow other recently published series.

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P- Reviewer Sangro B S- Editor Gou SX  
L- Editor Stewart GJ E- Editor Li JY



## CT/<sup>99m</sup>Tc-GSA SPECT fusion images demonstrate functional differences between the liver lobes

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Received: January 3, 2013 Revised: March 19, 2013

Accepted: April 9, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To evaluate the functional differences between the 2 liver lobes in non-cirrhotic patients by using computed tomography/<sup>99m</sup>Tc-galactosyl human serum albumin (CT/<sup>99m</sup>Tc-GSA) single-photon emission computed tomography (SPECT) fusion images.

**METHODS:** Between December 2008 and March 2012, 264 non-cirrhotic patients underwent preoperative liver function assessment using CT/<sup>99m</sup>Tc-GSA SPECT fusion images. Of these, 30 patients, in whom the influence of a tumor on the liver parenchyma was estimated to be negligible, were selected. Specifically, the selected patients were required to meet either of the following criteria: (1) the presence of an extrahepatic tumor; or (2) presence of a single small intrahepatic tumor. These 30 patients were retrospectively analyzed to calculate the percentage volume (%Volume) and the percentage function (%Function) of each lobe. The ra-

tio between the %Function and %Volume (function-to-volume ratio) of each lobe was also calculated, and the ratios were compared between the 2 lobes. Furthermore, the correlations between the function-to-volume ratio and each of 2 liver parameters [lobe volume and diameter ratio of the left portal vein to the right portal vein (LPV-to-RPV diameter ratio)] were investigated.

**RESULTS:** The median values of %Volume and %Function were 62.6% and 67.1% in the right lobe, with %Function being significantly higher than %Volume ( $P < 0.01$ ). The median values of %Volume and %Function were 31.0% and 28.7% in the left lobe, with %Function being significantly lower than %Volume ( $P < 0.01$ ). The function-to-volume ratios of the right lobe (1.04-1.14) were significantly higher than those of the left lobe (0.74-0.99) ( $P < 0.01$ ). The function-to-volume ratio showed no significant correlation between the lobe volume in either lobe. In contrast, the function-to-volume ratio showed significant correlations with the LPV-to-RPV diameter ratio in both lobes (right lobe: negative correlation,  $r_s = -0.37$ ,  $P = 0.048$ ; left lobe: positive correlation,  $r_s = 0.71$ ,  $P < 0.001$ ). The function-to-volume ratio in the left lobe tended to be higher, and that in the right lobe tended to be lower, in accordance with the increase in the LPV-to-RPV diameter ratio.

**CONCLUSION:** CT/<sup>99m</sup>Tc-GSA SPECT fusion images demonstrated that the function of the left lobe was significantly decreased compared with that of the right lobe in non-cirrhotic livers.

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**Key words:** Computed tomography; <sup>99m</sup>Tc neogalactosyl albumin; Single-photon emission computed tomography; Fusion image; Liver; Portal system

**Core tip:** This study describes that lobular differences in tracer accumulation can be observed even in pa-

tients with an apparently homogeneous liver on <sup>99m</sup>Tc-galactosyl human serum albumin (<sup>99m</sup>Tc-GSA) single-photon emission computed tomography (SPECT). computed tomography/<sup>99m</sup>Tc-GSA SPECT fusion images demonstrated that the function of the left lobe was significantly decreased compared with that of the right lobe in non-cirrhotic livers.

Sumiyoshi T, Shima Y, Tokorodani R, Okabayashi T, Kozuki A, Hata Y, Noda Y, Murata Y, Nakamura T, Uka K. CT/<sup>99m</sup>Tc-GSA SPECT fusion images demonstrate functional differences between the liver lobes. *World J Gastroenterol* 2013; 19(21): 3217-3225 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3217.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3217>

## INTRODUCTION

Preoperative evaluation of future remnant liver function is critical for patients undergoing hepatic surgery<sup>[1]</sup>. Overestimation of the remnant liver function can lead to life-threatening postoperative hepatic failure, and its underestimation can lead to a lost opportunity for potentially curative surgery. Conventionally, post-hepatectomy remnant liver function has been estimated preoperatively using computed tomography (CT) volumetry<sup>[1-4]</sup>. CT can provide precise anatomical information, and the remnant liver volume, measured by CT volumetry, has been reported to be an effective predictor of hepatic dysfunction after hepatectomy<sup>[1,5-8]</sup>. However, the indirect estimation of liver function by CT volumetry is reliable only when the function is assumed to be homogenous over the whole liver<sup>[6,7]</sup>. Recent reports on liver scintigraphy showed that regional hepatic function could be significantly decreased under various conditions, such as unilateral portal flow decrease or biliary stenosis<sup>[9-15]</sup>, although regional liver volume was maintained. Such observations indicate the difficulty associated with assuming strict homogeneity of the whole liver function. Therefore, accurate estimation of residual liver function, rather than estimation of residual liver volume, is more important for predicting the postoperative hepatic functional reserve<sup>[16,17]</sup>.

<sup>99m</sup>Tc-galactosyl human serum albumin (<sup>99m</sup>Tc-GSA) single-photon emission CT (SPECT) was developed to facilitate investigations of regional hepatic function. Several reports have proved that <sup>99m</sup>Tc-GSA SPECT is more suitable for evaluating remnant liver function than CT volumetry<sup>[14,18-20]</sup>. Further improvements have resulted in the recent development of a new SPECT examination method, CT/<sup>99m</sup>Tc-GSA SPECT fusion examination. A disadvantage of conventional <sup>99m</sup>Tc-GSA SPECT was poor anatomical resolution, and this newly developed SPECT has a tremendously improved anatomical resolution owing to the fusion of CT and <sup>99m</sup>Tc-GSA SPECT. Recent reports have indicated that accurate regional function, based on precise anatomical information, could be evaluated using this technique<sup>[11,6,21,22]</sup>.

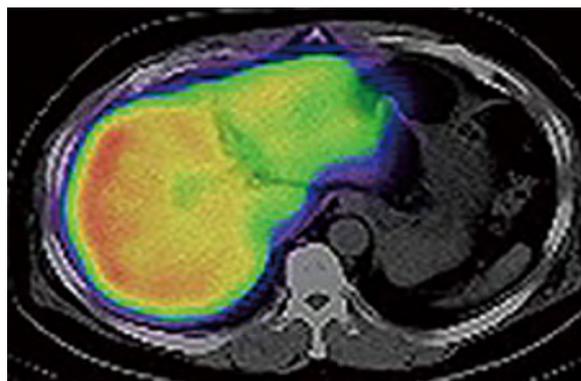


Figure 1 Computed tomography/<sup>99m</sup>Tc-galactosyl human serum albumin single-photon emission computed tomography fusion image in a 58-year-old male with early gallbladder cancer. The uptake of <sup>99m</sup>Tc-galactosyl human serum albumin was markedly decreased in the interior portion of the left lobe. This computed tomography image was at the level of the center of the left lobe, and was neither the cranial edge nor the caudal edge of that lobe.

In our department, remnant liver volume and function have been routinely estimated preoperatively by using CT/<sup>99m</sup>Tc-GSA SPECT fusion images since December 2008, resulting in the realization that the function of the interior portion of the left lobe was diminished compared with that of the right lobe, despite the absence of anatomical conditions suggestive of liver function heterogeneity (Figure 1). This study aimed to evaluate the functional differences between the 2 main lobes in non-cirrhotic livers. The percentage volume (%Volume) and percentage function (%Function) of each lobe were calculated using fusion images, and these data were compared between lobes.

## MATERIALS AND METHODS

### Patients

Between December 2008 and March 2012, 264 non-cirrhotic patients underwent preoperative liver function assessment by using CT/<sup>99m</sup>Tc-GSA SPECT fusion imaging. Cirrhotic patients were not included in this study because it is well known that the hemodynamics of bilateral portal flow and the bilateral lobe volumes are clearly different between non-cirrhotic and cirrhotic livers<sup>[23,24]</sup>. These differences might greatly influence the function of each lobe, and therefore, we investigated only non-cirrhotic livers in the current study. Each patient provided informed consent and our local ethics review committee approved the study. Of these patients, those in whom the influence of a lesion on the liver parenchyma was estimated to be negligible were selected for further study. Specifically, the selected patients were required to meet either of the following criteria: (1) presence of an extrahepatic tumor, where the tumor was close to the liver surface and had been preoperatively estimated to require hepatectomy, but both intraoperative and pathological findings showed the absence of direct tumor invasion into the liver; or (2) presence of small, intrahepatic tumors; those with large tumors were eliminated because

**Table 1 Clinical characteristics of the patients**

| Clinical characteristics   | Statistics                   |
|--|------------------------------|
| Age, yr, (median)  | 37-87 (69)                   |
| Sex (male:female)  | 17:13                        |
| Hepatitis virus  |                              |
| Hepatitis B  | 1                            |
| Hepatitis C  | 5                            |
| Serum liver function test: median (range)                            |                              |
| Albumin level, g/dL  | 4.2 (3.8-4.7)                |
| Bilirubin level, mg/dL   | 0.6 (0.3-1)                  |
| Platelet count, × 10 <sup>3</sup> /mL                                | 19.9 (14.9-41.4)             |
| Prothrombin time, s  | 11.8 (10.1-12.8)             |
| CT attenuation value of the liver on non-enhanced CT: median (range) |                              |
| Right lobe, HU   | 56.3 (35.0-70.5)             |
| Left lobe, HU  | 57.5 (39.5-73.0)             |
| Diameter of portal vein: median (range)                              |                              |
| Right portal vein, mm  | 10.2 (6.6-13.6) <sup>b</sup> |
| Left portal vein, mm   | 8.4 (4.5-10.8) <sup>b</sup>  |
| LPV-to-RPV diameter ratio: median (range)                            | 0.78 (0.5-1.26)              |
| Disease  |                              |
| Criterion 1: Patients with extrahepatic tumors ( <i>n</i> )          | 15                           |
| Early gallbladder cancer without liver invasion                      | 11                           |
| Gastric gastrointestinal stromal tumor                               | 1                            |
| Peritoneal dissemination of ovarian cancer                           | 1                            |
| Intra-abdominal recurrence of gastric cancer                         | 1                            |
| Extrahepatic bile duct cancer without bile duct dilatation           | 1                            |
| Criterion 2: Patients with intrahepatic small tumors ( <i>n</i> )    | 15                           |
| Hepatocellular carcinoma   | 4                            |
| Liver metastasis   | 9                            |
| Intrahepatic cholangiocarcinoma                                      | 2                            |
| Tumor location in patients within criterion 2 ( <i>n</i> )           |                              |
| Segment 1/segment 2/segment 3/segment 4/<br>Segment 6/segment 8      | 3/1/1/2/4/4                  |

HU: Hounsfield units; CT: Computed tomography; LPV: Left portal vein; RPV: Right portal vein. <sup>b</sup>*P* < 0.001.

such tumors might compress the surrounding liver parenchyma, resulting in a significant decrease in function. Eligible patients who met criterion 2 were limited to those with a single, small (< 3 cm diameter) tumor. Furthermore, the tumor had to be located on the liver surface and not in contact with a major branch of portal vein or hepatic vein. Thirty-six patients met either of these 2 criteria. Three patients treated by transcatheter arterial embolization and another 3 patients in whom the diagnosis could not be confirmed histopathologically because of the absence of surgery were excluded; thus, a total of 30 patients were included in this retrospective study. All these 30 patients underwent 4-phase multidetector-row CT (1 unenhanced image and 3 contrast-enhanced images) preoperatively, which confirmed that none had anatomical abnormalities, such as portal venous occlusion, hepatic artery stenosis, or intrahepatic biliary stenosis. Furthermore, it was also confirmed that there was no heterogeneous hepatic parenchymal enhancement in 3 contrast-enhanced images from each patient. The clinical characteristics of the 30 patients are shown in Table 1. Fifteen patients met criterion 1 [11 with early gall bladder cancer without liver invasion (Figure 2A), 1 with a gastric gastrointestinal stromal tumor (Figure 2B), 1 with peritoneal dissemination of ovarian cancer (Figure 2C), 1 with intra-abdominal recurrence of gastric cancer, and 1 with extrahepatic bile duct cancer, without jaundice]. Fifteen

patients met criterion 2 [4 with hepatocellular carcinoma, 9 with liver metastasis (Figure 2D), and 2 with intrahepatic cholangiocarcinoma]. Each tumor was confirmed to meet the inclusion criteria, on the basis of the examination of surgically resected specimens.

### CT/<sup>99m</sup>Tc-GSA SPECT fusion examination

All patients underwent CT/<sup>99m</sup>Tc-GSA SPECT fusion examination with a Symbia T6 scanner (Siemens, Munich, Germany) (Figure 3). This instrument combines variable angle dual detector SPECT with 6-slice CT for rapid, accurate attenuation correction and precise localization. The instrument also enables the seamless transition from a SPECT examination to a CT examination, and both SPECT and CT images could be obtained in a single examination without the need for a position change.

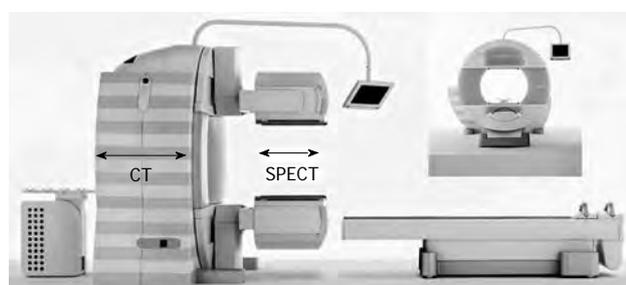
After overnight fasting, the patient was placed in a supine position. Cardiac and respiratory synchronization were not used in this modality. Instead, to minimize the possibility of artifacts due to cardiac pulsation and respiratory motion, the patients were encouraged to rest and take a small, slow breath before image acquisition. <sup>99m</sup>Tc-diethylenetriaminepentaacetic acid-GSA (Nihon Medi-Physics, Tokyo, Japan) (185 MBq/3 mg) was injected into an antecubital vein. SPECT data acquisition (60 steps of 20 s/step, 360°, 128 × 128 matrix) was started 20 min after the injection with a low-energy, high-resolution collimator; the entire study duration was approximately 30 min. The reconstruction algorithm for SPECT was a 3-dimensional ordered subset expectation maximization, with attenuation and scatter corrections. Following SPECT examination, non-enhanced CT images were obtained under standard conditions of 130 kV, 345 mA, 12 mm table feed per rotation, 0.6-s gantry rotation time, 0.6-mm collimation, and 1-mm reconstruction. CT images were reconstructed using a standard reconstruction algorithm with a 166-cm field-of-view of the target sites. The SPECT and CT images were fused automatically using the embedded Siemens common platform software Syngo MI workplace. SPECT slice data were retrieved through Digital Imaging and Communications in Medicine (DICOM), and SPECT slices were converted to a CT-like data volume for the fusion of the SPECT and CT images.

### Image analysis

All fusion images were evaluated retrospectively by hepatobiliary pancreatic surgeons with 13 and 24 years of experience. The extent of each lobe was manually circled on the non-enhanced CT image, with consensus between the evaluators, and 3 radiologists in our institution checked the accuracy in each case (Figure 4A and B). The extent of the left and right lobes was determined using anatomical landmarks, such as the middle hepatic vein and gallbladder bed, and by confirming the portal branch of segments 4, 5 and 8. The caudate lobe was excluded from the range of each lobe. The boundary between the right lobe and the caudate lobe was defined by the line connecting the bifurcation of the right portal vein (RPV)



**Figure 2** Computed tomography images of patients meeting criterion 1 or 2. A: An 82-year-old man with early gallbladder cancer, in whom the preoperative computed tomography (CT) scan showed a tumor localized in the gallbladder (white arrow); B: A 59-year-old man with gastric gastrointestinal stromal tumor, in whom a preoperative CT scan showed that the tumor had invaded the left lateral segment of the liver. However, no invasion into the liver was detected intraoperatively (circle); C: A 64-year-old woman with peritoneal dissemination of ovarian cancer, in whom a preoperative CT scan showed an implanted small tumor on the surface of the left caudate lobe (black arrow); D: A 62-year-old woman with liver metastasis, in whom a preoperative magnetic resonance image showed a small (5 mm) tumor on the liver surface (circle).



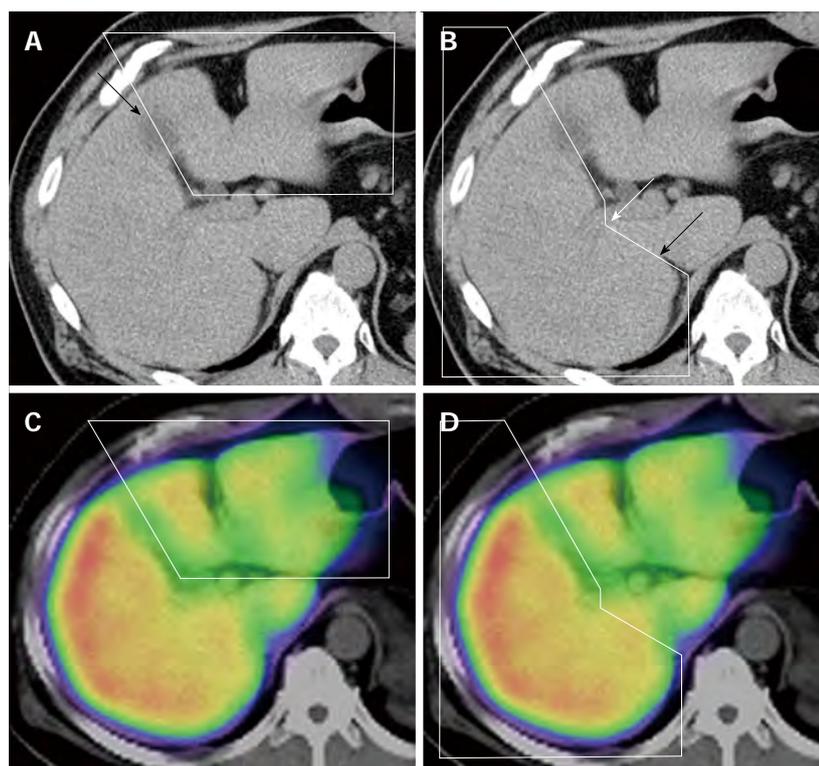
**Figure 3** Integrated computed tomography and single-photon emission computed tomography system, consisting of variable angle dual-detector single-photon emission computed tomography and 6-slice computed tomography. The system allows the seamless transition from a single-photon emission computed tomography (SPECT) examination to a computed tomography (CT) examination.

and the right wall of the inferior vena cava<sup>[25]</sup> (Figure 4B). The extent of each lobe, determined from the CT image, was automatically projected on the SPECT/CT fusion image (Figure 4C and D). The volume of each lobe was automatically determined using the Syngo software. The %Volume of each lobe was also automatically determined by dividing the volume of each lobe by the total liver volume. In the case of intrahepatic small tumors (criterion 2), tumor volumes were subtracted from the liver volumes. The %Function of each lobe was also automatically calculated by dividing the scintillation counts

in each lobe by the total scintillation counts in the whole liver. The ratio between the %Function and %Volume (function-to-volume ratio) of each lobe was calculated, and the ratios were compared between the 2 lobes.

#### Clinical liver function parameter

Serum liver function tests, including albumin level, bilirubin level, platelet count, prothrombin time, and the presence or absence of hepatitis virus were investigated in all patients. Two other parameters that might influence <sup>99m</sup>Tc-GSA accumulation in the liver were also measured on CT images. The first parameter was the degree of fat deposition, which was assessed by measuring the CT attenuation values [in Hounsfield units (HU)] on unenhanced CT images<sup>[26,27]</sup>. We delineated 12 regions of interest (ROIs) of 1 cm diameter within the liver. One ROI was defined in 4 sectors (right posterior, right anterior, left medial, and left lateral) at 3 representative levels. The levels consisted of the confluence of the right hepatic vein, the umbilical portion, and the right posterior portal vein. While defining ROIs, special attention was placed on excluding cystic areas and the vessels. The mean attenuation value of 6 ROIs in the right posterior and right anterior sectors was defined as the attenuation value of the right lobe, and that of the 6 ROIs in the left medial and lateral sector was defined as the attenuation value of the left lobe. The second parameter was the left portal vein (LPV)-to-RPV diameter ratio. The diameters of the portal veins were measured on



**Figure 4** Range of each lobe on the computed tomography and computed tomography/single-photon emission computed tomography fusion image. A: The extent of the left lobe is manually circled on the non-enhanced computed tomography (CT) image. The arrow shows the gallbladder bed; B: The extent of the right lobe on the non-enhanced CT image. The boundary between the right lobe and the caudate lobe was defined by the line connecting the bifurcation of the right portal vein (white arrow) and the right wall of the inferior vena cava (black arrow); C and D: The extent of each lobe determined from the CT image was automatically projected on the single-photon emission computed tomography/CT fusion image (C: Left lobe; D: Right lobe).

the contrast-enhanced CT images, and the LPV-to-RPV diameter ratio was calculated.

### Statistical analysis

The Wilcoxon signed-rank test was used for the following comparisons: (1) comparison of 2 liver parameters (the attenuation value on an unenhanced CT image and the diameter of the portal veins on an enhanced CT image) between the 2 lobes; (2) comparison of the diameters of the LPV and the RPV; (3) comparison of the %Volume and %Function values in each lobe; and (4) comparison of the function-to-volume ratio between the 2 lobes. The correlations between the function-to-volume ratio and the 2 liver parameters (lobe volume and LPV-to-RPV diameter ratio) were assessed using Spearman's correlation coefficient. Data were analyzed using SPSS Statistics 19 (IBM, Armonk, NY, United States), and  $P < 0.05$  was considered statistically significant.

## RESULTS

### Serum liver function test

All values of the serum liver function tests were within the reference ranges for all patients (Table 1). Hepatitis B virus antigen and hepatitis C virus antibody were positive in 1 and 5 patients, respectively. However, none of them were associated with active hepatitis, and none of them showed clinical symptoms of hepatitis. There were no cases of non-B, non-C hepatitis.

### Degree of fat deposition in each lobe

The attenuation value of the right lobe ranged from 35.0 to 70.5 HU (median, 56.3 HU), and that of the left lobe ranged from 39.5 to 73.0 HU (median, 57.5 HU). Only 2

of 30 patients showed severe fatty change with an attenuation value of  $< 40$  HU. There were no significant differences between the attenuation values of the 2 lobes.

### Diameter of the LPV and the RPV

The diameter of the RPV ranged from 6.6 to 13.6 mm (median, 10.2 mm), and that of the LPV ranged from 4.5 to 10.8 mm (median, 8.4 mm). The diameters of the RPV were significantly larger than those of the LPV ( $P < 0.001$ ). The LPV-to-RPV diameter ratios ranged from 0.5 to 1.26.

### Comparison of the %Volume and %Function in each lobe

The extent of each lobe could be clearly defined on the CT image, and both %Volume and %Function in each lobe could be calculated for all patients. The %Volume and %Function of the right lobe ranged from 51.5% to 77.8% (median, 62.6%) and from 55.6% to 83.8% (median, 67.1%), respectively (Figure 5); the %Function was significantly higher than the %Volume in the right lobe ( $P < 0.01$ ). The %Volume and %Function in the left lobe ranged from 20.2% to 44.2% (median, 31.0%) and from 17.4% to 42.0% (median, 28.7%), respectively (Figure 5); the %Function was significantly lower than the %Volume in the left lobe ( $P < 0.01$ ).

### Comparison of the function-to-volume ratio between lobes

The function-to-volume ratio of the right lobe ranged from 1.04 to 1.14 (median, 1.08), and this ratio was  $> 1.0$  in all patients (Figure 6). The function-to-volume ratio of the left lobe ranged from 0.74 to 0.99 (median, 0.92), and this ratio was  $< 1.0$  in all patients. The function-to-volume ratios of the right lobe were significantly higher than those of the left lobe ( $P < 0.01$ ).

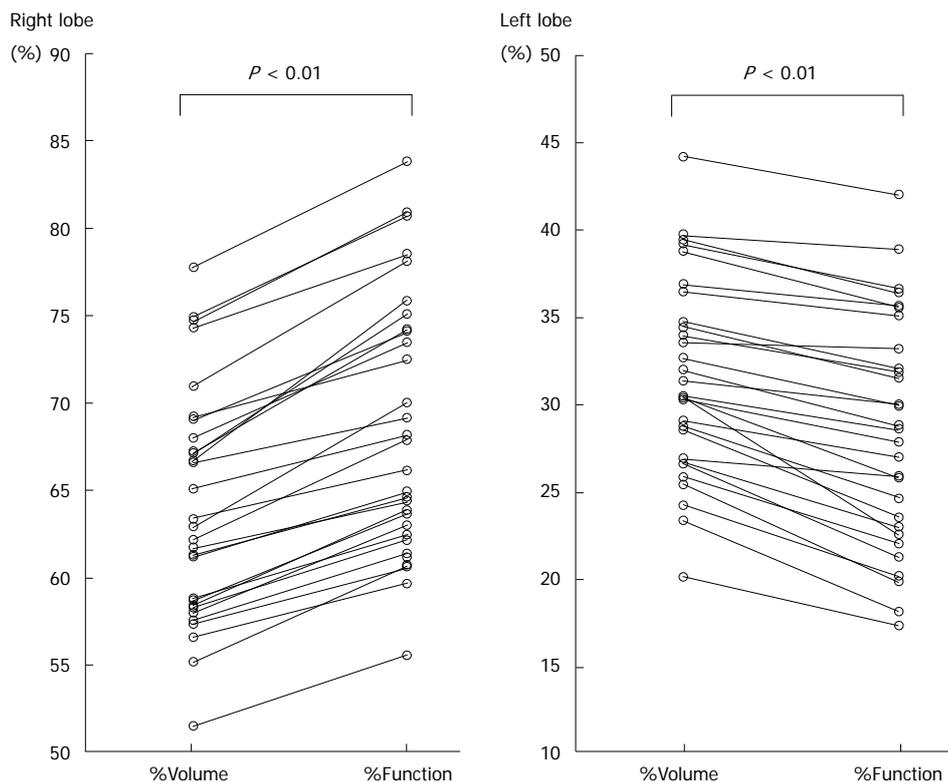


Figure 5 Percentage volume and percentage function of each lobe. The %Function was significantly higher than the %Volume in the right lobe ( $P < 0.01$ ). The %Function was significantly lower than the %Volume in the left lobe ( $P < 0.01$ ).

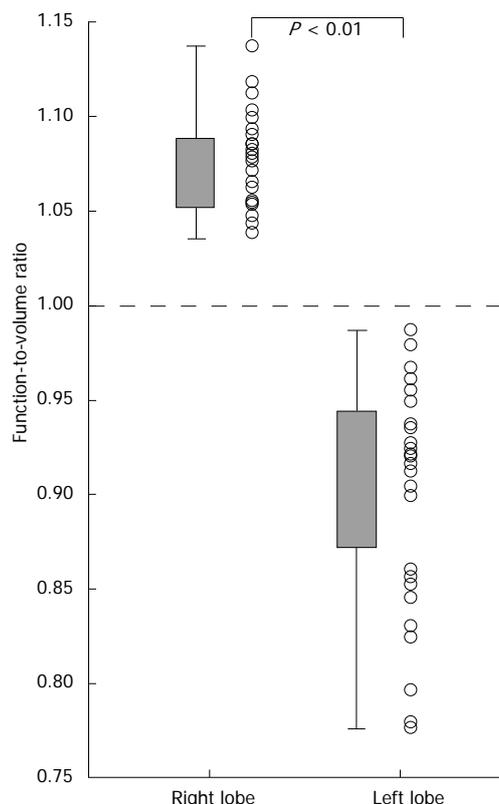


Figure 6 Comparison of the function-to-volume ratio between the right and left lobes. The function-to-volume ratios of the right lobe were significantly higher than those of the left lobe ( $P < 0.01$ ).

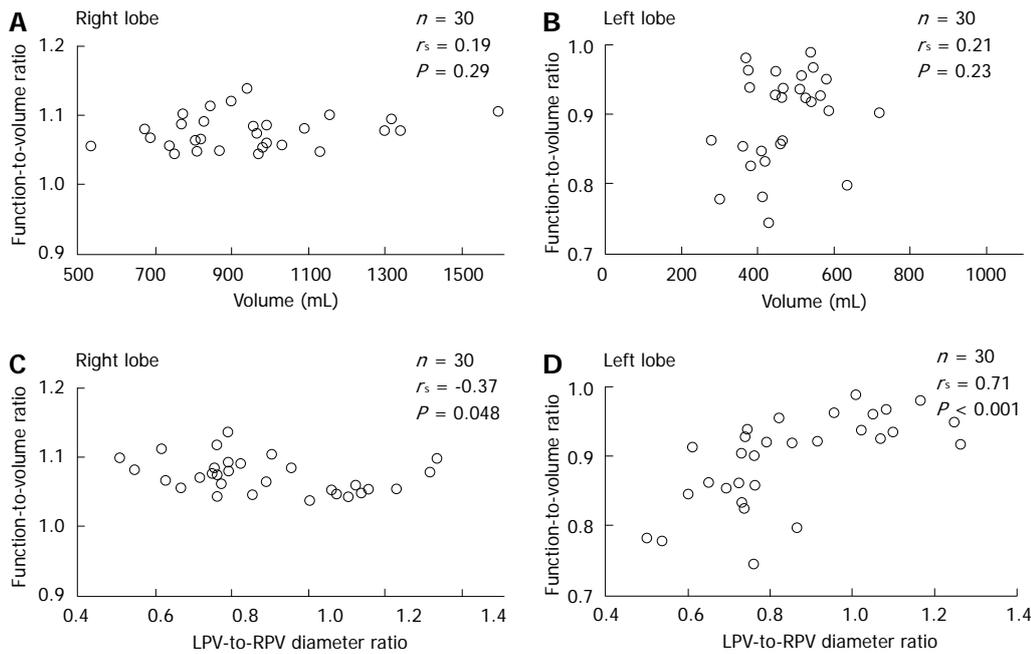
**Correlation between the function-to-volume ratio and 2 liver parameters (lobe volume and LPV-to-RPV diameter ratio)**

The function-to-volume ratio showed no significant cor-

relation with lobe volume in either lobe (right lobe;  $r_s = 0.19$ ,  $P = 0.29$ , left lobe;  $r_s = 0.21$ ,  $P = 0.23$ ). In contrast, the function-to-volume ratio showed a significant correlation with LPV-to-RPV diameter ratio in both lobes (right lobe; negative correlation,  $r_s = -0.37$ ,  $P = 0.048$ , left lobe; positive correlation,  $r_s = 0.71$ ,  $P < 0.001$ ) (Figure 7).

**DISCUSSION**

Conventionally, future remnant liver function after hepatectomy has been estimated using CT volumetry. Simulation by CT volumetry is satisfactory because it has better resolution and can provide precise anatomical information, if the function of the whole liver can be assumed to be homogeneous<sup>[1,5-8]</sup>. However, recent reports on the use of <sup>99m</sup>Tc-GSA scintigraphy have shown that regional hepatic function is significantly decreased when conditions such as biliary stenosis or unilaterally decreased portal flow exist<sup>[9-15]</sup>. The liver parenchyma, the area surrounding the tumor, has also been reported to be damaged by mechanical compression caused by intrahepatic tumors<sup>[5]</sup>. Therefore, accurate estimation of residual liver function, rather than estimation of residual liver volume, is more important for predicting postoperative hepatic functional reserve, and this has led to the development of <sup>99m</sup>Tc-GSA SPECT<sup>[1,6,16,20,28]</sup>. Several studies have shown that preoperative estimates of remnant liver function from <sup>99m</sup>Tc-GSA scintigraphy show better correlation than those from CT volumetry with postoperative liver function tests; therefore, <sup>99m</sup>Tc-GSA scintigraphy provides a better prediction of postoperative liver function<sup>[14,18-20]</sup>. Although this scintigraphic examination is not yet widely used worldwide, it is recognized as a useful modality for



**Figure 7** Correlation between the function-to-volume ratio and 2 liver parameters in each lobe. A and B: The function-to-volume ratio showed no significant correlation with lobe volume in either lobe; C and D: The function-to-volume ratio showed a significant correlation with the ratio of the diameter of the LPV to that of the RPV in both lobes (C: Right lobe, negative correlation; D: Left lobe, positive correlation). LPV: Left portal vein; RPV: Right portal vein.

preoperative liver function assessment in Japan, and we have decided the surgical indication on the basis of the estimated remnant liver function ratio measured by this modality. The plasma clearance rate of indocyanine green (ICGK) was measured preoperatively in each patient, and a patient with a future liver remnant ICGK value (ICGK  $\times$  remnant liver function ratio) of  $> 0.05$  was considered a candidate for hepatectomy<sup>[29]</sup>.

In this study, the volume ratio and the functional ratio of the 2 lobes were accurately measured using the newly developed CT/<sup>99m</sup>Tc-GSA SPECT fusion imaging technique. Although contrast-enhanced CT has usually been employed for volumetry, the anatomical landmarks could be confirmed in all cases in this non-enhanced CT. Therefore, setting the boundary between the 2 lobes was not complicated, and it could be performed reliably with confirmation by radiologists. The functional ratio was approximately 8% higher than the volume ratio in the right lobe and approximately 8% lower than the volume ratio in the left lobe. Furthermore, there were significant correlations between the function-to-volume ratio and the LPV-to-RPV diameter ratio in both lobes. The function-to-volume ratio in the left lobe tended to be higher, and that in the right lobe tended to be lower, in accordance with the increase of the diameter ratio.

This appears to be the first report to describe the functional differences between the 2 hepatic lobes using <sup>99m</sup>Tc-GSA scintigraphy. A few reports on liver scintigraphy with other agents, such as <sup>99m</sup>Tc-dimethyliminodiacetic acid for biliary scintigraphy and radiolabeled colloids for depiction of the reticuloendothelial system, have also shown lower uptake in the left liver lobe than in the right lobe<sup>[30,31]</sup>. The results of this study indicate that postsurgical remnant liver function and the tolerable extent of hepatectomy might change depending on the region of the hepatectomy. Residual liver function might be less after right-sided hepatectomy than after left-sided hepatec-

tomy, even if the hepatectomy volumes were equivalent.

Four possible causative factors for the functional discrepancy between the 2 lobes were estimated. First, the partial volume effect (PVE) of the peripheral part of the liver: the scintillation counts in the left lobe might have been underestimated owing to it being relatively thinner than the right lobe. However, we originally began this research because we realized there was decreased function in the interior portion of the left lobe on the fusion images. Furthermore, if PVE is the main cause of the functional decrease in the left lobe, the function-to-volume ratio should become higher in accordance with the increase in the lobe volume. However, there was no correlation between the lobe volume and the function-to-volume ratio. Second, the effect of tracer accumulation in the heart: to confirm the presence or absence of artifacts from tracer accumulation in the heart, we checked all SPECT images of all 30 patients, and no apparent artifact could be confirmed. Third, heterogeneous fat deposition: we investigated the mean CT attenuation values of the 2 lobes on non-enhanced CT images because the degree of fat deposition might influence the tracer accumulation. However, there were no differences in the degree of fat deposition between the 2 lobes. We concluded that the influences of these 3 factors were not significant, even if they could not be completely excluded from contributing to the functional discrepancy between the 2 lobes. Fourth, the portal flow difference: it is speculated that the difference in the bilateral portal flow might be the cause of the functional discrepancy. It is well known that the bilateral portal veins are developmentally distinct<sup>[32]</sup> and that the blood flow of the LPV is decreased compared with that of the RPV in non-cirrhotic livers<sup>[23,24]</sup>. During the early fetal period, the right and left hepatic lobes are not connected<sup>[32]</sup>. The main portal vein, which flows into the RPV, supplies blood only to the right lobe. The LPV, which is not connected to the portal vein, receives blood

from the left umbilical vein. Subsequently, the LPV joins the RPV through an almost 90° right turn. Within a week of birth, the infant's umbilical vein is completely obliterated, and the placental blood supply to the left lobe is cut off with the main circulation to the liver being taken over by the portal vein that was previously supplying only the right lobe. The left lobe is then supplied with blood only from the LPV, and blood flow in the LPV is less than that of the RPV<sup>[23]</sup>. Although the liver receives a dual blood supply from the portal vein and the hepatic artery, a decrease in portal flow without an arterial flow change can cause significant decreases in regional function<sup>[14,15,22]</sup>. Previous studies showed that a reduction in unilateral portal flow induced a decrease in both the function and the volume of the ipsilateral lobe, and the degree of the functional decrease was significantly higher than the degree of the volume decrease<sup>[14,15,22]</sup>. The decreased function observed in the left lobe in this study was also thought to be attributed to the lower flow volume in the LPV compared with that in the RPV. Although the measurement of the portal flow volume by Doppler ultrasound would be most appropriate to prove this speculation, we had no such data. Instead, we measured the diameter of the bilateral portal veins on CT images. Although the diameter of the portal vein could not directly indicate the portal flow volume, it is one of 2 factors that define the blood flow volume<sup>[23,24]</sup>. In this study, the LPV-to-RPV diameter ratio showed a significant correlation with the function-to-volume ratio in both lobes, indicating that portal flow significantly influenced the function of the 2 lobes.

Despite the novel findings of this study, it does have some limitations. First, although intrahepatic tumors in the surgical cases were limited to small tumors and the influence of the tumor on the surrounding parenchyma was estimated to be negligible, we cannot be certain that these tumors had no effect. However, the number of cases of right-sided tumors was higher than the number of cases of left-sided tumors (8 tumors in the right lobe and 4 tumors in the left lobe). Thus, although these tumors might have caused some degree of functional decrease, the decrease would more often have been associated with the right lobe. Second, although the lower function in the left lobe can be explained by decreased left portal flow, the mechanism of the functional discrepancy between the 2 lobes has not been sufficiently elucidated, and further investigation is necessary.

In conclusion, the results of this study on the use of CT/<sup>99m</sup>Tc-GSA SPECT fusion images indicated that the function of the left lobe was significantly decreased compared with that of the right lobe in non-cirrhotic livers. The function-to-volume ratio of each lobe showed a significant correlation with the LPV-to-RPV diameter ratio.

## COMMENTS

### Background

Preoperative evaluation of future remnant liver function is critical for patients undergoing hepatic surgery. In authors' department, remnant liver volume and function have been routinely estimated preoperatively using computed tomography/<sup>99m</sup>Tc-galactosyl human serum albumin (CT/<sup>99m</sup>Tc-GSA) single-photon emission computed tomography (SPECT) fusion imaging since December 2008, resulting in the

realization that the function of the interior portion of the left lobe was diminished compared with that of the right lobe, despite the absence of anatomical conditions suggestive of liver function heterogeneity. This study aimed to evaluate the functional differences between the 2 main lobes in non-cirrhotic livers.

### Research frontiers

Although recent reports have indicated the effectiveness of this newly developed CT/<sup>99m</sup>Tc-GSA SPECT fusion imaging technique for estimating the future remnant liver function, there have been no reports on the heterogeneous GSA accumulation between the 2 lobes.

### Innovations and breakthroughs

The results of this study on the use of CT/<sup>99m</sup>Tc-GSA SPECT fusion imaging indicated that the function of the left lobe was significantly decreased compared with that of the right lobe in non-cirrhotic livers. Furthermore, the function-to-volume ratio of each lobe showed a significant correlation with the left portal vein-to-right portal vein diameter ratio.

### Applications

The results of this study indicate that postsurgical remnant liver function and the tolerable extent of hepatectomy might change depending on the region of the hepatectomy. The residual liver function might be less after right-sided hepatectomy than after left-sided hepatectomy, even if the hepatectomy volumes were equivalent.

### Terminology

CT/<sup>99m</sup>Tc-GSA SPECT fusion examination is a newly developed SPECT examination method. Conventional <sup>99m</sup>Tc-GSA SPECT has the disadvantage of poor anatomical resolution, and this newly developed SPECT has a tremendously improved anatomical resolution owing to the fusion of CT and <sup>99m</sup>Tc-GSA SPECT.

### Peer review

This study describes that lobular differences in tracer accumulation can be observed even in patients with an apparently homogeneous liver on <sup>99m</sup>Tc-GSA SPECT. This finding is potentially important in the preoperative evaluation of future remnant liver function.

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P- Reviewers Dumitrascu DL, Nilsson H, Yamada A  
S- Editor Wen LL L- Editor Cant MR E- Editor Ma S



## Mechanisms of trichostatin A inhibiting AGS proliferation and identification of lysine-acetylated proteins

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Supported by Shanghai Municipal Health Bureau Key Disciplines Grant, No. ZK2012A05; National Natural Science Foundation, No. 81070344

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Received: January 16, 2013 Revised: March 21, 2013

Accepted: April 9, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To explore the effect of lysine acetylation in related proteins on regulation of the proliferation of gastric cancer cells, and determine the lysine-acetylated proteins and the acetylated modified sites in AGS gastric cancer cells.

**METHODS:** The CCK-8 experiment and flow cytometry were used to observe the changes in proliferation and cycle of AGS cells treated with trichostatin A (TSA). Real time polymerase chain reaction and Western blotting were used to observe expression changes in p21, p53, Bax, Bcl-2, CDK2, and CyclinD1 in gastric cancer cells exposed to TSA. Cytoplasmic proteins in gastric cancer cells before and after TSA treatment were immunoprecipitated with anti-acetylated lysine antibodies, separated using sodium dodecyl sulfate polyacrylamide

gel electrophoresis gel and silver-stained to detect the proteins by mass spectrometry after removal of the gel. The acetylated proteins in AGS cells were enriched with lysine-acetylated antibodies, and a high-resolution mass spectrometer was used to detect the acetylated proteins and modified sites.

**RESULTS:** TSA significantly inhibited AGS cell proliferation, and promoted cell apoptosis, leading to AGS cell cycle arrest in G0/G1 and G2/M phases, especially G0/G1 phase. p21, p53 and Bax gene expression levels in AGS cells were increased with TSA treatment duration; Bcl-2, CDK2, and CyclinD1 gene expression levels were decreased with TSA treatment duration. Two unknown protein bands, 72 kDa (before exposure to TSA) and 28 kDa (after exposure to TSA), were identified by silver-staining after immunoprecipitation of AGS cells with the lysine-acetylated monoclonal antibodies. Mass spectrometry showed that the 72 kDa protein band may be PKM2 and the 28 kDa protein band may be ATP50. The acetylated proteins and modified sites in AGS cells were determined.

**CONCLUSION:** TSA can inhibit gastric cancer cell proliferation, which possibly activated signaling pathways in a variety of tumor-associated factors. ATP50 was obviously acetylated in AGS cells following TSA treatment.

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**Key words:** Trichostatin A; Acetylation modification; Gastric cancer; Mass spectrometry; ATP50; PKM2

**Core tip:** Previous research has shown that deacetyltransferase inhibitors not only induce cell cycle arrest, differentiation and apoptosis of many tumor cells *in vitro*, but also inhibit tumor growth in tumor-bearing animals. They are through the acetylation modification of deacetyltransferase inhibitor on histone. Only a few studies have focused on the acetylation modification by deacetyltransferase on non-histone. This is the first study to identify acetylated proteins in gastric cancer

cells before and after exposure to trichostatin A to explore the effect of lysine acetylation of related proteins on the regulation of gastric cancer cell proliferation. Moreover, the lysine-acetylated proteins and the modified sites in AGS cells were assessed. We explored whether ATP5O was obviously acetylated after trichostatin A treatment in AGS cells.

Wang YG, Wang N, Li GM, Fang WL, Wei J, Ma JL, Wang T, Shi M. Mechanisms of trichostatin A inhibiting AGS proliferation and identification of lysine-acetylated proteins. *World J Gastroenterol* 2013; 19(21): 3226-3240 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3226.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3226>

## INTRODUCTION

Gastric cancer has a high incidence and mortality worldwide, especially in East Asia<sup>[1,2]</sup>. More than 400000 new patients with gastric cancer are diagnosed in China every year. The prevalence and mortality of this disease in China are higher than the world average values<sup>[3]</sup>. In the absence of targets, traditional chemotherapy has severe side effects. Therefore, cancer treatment and research are now focusing on molecular targeted therapy due to its high selectivity, good efficacy and reduced side effects.

Histone acetylation/deacetylation modification, one of the essential mechanisms of gene transcriptional regulation, occurs mainly in conservative lysine residues on histone H3 and H4 tails, which are regulated by histone acetyltransferases and histone deacetylases (HDACs). Significantly increased activity of HDACs leads to an expression imbalance of some molecules affecting cell proliferation, apoptosis and cell cycle, thus causing cancer<sup>[4]</sup>. A large number of studies have shown that deacetyltransferase inhibitors not only induce cell cycle arrest, differentiation and apoptosis of many tumor cells *in vitro*, but also inhibit tumor growth in tumor-bearing animals<sup>[5,6]</sup>. There have been numerous studies on the acetylation modification by deacetyltransferase inhibitors on histone, however, studies focusing on the acetylation modification by deacetyltransferase on non-histones are rare. Further studies are needed to investigate the acetylated non-histones involved in tumor growth and metabolism, and the signaling pathways through which these proteins induce tumor apoptosis. We treated AGS gastric cancer cells with the histone deacetyltransferase inhibitor, trichostatin A (TSA), to identify differentially acetylated non-histones before and after TSA treatment. We also explored the apoptosis and proliferation mechanisms of gastric cancer cells.

## MATERIALS AND METHODS

### Materials

AGS cells were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences; Ham's F12 medium was from

HyClone; trypsin-EDTA solution and fetal bovine serum from Invitrogen; the cell counting kit-8 (CCK-8) from Dojindo Company; TSA from Sigma (batch number T1952); the Annexin V-FITC Apoptosis Detection Kit, FACS Calibur and LSR™ II Flow Cytometer from BD Pharmingen; the primer was designed by Shanghai Sangon Biotech Co., Ltd.; Agarose I™ was from Amresco, the RNeasy Mini Kit from Qiagen; the Reverse Transcription System from Promega; SYBR® Premix Ex Taq™ from TaKaRa; ABI prism 7300 polymerase chain reaction (PCR) from ABI; Amersham ECL plus the Western blotting Detection System and CNBr Activated Sepharose 4B from GE; Pierce BCA Protein Assay Kit from Thermo; Acetyl- $\alpha$ -Tubulin (Lys40) (D20G3) XP® Rabbit mAb from CST; Goat anti-rabbit IgG-HRP from Sigma; LTQ VELOS from Thermo Finnigan, and anti-ATP5O and anti-PKM2 antibodies from Sigma.

### CCK-8 experiment

AGS cell strains were cultured in Ham's F12 medium + 10% FBS for 24 h and divided into 8 groups (3 holes in each group). The media in the holes were added to complete media containing TSA at final concentrations of 0, 0.015, 0.03, 0.06, 0.1, 0.25, 0.5 and 1  $\mu\text{mol/L}$ , respectively. The complete media were incubated with 5% CO<sub>2</sub> at 37 °C for 72 h, and then added to CCK-8 solution in the proportion of 100  $\mu\text{L}/10 \mu\text{L}$ , and left to stand at 37 °C for 1 h. Absorbance was then read at a wavelength of 450 nm using a microplate reader.

### Detection of cell apoptosis and cycle by flow cytometry

Two dishes of AGS cells cultured for 24 h were added to complete medium containing TSA at a final concentration of 0.25  $\mu\text{mol/L}$ , and a further two dishes of cultured cells were added to new medium as a control. The media were incubated with 5% CO<sub>2</sub> at 37 °C for 24 h, centrifuged, transferred to a 5 mL culture tube and the supernatant was removed. The cells were re-suspended, and 5  $\mu\text{L}$  Annexin V-FITC and propidium iodide (PI) were added, incubated in the dark at 20-25 °C for 15 min and then 400  $\mu\text{L}$  Annexin V binding solution was added for flow cytometry. Annexin V-FITC had green fluorescence and PI had red fluorescence. The wavelength of light excited by flow cytometry was adjusted to 488 nm. FITC fluorescence was detected with a band-pass filter of 515 nm and PI fluorescence was detected with a filter of more than 560 nm. In addition, the cell sediments were added to 1 mL of 70% ethanol, fixed, washed, centrifuged twice, re-suspended in 0.5 mL PBS containing 50  $\mu\text{g}/\text{mL}$  PI and 100  $\mu\text{g}/\text{mL}$  RNase A, and incubated in the dark at 37 °C for 30 min to determine the cell cycle using a flow cytometer according to standard procedures. The results were analyzed using a cycle meter and the software FlowJo6.3<sup>[7]</sup>.

### Real-time polymerase chain reaction

AGS cells cultured for 24 h were added to complete medium containing TSA at final concentrations of 0 and 0.25  $\mu\text{mol/L}$ , respectively (the former for the control).

**Table 1** Oligonucleotide sequences used in real-time polymerase chain reaction

| Gene            | Primer (5' to 3')                | Length (bp) |
|-----------------|----------------------------------|-------------|
| <i>β-actin</i>  | F: 5'TGGAGAAAATCTGGCACCA3'       | 189         |
|                 | R: 5'CAGGCGTACAGGGATAGCAC3'      |             |
| <i>p21</i>      | F: 5'TCCAAGAGGAAGCCCTAATCC3'     | 101         |
|                 | R: 5'ACAACTGAGACTAAGGCAGAAGATG3' |             |
| <i>p53</i>      | F: 5'TCAGTCTACCTCCCGCCATAA3'     | 231         |
|                 | R: 5'GTGAGGCCAACTTGTTCAGT3'      |             |
| <i>Bcl-2</i>    | F: 5'CTTTTCTACTTTGCCAGCAAAC3'    | 149         |
|                 | R: 5'GAGGCCGTCCCAACCAC3'         |             |
| <i>CDK2</i>     | F: 5'GCTAGCAGACTTTGGACTAGCCAG3'  | 85          |
|                 | R: 5'AGCTCGGTACCACAGGGTCA3'      |             |
| <i>CyclinD1</i> | F: 5'AACAGAAGTGCAGGAGGAG3'       | 299         |
|                 | R: 5'CTGGCATTTGGAGAGGAAG3'       |             |
| <i>Bax</i>      | F: 5'CCAGGGTGGTGGGTGAGACT3'      | 231         |
|                 | R: 5'TGGGAGGTCAGCAGGGTAGAT3'     |             |

Bcl-2: B cell lymphoma-2; CDK2: Cyclin-dependent kinase 2; Bax: Bcl-associated X protein.

The total RNA in all samples was extracted, quantified and reversely transcribed according to the Qiagen kit instructions. Fluorescence quantitative PCR was carried out on p21, p53, Bax, Bcl-2, CDK2 and CyclinD1, followed by data collection and analysis. The PCR primer sequences and fragment lengths are shown in Table 1.

### Western blotting

One dish of AGS cells cultured for 24 h was used as the 0 h sample, and a further 2 dishes of cells were added to medium containing a final concentration of 0.25 μmol/L TSA, and incubated with 5% CO<sub>2</sub> at 37 °C for 12 and 24 h, respectively. The cells were collected after digestion with pancreatin, washed twice with PBS, centrifuged to remove the supernatant, collected and placed on ice for lysis. The proteins were quantified using the BCA method. Protein electrophoresis sodium dodecyl sulfate polyacrylamide gel electrophoresis, membrane-transfer, immunoreactions, development and gel electrophoresis image analysis were performed for p21, p53, Bax, Bcl-2, CDK and CyclinD1.

### Enrichment of lysine-acetylated proteins

Five dishes of AGS cells were added to complete medium containing a final concentration of 0.5 μmol/L TSA, and another five dishes of cells were directly placed in new medium as the control. Cell lysis was performed after incubation in the medium for 24 h, and all protein concentrations were adjusted to 5 mg/mL after determination using the BCA method. Total protein of 20 mg and lysine-acetylated mAb of 0.5 mL (CNBr Activated Sepharose 4B) were mixed, incubated in a table concentrator at 4 °C for 5 h, washed 3 times and collected for vacuum drying. The lysine-acetylated proteins were enriched and dissolved in PBS. Electrophoresis, silver-staining and photographs of the total proteins of 2 μg taken from each dish after the proteins were quantified with BCA were carried out. Western blotting was performed

on all proteins in each group to determine the effect of acetylation (20 μg total protein from AGS cells not treated and treated with 0.5 μmol/L TSA, respectively; 20 μg flow-through proteins incubated with the antibody gel column in AGS cells not treated and treated with 0.5 μmol/L TSA respectively; and 100 ng total proteins enriched after incubation with antibody gel column in AGS not treated and treated with 0.5 μmol/L TSA, respectively). Acetyl-α-tubulin (Lys40) (D20G3) XP<sup>®</sup> Rabbit mAb was the primary antibody and Goat anti-rabbit IgG-HRP was the secondary antibody.

### Identification of in-gel protein with mass spectrometry

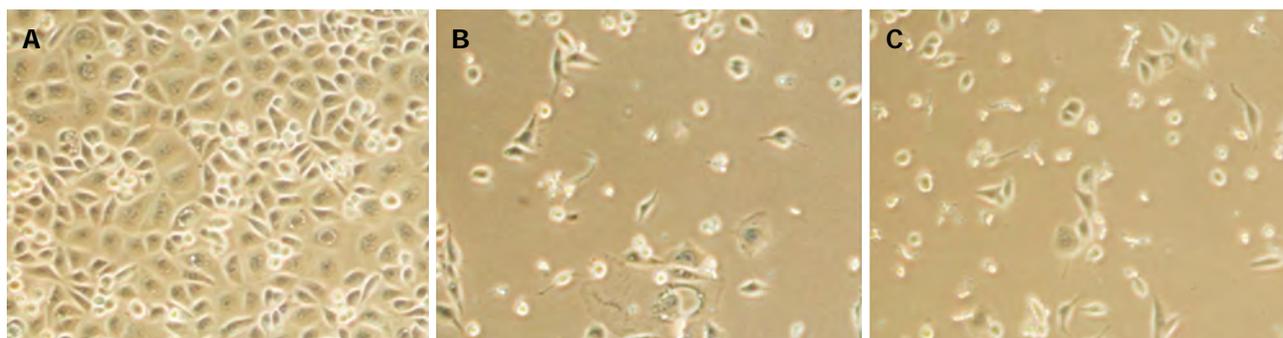
The enriched protein band on silver-stained gel (72 kDa before exposure to TSA and 28 kDa after exposure to TSA), was broken down in the gel with enzyme (trypsin for 20 h), and the decomposed peptide was extracted for ESI MS detection. After the chromatographic column was equilibrated with 95% solution A (0.1% formic acid solution), the sample was fed into a Trap column. From 0 to 50 min, the linear gradient of solution B (78% acetonitrile solution containing 0.1% formic acid) increased from 4% to 50%; from 50 to 54 min, the linear gradient of solution B increased from 50% to 100%; from 54 to 60 min, the linear gradient of solution B was maintained at 100%. Twenty fragmentographies (MS2 scan) were collected by mass-to-charge ratio of the polypeptides and polypeptide fragments after full scan. The raw file was searched with BIOWORKS software in the relevant database to determine the protein. The database was ipi. HUMAN.v3.53. SEQUEST screening parameters were as follows: when Charge + 1, Xcorr ≥ 1.9; when Charge + 2, Xcorr ≥ 2.2; and when Charge + 3, Xcorr ≥ 3.75; wherein DelCN ≥ 0.1.

### Identification of acetylated sites using mass spectrometry

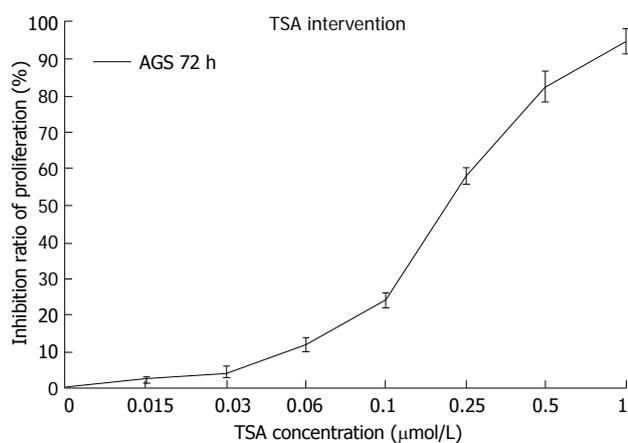
Experimental methods for cell lysis, protein extraction and acetylated peptides affinity enrichment were obtained from published techniques<sup>[8]</sup>. The resulting peptides were assayed by continuous separation using SCX followed by C18 columns (Dionex, Sunnyvale, CA, United States) before being subjected to MS/MS analysis using an LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany).

### Protein sequence database search and manual verification

The mass spectrometry data were initially searched against the NCBI database with the aid of the Sequest search engine. Searches for acetylated peptides were done against the *Homo sapiens* proteins database. The search engine MASCOT (Matrix Science, London, United Kingdom) was used for the database search, and extract\_msn.exe version 4.0 was used for peaklist generation. A low cutoff of the peptide score of 20 was selected to maximize the identification of lysine-acetylated peptides. Trypsin was specified as the proteolytic enzyme, and up to 6 missed cleavage sites per peptide were allowed. Carbamidomethylation of cysteine was set as a fixed modification and



**Figure 1** Proliferation of AGS cells exposed to different concentrations of trichostatin A for 72 h. A: AGS cells after treatment with 0  $\mu\text{mol/L}$  trichostatin A (TSA); B and C: AGS cells were significantly reduced after exposed to 0.25  $\mu\text{mol/L}$  TSA (B) and further reduced after treated with 0.5  $\mu\text{mol/L}$  TSA (C).



**Figure 2** Effect of different concentrations of trichostatin A on inhibition of AGS cell proliferation in the cell counting kit-8 experiment. The inhibition of AGS cells was gradually increased with increasing trichostatin A concentration 0, 0.015, 0.03, 0.06, 0.1, 0.25, 0.5 and 1  $\mu\text{mol/L}$ . TSA: Trichostatin A.

oxidation of methionine and acetylation of lysine as variable modifications. Charge states of +1, +2 or +3 were considered for parent ions. Mass tolerance was set to  $\pm 4.0$  Da for parent ion masses and  $\pm 0.6$  Da for fragment ion masses. Acetylated lysine-containing peptides identified with a MASCOT score of 25 were manually verified by the method described by Chen *et al*<sup>[9]</sup>.

#### Detection of acetylated proteins

One dish of normal AGS cells was collected as the 0 h sample after digestion with pancreatin, and a further 3 dishes of cells were added with a final concentration of 0.5  $\mu\text{mol/L}$  TSA and incubated with 5%  $\text{CO}_2$  in an incubator at 37  $^\circ\text{C}$  for 6, 12 and 24 h. The collected cells were digested with pancreatin, re-suspended, and decomposed by ultrasound on ice. The decomposed cells were centrifuged at 15000  $g$  and 4  $^\circ\text{C}$  for 30 min and the supernatant was obtained for identification of protein concentration using the BCA. Five mg of total protein was mixed with 50  $\mu\text{g}$  of the M2 isoform of pyruvate kinase antibody (anti-PKM2) and the ATP synthase subunit O antibody (anti-ATP5O) (covalently cross-linked with CNBr Activated Sepharose 4B) and incubated in an incubator at 4  $^\circ\text{C}$  for 5 h. The gel column was washed 3 times and the

washed proteins were collected. The protein content of ATP5O<sup>[10]</sup> and PKM2<sup>[11]</sup> in the 4 samples was determined using Western blotting. After binding, the washed proteins with the anti-acetylated lysine antibodies, and the protein content of ATP5O and PKM2 in the 4 samples were detected using Western blotting.

## RESULTS

### Effect of TSA on AGS cell proliferation, apoptosis and cell cycle

CCK-8 experiments showed that AGS cells were significantly reduced after the addition of 0.25  $\mu\text{mol/L}$  TSA and AGS cell proliferation was more obviously inhibited after the addition of 0.5  $\mu\text{mol/L}$  TSA. Therefore, TSA significantly inhibited proliferation of the gastric cancer cell line (Figures 1 and 2). The bivariate scatter diagram of flow cytometry showed more apoptotic and necrotic AGS cells after treatment with 0.25  $\mu\text{mol/L}$  TSA (Figure 3). The flow cytometry cycle diagrams showed that the AGS cell cycle ratio before TSA treatment was as follows: %G1 = 26, %S = 53.5, %G2 = 17.7, and the AGS cell cycle ratio after 0.25  $\mu\text{mol/L}$  TSA treatment was as follows: %G1 = 44.6, %S = 20.9, %G2 = 31.3. Therefore, TSA induced apoptosis and necrosis of AGS cells, and cycle arrest mainly occurred in G0/G1 and G2/M phases, especially in G0/G1 phase (Figure 4).

### Observation of p21, p53, Bax, Bcl-2, CDK2 and CyclinD1 expression levels after TSA treatment using real-time PCR and Western blotting

Real-time PCR results showed that more p21, p53 and Bax mRNA was expressed after AGS cells were exposed to 0.25  $\mu\text{mol/L}$  TSA, and the expression levels were increased with TSA treatment duration, while less Bcl-2, CDK2 and CyclinD1 mRNA was expressed after TSA treatment, and the expression levels were decreased with TSA treatment duration (Figure 5). The expression levels of the above six cell cycle-related proteins in AGS cells shown in Western blotting were the same as the levels shown in real-time PCR (Figure 6).

### Enrichment of lysine-acetylated proteins

In AGS cells enriched with lysine-acetylated monoclonal

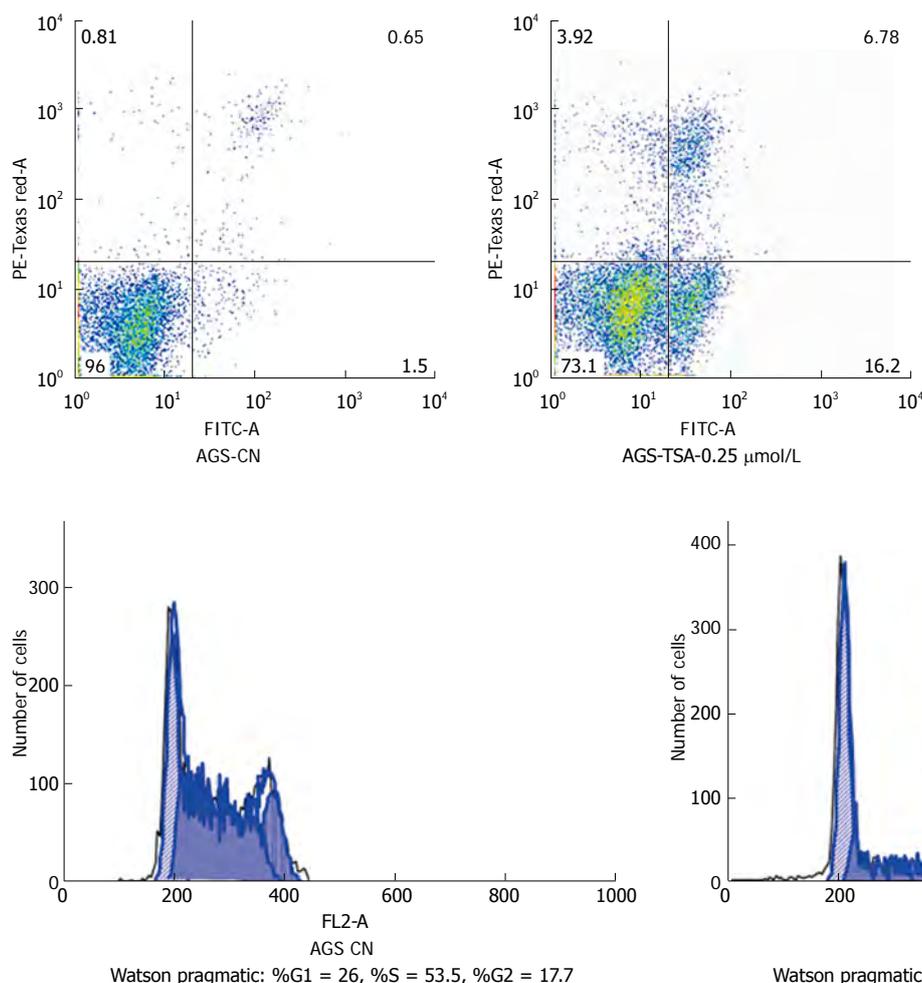


Figure 3 AGS cell apoptosis changes before and after trichostatin A exposure by flow cytometry. Fluorescence staining was mainly seen in normal areas, but rarely in apoptotic and necrotic areas of unexposed AGS cells; staining appeared in the apoptotic and necrotic areas of AGS cells exposed to 0.25  $\mu\text{mol/L}$  trichostatin A (TSA).

Figure 4 AGS cell cycle changes before and after trichostatin A exposure by flow cytometry. The AGS cell cycle ratio before TSA treatment was: %G1 = 26, %S = 53.5, %G2 = 17.7, and the AGS cell cycle ratio after 0.25  $\mu\text{mol/L}$  trichostatin A treatment was: %G1 = 44.6, %S = 20.9, %G2 = 31.3. Cycle arrest occurred in G0/G1, G2/M phases, especially in G0/G1 phase. TSA: Trichostatin A.

antibodies, the enriched proteins were located at 72 kDa before exposure to 0.5  $\mu\text{mol/L}$  TSA shown by silver-staining, but appeared at 55, 28 and 17 kDa after exposure to 0.5  $\mu\text{mol/L}$  TSA, which was consistent with the Western blotting results (Figure 7). Some studies have shown that the enriched proteins at 55 and 17 kDa were tubulin and histone protein, respectively. In our experiments which were designed to determine the modified proteins enriched by lysine-acetylated monoclonal antibodies, total protein in the cytoplasm, flow-through proteins, and enriched proteins all showed obvious bands. No obvious bands for these three proteins were found before TSA treatment (Figure 8), which indicated that the protein enrichment method with lysine-acetylated monoclonal antibodies was effective and credible.

#### Identification of in-gel proteins by mass spectrometry

Mass spectrometry was carried out on the unknown protein bands, 72 kDa (before TSA treatment) and 28 kDa (after TSA treatment), which were enriched and modified by lysine acetylation to obtain ESI MS total ion chromatography (Figure 9). We searched the protein database ipi.

HUMAN.v3.53 with the SEQUEST program according to the set screening parameters. The results indicated that 72 kDa (before TSA treatment) was PKM2, and 28 kDa (after TSA treatment) was ATP5O in AGS cells (Figure 10).

#### Identification of acetylated sites using mass spectrometry and detection of acetylated proteins

We screened all peptide sequences obtained by detection of the acetylated sites using mass spectrometry. In the plasmosin of normal AGS cells, we found 602 acetylated peptides, 171 unique peptides and 136 acetylated sites (Table 2). Cell cycle G0 analysis showed that the identified proteins contained chromatin, nucleosome, DNA components as well as chromatin modification, protein acetylation, glucose metabolism and other biological processes. These components were mainly involved in cellular components such as chromatin, nucleoplasm and organelles, and these molecular functions were mainly associated with cell proliferation and apoptosis (Figure 11). In these acetylated peptides, the presence of ATP5O indicated that ATP5O had modified sites (Figure 12). In the mass spectrometry results, most of the identified proteins had a

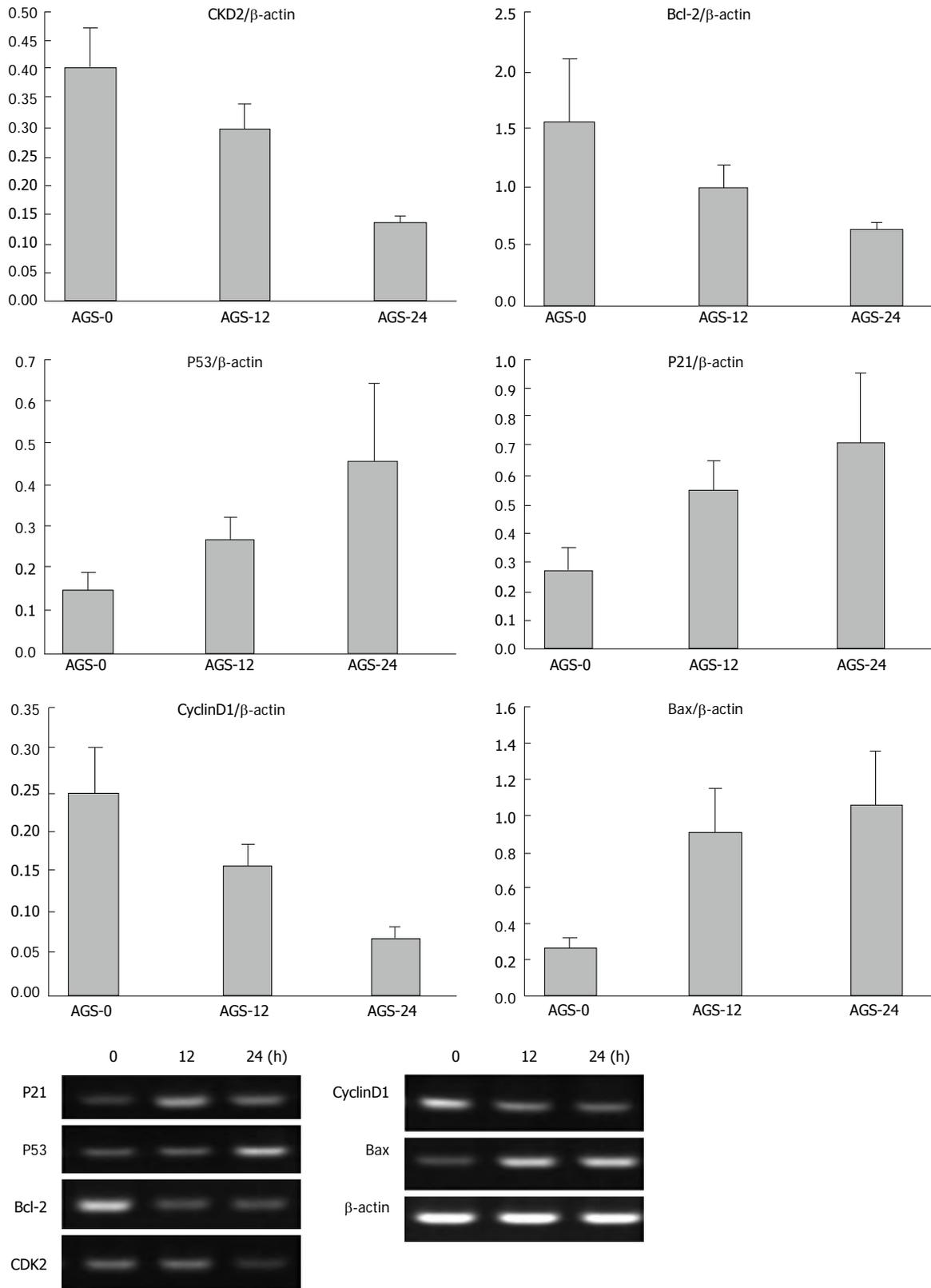
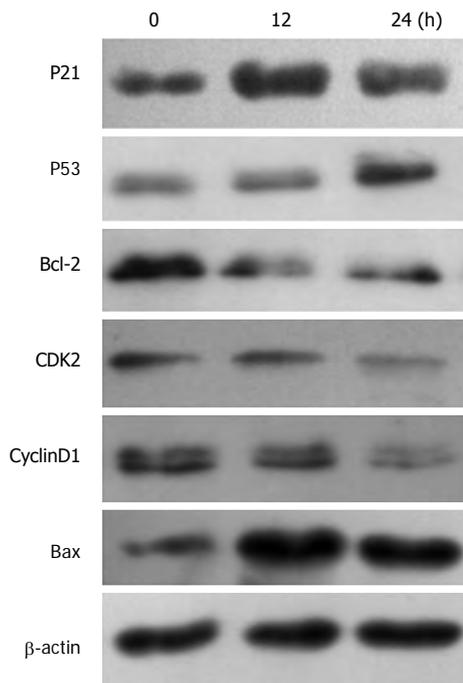


Figure 5 mRNA expression levels of p21, p53, Bax, Bcl-2, CDK2 and CyclinD1 in AGS cells exposed to 0.25  $\mu\text{mol/L}$  trichostatin A shown by real-time polymerase chain reaction. The mRNA expression levels of Bcl-2, CDK2 and CyclinD1 were decreased and the mRNA expression levels of p21, p53 and Bax were increased 12 h after AGS cells were exposed to 0.25  $\mu\text{mol/L}$  trichostatin A. The mRNA expression levels of Bcl-2, CDK2 and CyclinD1 were further decreased and the mRNA expression levels of p21, p53 and Bax were further increased 24 h after exposure.

cover percent greater than 20%, and a score greater than 25, which proved that the mass spectrometry identifica-

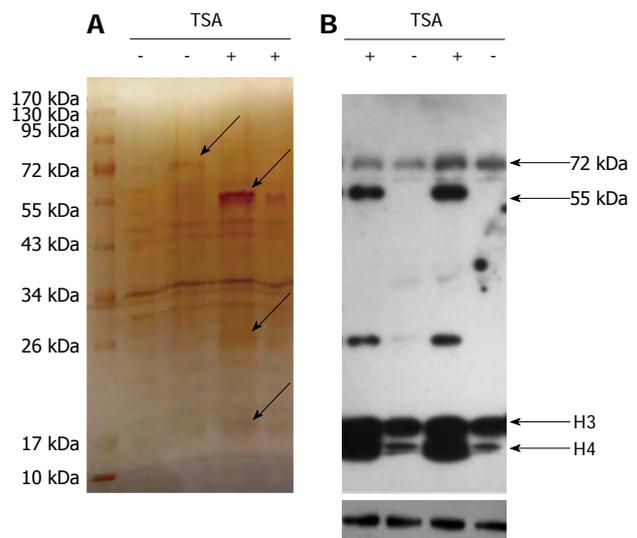


**Figure 6** Protein expression levels of p21, p53, Bax, Bcl-2, CDK and cyclin after AGS cells were exposed to 0.25 μmol/L trichostatin A shown by Western blotting. The protein expression levels of Bcl-2, CDK2 and CyclinD1 were decreased and the protein expression levels of p21, p53 and Bax were increased 12 h after AGS cells were exposed to 0.25 μmol/L trichostatin A. The protein expression levels of Bcl-2, CDK2 and CyclinD1 were further decreased and the protein expression levels of p21, p53 and Bax were further increased 24 h after exposure.

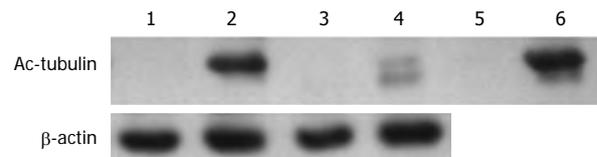
tion results were correct. Further validation of the acetylated proteins, ATP5O and PKM2, showed that the total amount of ATP5O and PKM2 proteins did not change with the treatment duration of 0.5 μmol/L TSA, however, more ATP5O was acetylated than PKM2 (Figure 13), which indicated acetylation of ATP5O and deacetylation of PKM2 after TSA treatment.

## DISCUSSION

Modern oncology theories have revealed that genetic defects and gene epigenetic changes lead to malignant tumors. Epigenetics has shown acetylation of DNA methylation and histone, which are involved in gene transcription and expression, thus regulating DNA functions<sup>[12-14]</sup>. TSA derives from a natural hydroxamic acid, HDACi, which inhibits the activity of HDACs by binding the hydroxamic acid ligand with zinc ions at the bottom of the HDAC tubular structure<sup>[15]</sup>. Cancer research has discovered that TSA can arrest cell cycle, induce cell apoptosis, regulate cell differentiation and inhibit cell migration in the absence of cytotoxicity<sup>[16-18]</sup>. We found that the proliferation of normally grown AGS gastric cancer cells was significantly inhibited after exposure to 0.25 μmol/L TSA, *i.e.*, more apoptotic and necrotic cells. In addition, flow cytometry showed that the cycle arrest of AGS cells exposed to TSA occurred in G0/G1 and G2/M phases, which is consistent with other previous reports<sup>[19-22]</sup>. In



**Figure 7** Identification of differential proteins of lysine acetylation after trichostatin A treatment. A: Silver staining showed the differential proteins of lysine acetylation in AGS cells after trichostatin A (TSA) treatment; B: Western blotting showed the differential proteins of lysine acetylation in AGS cells after TSA treatment, “-” before TSA intervention; “+” after TSA intervention, Acetyl-α-tubulin (Lys40) (D20G3) XP<sup>®</sup> Rabbit mAb was the primary antibody and Goat anti-rabbit IgG-HRP was the secondary antibody.



**Figure 8** Identification of the effectiveness of lysine-acetylated antibodies enriching acetylated proteins. Line 1: 20 μg total protein from AGS cells unexposed to trichostatin A (TSA); Line 2: 20 μg total protein from AGS cells exposed to 0.5 μmol/L TSA; Line 3: 20 μg flow-through protein from AGS cells unexposed to TSA, which was incubated with an antibody gel column; Line 4: 20 μg flow-through protein from AGS cells exposed to 0.5 μmol/L TSA, which was incubated with an antibody gel column; Line 5: 100 ng enriched protein from AGS cells unexposed to TSA, which was incubated with an antibody gel column; and Line 6: 100 ng enriched protein from AGS cells exposed to 0.5 μmol/L TSA, which was incubated with an antibody gel column.

the present study, cycle arrest in G0/G1 phase was more obvious.

Current studies indicate that TSA can activate histone acetylation to loosen the chromosome structure, thus endonuclease can easily access DNA, and TSA can block signal transduction pathways associated with cell proliferation by activating death receptors and mitochondrial apoptotic pathways, promoting transcription of tumor suppressor genes, which directly or indirectly induces tumor cell apoptosis<sup>[23,24]</sup>. Our research showed that the expression levels of tumor suppressor genes, *p21*, *p53* and *Bax*, were increased after AGS cells were exposed to 0.25 μmol/L TSA, which increased with treatment duration, and the protooncogenes *Bcl-2*, *CDK2* and *CyclinD1* showed the opposite trend. These results are consistent with those of previous publications<sup>[22,25-29]</sup>, but different from some reports. The research of Suzuki *et al.*<sup>[20]</sup> showed that TSA could reduce *p53* expression level, although

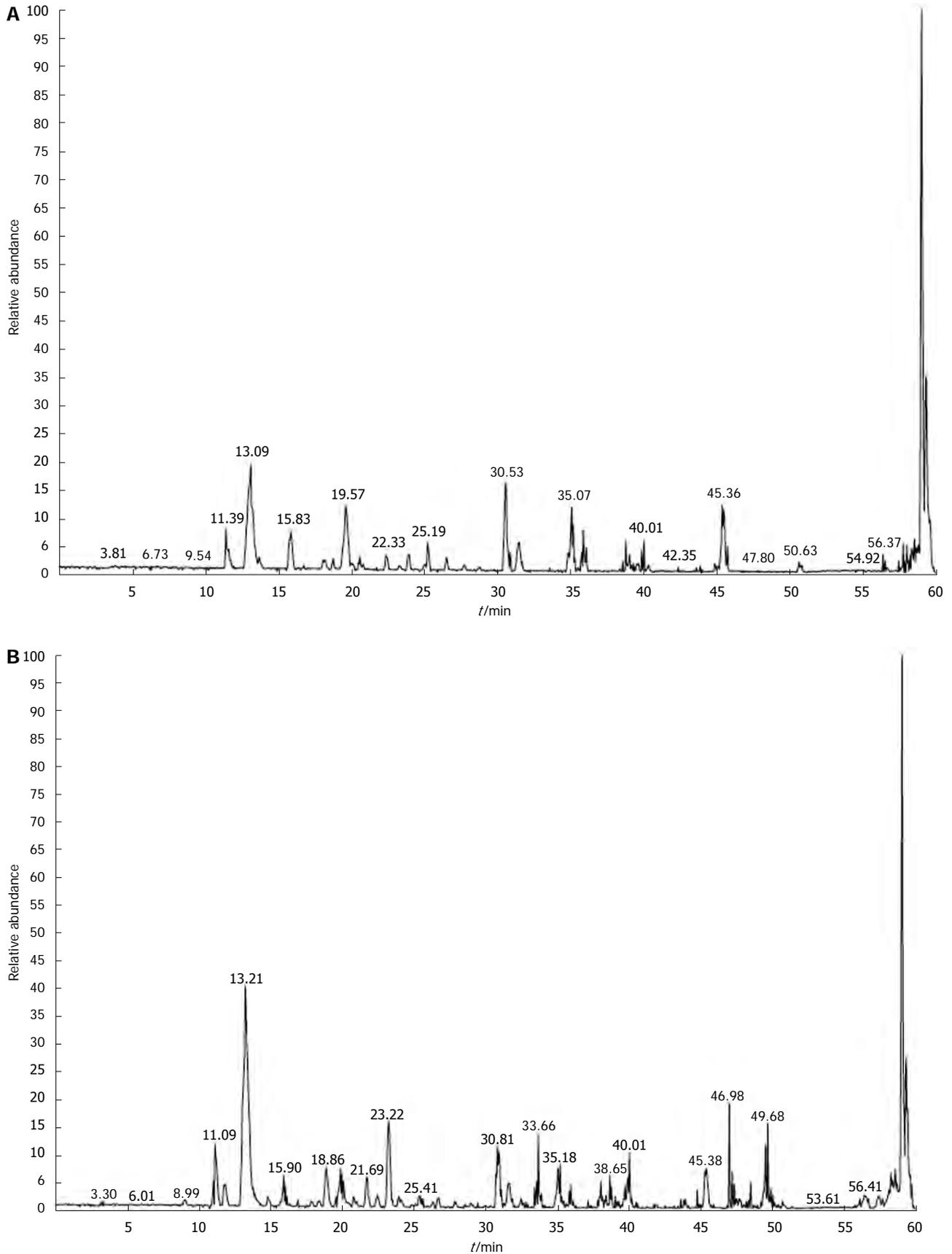
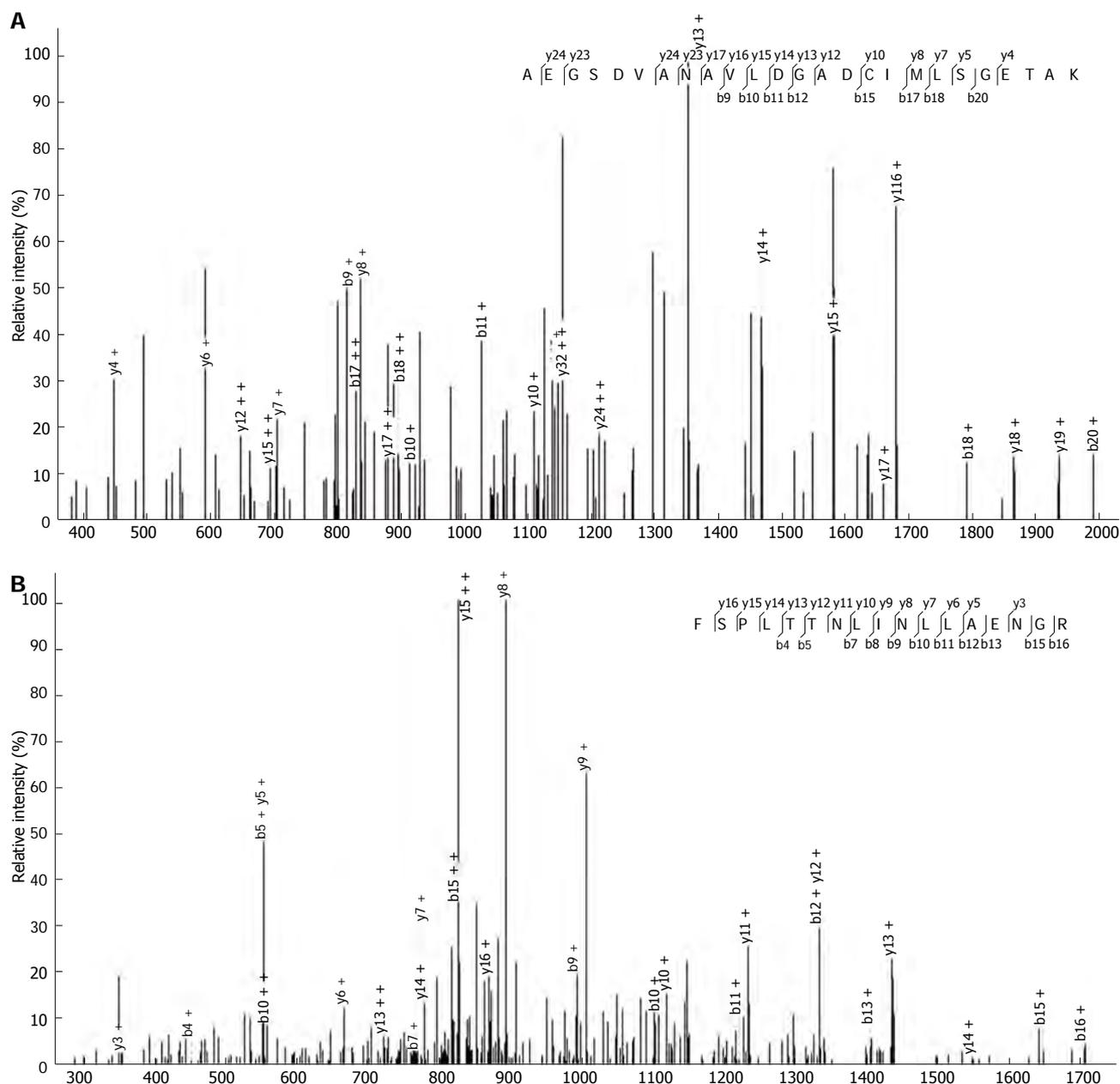


Figure 9 Mass spectrometry total ion chromatography. A: 72 kDa band of lysine-acetylated protein in AGS cells before trichostatin A (TSA) treatment; B: 28 kDa band of lysine-acetylated protein in AGS cells after TSA treatment.



**Figure 10** Mass spectrometry identification of peptides from silver staining gel. Mass spectra of 2 acetylated peptides from PKM2 and ATP5O are presented. A: PKM2 “AEGSDVANAVLDGADCIMLSGETAK”; B: ATP5O “FSPLTTNLLAENGR”.

p21 and Bax expression levels were enhanced. The research of Juan *et al*<sup>[30]</sup> showed that deacetyltransferase can specifically lower *p53* and *p53*-dependent genes. These different results may be the result of different group designs and study objectives, which need to be confirmed. In most research studies, *P21waf1/cip1* is used as the transcript of target gene at p53 downstream, a suppressor of cyclin and cyclin-dependent kinase, which can be combined with a variety of cyclins/CDK complexes by phosphorylation to inhibit cell growth in G2/M phase, thus inhibiting proliferation of tumor cells. It is believed that *P21waf1/cip1* silencing mechanisms in tumor cells may be decided by epigenetic modifications of their chromatins, and their expression levels are regulated by histone acetylation<sup>[31]</sup>. Some research studies confirmed that HDACi-

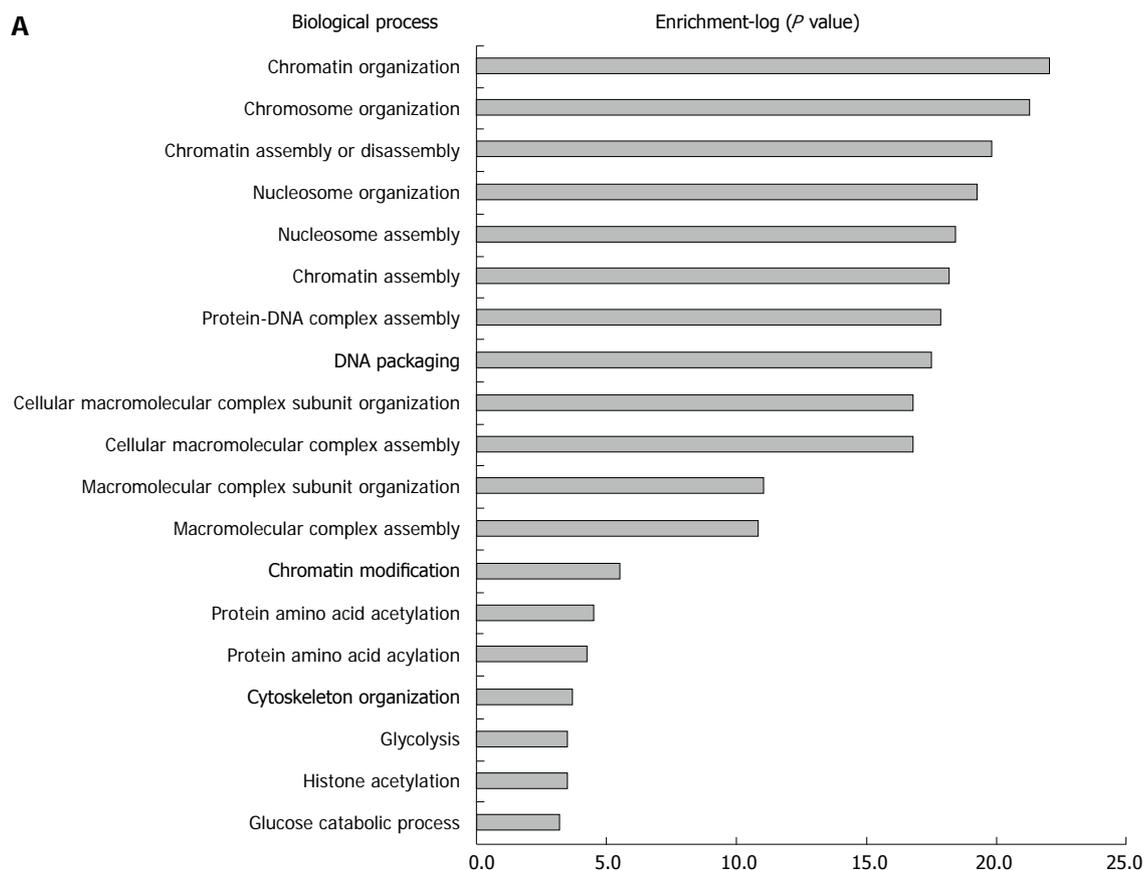
induced histone is acetylated in the *P21waf1/cip1* gene promoter region, and *P21waf1/cip1* may be a direct target of TSA<sup>[32]</sup>.

It is clear that TSA plays a role in histone acetylation, however, the function of TSA in non-histone acetylation in the inhibition of tumor growth has rarely been reported. Therefore, we immunoprecipitated AGS cells before and after exposure to TSA with lysine-acetylated monoclonal antibodies, and found an enriched protein band at 72 kDa before exposure and three enriched protein bands at 55, 28 and 17 kDa after exposure. The enriched proteins at 55 and 17 kDa were found to be tubulin and histone, respectively. We extracted two unknown protein bands at 72 and 28 kDa with gel and identified them by in-gel mass spectrometry and found that the 28 kDa

**Table 2 Proteomic identification of acetylated proteins in AGS cells**

| Reference  | Sequence  |
|--|---|
| Filamin A, alpha   | K.TGVAVNKPAEFTVDAK*HGGK.A   |
| Heat shock cognate 71 kDa protein                            | R.RFDDAVVQSDMK*HWPFMVVNDAGRPK.H   |
| Heat shock protein HSP 90-alpha                              | R.MKENQK*HIYYITGETK.D<br>R.MK*ENQKHIYYITGETK.D  |
| Histone H2B type 1-C/E/F/G/I                                 | M.PEPAK*SAPAPK*K*GSK*K*AVTK*AQK.K<br>R.LLLPGELAK*HAVSEGTK.A   |
| Histone H2B type 1-D   | K.SAPAPK*K*GSK*K*AVTK*AQK*K.D   |
| Histone H2B type 1-H   | K.#KGSK*K*AVTK*AQK*K.D  |
| Histone H2B type 2   | K.SAPAPK*K*GSK*K*AVTK*VQK.K   |
| Ezrin  | R.QAVDQIK*SQEQLAAELAEYTAI.I   |
| Histone H4   | M.SGRGK*GGK*GLGK*GGAK*R.H   |
| Fructose-bisphosphate aldolase A                             | K.DGADFAK*WR.C  |
| Histone H2B type 1-B   | K.K*GSK*K*AITK*AQK*K.D<br>K.SAPAPK*K*GSK*K*AITK*AQK.K<br>R.LLLPGELAK*HAVSEGTK.A                         |
| Tubulin beta chain   | R.ISVYYNEATGGK*YVPR.A   |
| Histone H2B type 1-M   | K.K*GSK*K*AINK*AQK.K  |
| T-complex protein 1 subunit theta                            | K.EGAK*HFSGLEEAVYR.N  |
| Tubulin beta-4B chain  | R.INVYVNEATGGK*YVPR.A   |
| Uncharacterized protein                                      | K.HELQANCYEEVK*DR.C   |
| Histone H3   | R.K*QLATK*AAR.K<br>R.K*STGGK*APR.K  |
| Glyceraldehyde-3-phosphate dehydrogenase                     | R.VIISAPSADAPMFVMGVNHEK*YDNSLK.I  |
| Heterogeneous nuclear ribonucleoprotein Q                    | K.SAFLCGVMK*TYR.Q   |
| Phosphoglycerate kinase 1                                    | R.FHVEEKG*GK.D  |
| Histone H3.1t  | R.EIAQDFK*TDLR.F  |
| T-complex protein 1 subunit alpha                            | K.DDK*HGSYEDAVHSGALND.-   |
| Phosphoglycerate mutase 1                                    | K.AETAAK*HGEAQVK.I  |
| Nucleophosmin  | K.VEAK*FINYVK.N   |
| 60S ribosomal protein L3                                     | K.FIDTTSK*FGHGR.F   |
| ADP/ATP translocase 3  | K.QIFLGGVDK*HTQFWR.Y  |
| U1 small nuclear ribonucleoprotein 70 kDa                    | R.VNYDITESK*LR.R  |
| ATP5O ATP synthase subunit O, mitochondrial                  | GEVPCTVTSASPLEEATLSELK*TVLK   |
| CREB-binding protein   | K.#KK*NNK*K*TNK*NK*SSISR.A<br>K.SHAHK*MVK*WGLGLDDEGSSQGEPOSK*SPQESR.R<br>R.KKEESTAASETTEGSQDQSK*NAK*K.K |
| HUMAN Histone H2A.Z  | M.AGGK*AGK*DSGK*AK*TK.A   |
| Uncharacterized protein                                      | K.FK*YDDAER.R   |
| Uncharacterized protein                                      | K.SAFLCGVMK*TYR.Q   |
| Heat shock protein beta-1                                    | K.DGVVEITGK*HEER.Q  |
| T-complex protein 1 subunit beta                             | R.EALSSAVDHGSDEVK*FR.Q  |
| Galectin-1   | K.LPDGYEFK*FPNR.L   |
| Histone H2A type 1-H   | R.GK*QGGK*AR.A  |
| Asparagine synthetase  | K.VASVEMVK*YHHC.R   |
| Retinoblastoma binding protein 7                             | K.IECEIK*INHEGEVNR.A  |
| Glucose-6-phosphate isomerase                                | R.SGDWK*GYTGK.T   |
| Uncharacterized protein                                      | R.EQCCYNCCK*PGHLAR.D  |
| Uncharacterized protein                                      | K.IASK*YDHQAEEDLR.N   |
| Actin-related protein 3                                      | K.EFNK*YDIDGSK.W  |
| Histone H2A type 2-B   | R.GK*QGGK*AR.A  |
| Uncharacterized protein                                      | K.ITIMPK*.H   |
| Programmed cell death protein 5                              | K.HGDPGDAAQQAQK*HRE.E   |
| Fumarate hydratase, mitochondrial                            | K.VPNDK*YYGAQTVR.S  |
| Lamin-B receptor   | K.YGVAWEK*YCQR.V  |
| Uncharacterized protein                                      | K.VTGILETK*YK.W   |
| PC4 and SFRS1-interacting protein                            | K.TKDQGK*K*GPNK*K*.L  |
| 26S protease regulatory subunit 10B                          | K.VVSSIVDK*YIGESAR.L  |
| Hematological and neurological expressed 1 protein           | R.RNPPGGK*SSLVLG.-  |
| Uncharacterized protein                                      | R.LNQVIFPVSYNDK*FYK.D   |
| Acyl-CoA-binding protein                                     | K.TK*PSDEEM@LFIYGHYK.Q  |
| Probable global transcription activator SNF2L2               | K.K*GK*GGAK.T   |
| Chromodomain-helicase-DNA-binding protein 4                  | R.NLGK*GK.R   |
| Biorientation of chromosomes in cell division protein 1-like | K.SLLEEK*LVLK*SK*S  |
| Aspartate aminotransferase                                   | R.DVFLPK*PTWGNHTPIFR.D  |
| Histone-lysine N-methyltransferase MLL3                      | K.TLVLSDK*HSPQK*K.S   |
| Bromodomain-containing protein 1                             | R.HPSSPCSVK*HSPTR.E   |
| LINE-1 type transposase domain-containing protein 1          | R.KFQK*LKNKEEVK*.A  |

|  |                               |
|--|-------------------------------|
| B-cell CLL/lymphoma 9-like protein                         | R.GHCPPAPAK*PMHPENK*LTNHGK.T  |
| WD repeat-containing protein 46                            | M.ETAPK*PGK.D                 |
| Metastasis-associated protein MTA2                         | R.VGCK*YQAEIPDR.L             |
| Cyclin-dependent kinase 11B                                | R.SHSAEGGK*HAR.V              |
| Homeobox protein Hox-C8                                    | R.YQTLELEK*.E                 |
| Ubiquitin carboxyl-terminal hydrolase 8                    | R.KEEQFQK*AKK*K.Q             |
| 28S ribosomal protein S9, mitochondrial                    | K.AEAIVYK*HGSGR.I             |
| GDNF family receptor alpha-like                            | K.K*CINKSDNVK*EDK*FK.W        |
| Solute carrier organic anion transporter family member 4C1 | K.FGK*SIK.D                   |
| Ovochymase-1   | R.#GAFGISYIDLK*VLGPK.D        |
| Coiled-coil domain-containing protein 13                   | K.KKIEEDRFAFTGTAGVAGDVVATK*.I |
| Threonine synthase-like 1                                  | K.LSCGEWK*SLVGATYVER.A        |
| Secreted frizzled-related protein 1                        | K.K*K*DLKKLVLYLK*.N           |
| Dynein heavy chain 3, axonemal                             | R.LREAEGKLAQMOK*.L            |
| Histone acetyltransferase KAT6B                            | R.#QSPAKVQSK*NK*YLHSPESR.P!   |
| Polycomb protein SCMH1                                     | R.KPGK*K*R.G                  |
| Zinc finger protein 512B                                   | K.#EK*K*K*NLAGGK*K*.R         |
| Dedicator of cytokinesis protein 7                         | R.AHGELHEQFK*R.K              |
| Remodeling and spacing factor 1                            | R.GK*DISTITGHR.G              |
| HIRA-interacting protein 3                                 | R.#TQLKGGK*R.L                |
| Ubiquitin-fold modifier-conjugating enzyme 1               | K.ICLTDHFK*PLWAR.N            |
| Uncharacterized protein                                    | K.QNKTK*R.Q                   |
| Chromosome 1 open reading frame 149                        | R.YLTNQK*NSNSK*NDR.R          |
| Uncharacterized protein                                    | K.#EK*GDK*K*EGKDVK*.V         |
| Uncharacterized protein                                    | K.NLACEESK*R.K                |
| Ubiquitin carboxyl-terminal hydrolase                      | R.DLLQFFK*PR.Q                |
| Uncharacterized protein                                    | R.YLVASK*.E                   |



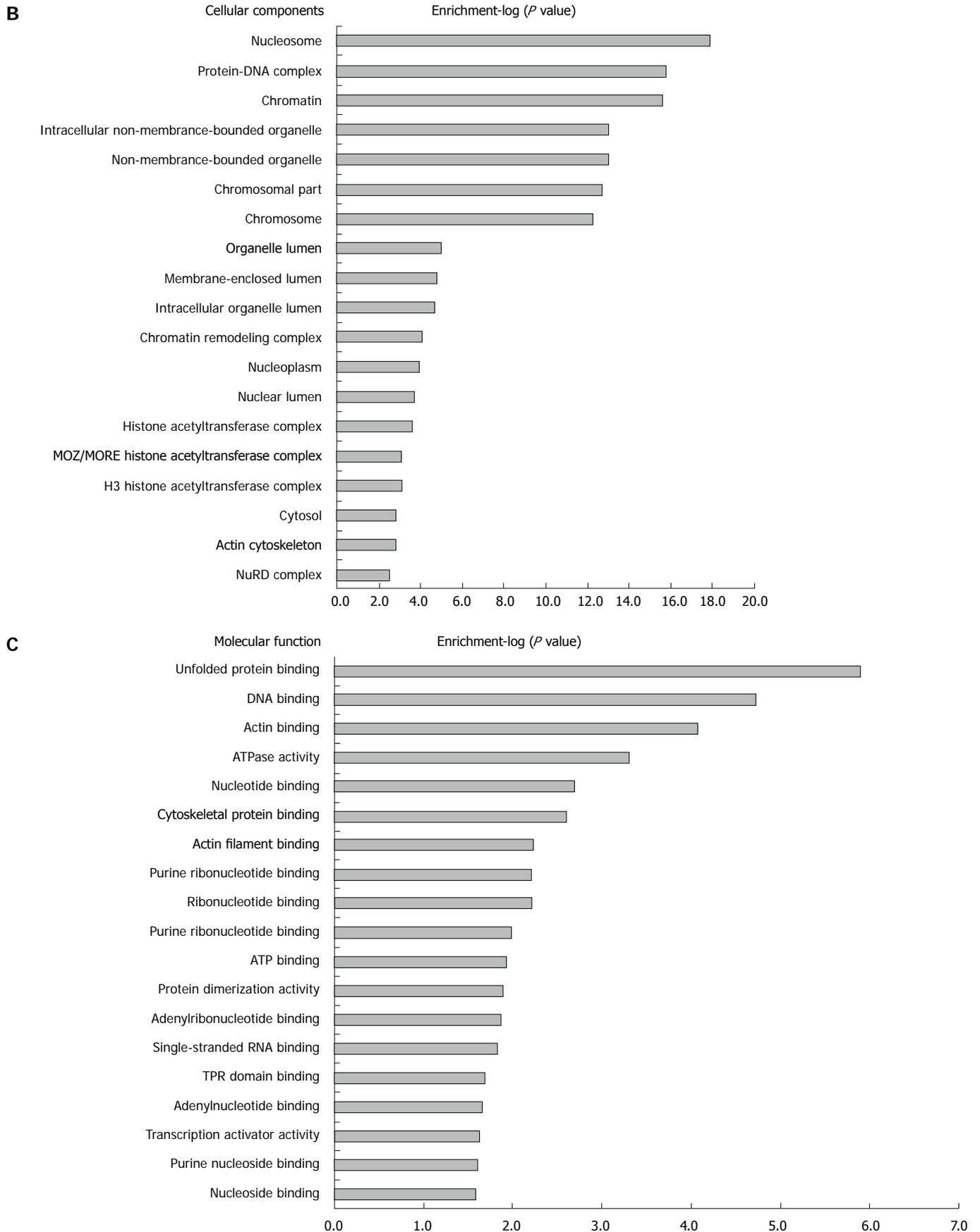


Figure 11 G0 contents based on biological process, cellular components and molecular function in which differently modified proteins were significantly enriched. A: Biological process; B: Cellular components; C: Molecular function.

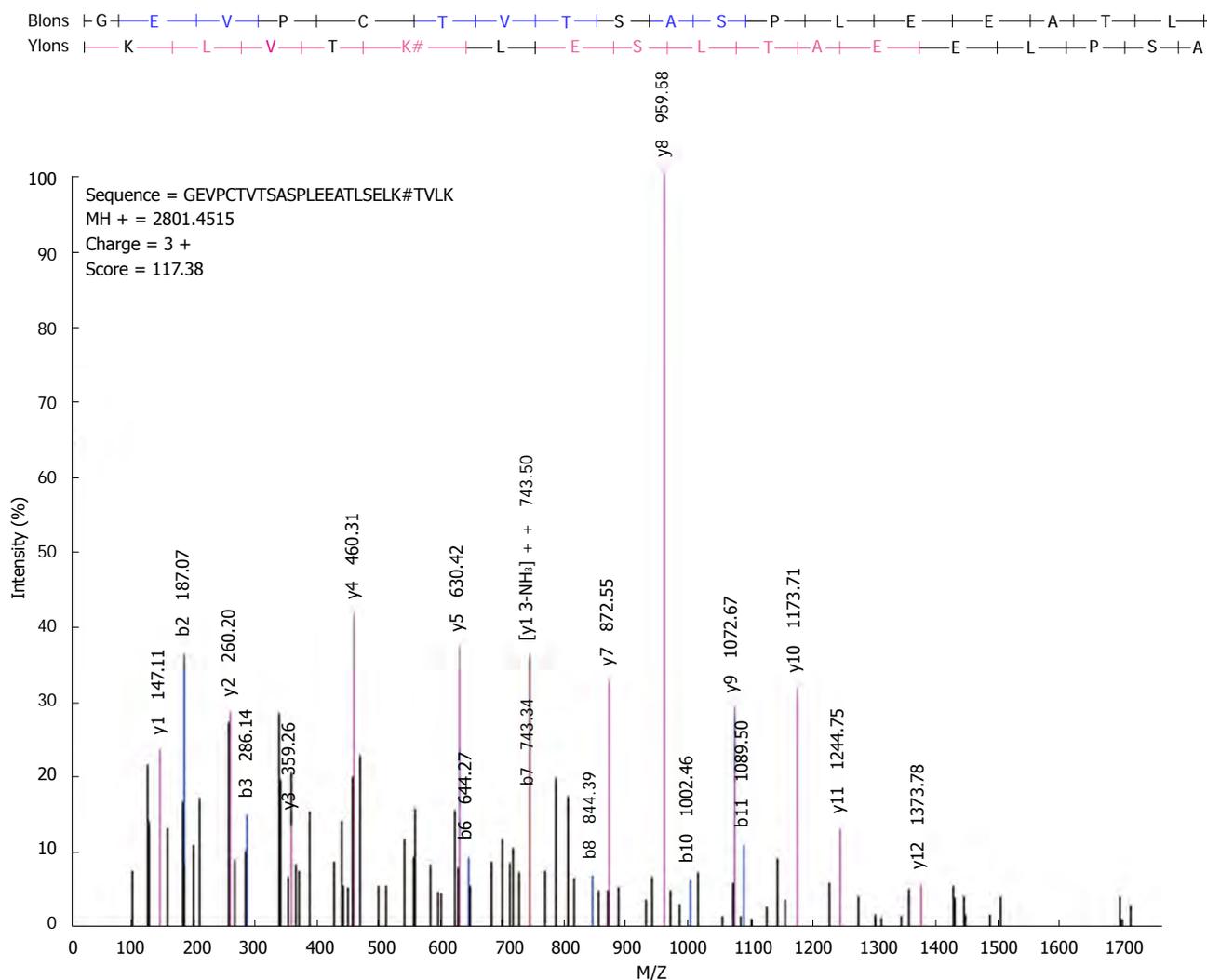


Figure 12 High-resolution MS/MS spectra of acetylated peptide at lysine 158 (GEVPCTVTSASPLEEATLSELK\*TVLK) in ATP5O.

protein band was ATP5O. To further determine whether ATP5O showed lysine acetylation, we performed mass spectrometry on the acetylated sites of normal AGS cells. The results confirmed that ATP5O had acetylated sites. The verification experiment of acetylated protein showed that the degree of acetylation of ATP5O was increased with exposure time, while ATP5O expression level was not changed during the process. ATP5O is the main component of the oligomycin-sensitivity donor protein subunit, ATP synthase, and located in human chromosome 21q22.1-Q22.2<sup>[33]</sup>. It is important for oxidation and phosphorylation. The component is not only associated with oxidative stress due to neurodegeneration, but also with human recombinant superoxide dismutase-1. Proteomics has become a main theme in life science research. Mass spectrometry has high sensitivity, high accuracy, and easy automation. Therefore, mass spectrometry-based identification methods have gradually become a standard for proteomics. We found that ATP5O was significantly acetylated after AGS cells were exposed to the deacetyltransferase inhibitor, TSA, using mass spectrometry technology, which indicated that the acetylation

of ATP5O was dynamically regulated in cells. At present, no ATP5O acetylation mechanisms have been reported in the domestic or international literature. Further studies are needed to determine what role this dynamic regulation plays in tumor cells and through which paths ATP5O affects tumor generation and growth after acetylation. In addition, mass spectrometry showed that a large amount of acetylated PKM2 (isoform of modified pyruvate kinase) existed in differential proteins before AGS cells were exposed to TSA, however, acetylated PKM2 was significantly reduced after exposure to TSA. PKM2 is an isoenzyme of pyruvate kinase, and a specific protein in embryos and differentiated cells<sup>[34]</sup>. Mazurek<sup>[35]</sup> revealed that PKM2 is a crucial factor in tumor metabolism, promoting cell proliferation and leading to tumors. Lv *et al*<sup>[36]</sup> confirmed that PKM2 K305 acetylation decreases PKM2 enzyme activity and promotes its lysosomal-dependent degradation *via* chaperone-mediated autophagy (CMA). Acetylation increases the interaction between PKM2 and HSC70, a chaperone for CMA, and its association with lysosomes. Ectopic expression of an acetylation mimetic K305Q mutant accumulates glycolytic intermediates and

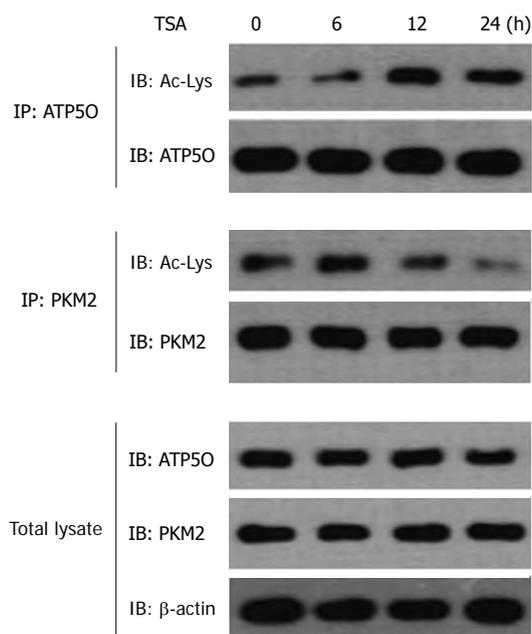


Figure 13 AGS cells were exposed to 0.5  $\mu\text{mol/L}$  trichostatin A for the indicated time periods and then ATP50 and PKM2 protein were immunoprecipitated using an anti-ATP50 antibody and an anti-PKM2 antibody. Total ATP50 and acetylation of ATP50 were detected using an anti-ATP50 antibody and an antibody specific to acetylated lysine, respectively. Total PKM2 and deacetylation of PKM2 were detected using an anti-PKM2 antibody and an antibody specific to deacetylated lysine, respectively. ATP50, PKM2 and  $\beta$ -Actin protein levels in the total lysate are also shown.

promotes cell proliferation and tumor growth. Further research is required on why acetylated PKM2 is reduced after AGS cells are exposed to TSA, what mechanisms affect the process, and whether TSA induces PKM2 deacetylation by activating other signaling pathways.

One field of tumor research is to explore deacetyltransferase inhibitors which have little toxicity and good efficacy, and combine deacetyltransferase inhibitors with clinical cancer treatment, including the combination of deacetyltransferase inhibitors with chemotherapeutics or with gene therapies or other tumor apoptosis or differentiation-inducing agents to determine better individual therapy for all tumors. Our experiments demonstrated that TSA played a role in inhibiting proliferation, promoting apoptosis and affecting the normal cell cycle of AGS cells. Besides activation of a variety of tumor-related signaling pathways and involvement in histone acetylation, TSA may also influence the growth and metabolism of gastric cancer cells by acetylation of non-histone, such as modification of ATP50. Exploring more deacetyltransferase inhibitors and their action sites is favorable in the development of new drugs.

## COMMENTS

### Background

The prevalence and mortality of gastric cancer in East Asia are higher than the world average values. In China, more than 400000 new patients are diagnosed every year. In absence of targets, the traditional chemotherapies have severe side effect. Therefore cancer treatment and research are now focusing on the

molecular targeted therapy due to its high selectivity, good efficacy and little side effects.

### Research frontiers

This study is the first to select acetylated differential proteins before and after gastric cancer cells are exposed to trichostatin A (TSA) to explore the effect of lysine acetylation of related proteins on regulating the proliferation of gastric cancer cells. Moreover, sketch the lysine-acetylated proteins and the modified sites of AGS cells.

### Innovations and breakthroughs

Previous researches are with the acetylation modification of deacetyltransferase inhibitor on histone, but rare studies focus on the acetylation modification of deacetyltransferase on non-histone. The study is on whether the acetylated non-histones is involved in tumor growth and metabolism, a high-resolution mass spectrometer is applied to detect the acetylated proteins and modified sites.

### Applications

The study provides an experimental basis for future studies on exploring more deacetyltransferase inhibitors and action sites thereof is favorable to the development of new drugs for cancer.

### Peer review

The authors explored TSA can inhibit gastric cancer cell proliferation and ATP50 was obviously acetylated after TSA intervenes. Simultaneously, they sketched the acetylated proteins and modified sites in AGS cells. The paper is well presented and the results are interesting.

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**P- Reviewers** Demonacos C, Mimeault M **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Li JY



## Survival after inflammatory bowel disease-associated colorectal cancer in the Colon Cancer Family Registry

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Supported by The American Society of Preventive Oncology/Prevent Cancer Foundation/American Society for Clinical Oncology Cancer Prevention Research Fellowship to SVA; the Australasian Colorectal Cancer Family Registry, No. U01 CA097735; the Familial Colorectal Neoplasia Collaborative Group, No. U01 CA074799; the Mayo Clinic Cooperative Family Registry for Colon Cancer Studies, No. U01 CA074800; the Ontario Registry for Studies of Familial Colorectal Cancer, No. U01 CA074783; the Seattle Colorectal Cancer Family Registry, No. U01 CA074794; the University of Hawaii Colorectal Cancer Family Registry, No. U01 CA074806; and the University of California, Irvine Informatics Center, No. U01 CA078296

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Received: December 13, 2012 Revised: March 12, 2013

Accepted: April 3, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate the survival of individuals with colorectal cancer (CRC) with inflammatory bowel disease (IBD-associated CRC) compared to that of individuals without IBD diagnosed with CRC.

**METHODS:** Epidemiologic, clinical, and follow-up data were obtained from the Colon Cancer Family Registry (Colon CFR). IBD-associated cases were identified from self-report of physician diagnosis. For a subset of participants, medical records were examined to confirm self-report of IBD. Cox proportional hazards regression was applied to estimate adjusted hazard ratios (aHR) and 95%CI of mortality, comparing IBD-associated to non-IBD-associated CRC, adjusted for age at CRC diagnosis, sex, Colon CFR phase, and number of prior endoscopies. Following imputation to complete CRC stage information, adjustment for CRC stage was examined.

**RESULTS:** A total of 7202 CRC cases, including 250 cases of IBD-associated CRC, were analyzed. Over a twelve year follow-up period following CRC diagnosis, 2013 and 74 deaths occurred among non-IBD associated CRC and IBD-associated CRC patients, respectively. The difference in survival between IBD-associated and non-IBD CRC cases was not statistically significant (aHR = 1.08; 95%CI: 0.85-1.36). However, the assumption of proportional hazards necessary for valid inference from Cox regression was not met over the entire follow-up period, and we therefore limited analyses to within five years after CRC diagnosis when the assumption of proportional hazards was met. Over this period, there was evidence of worse prognosis for IBD-associated CRC (aHR = 1.36; 95%CI: 1.05-1.76). Results were similar when adjusted for CRC stage, or restricted to IBD confirmed in medical records.

**CONCLUSION:** These results support the hypothesis

that IBD-associated CRC has a worse prognosis than non-IBD-associated CRC.

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**Key words:** Colorectal cancer; Inflammatory bowel disease; Outcomes research; Cancer survival; Inflammation

**Core tip:** Inflammatory bowel disease (IBD) - and more generally, inflammation - increases risk of colorectal cancer (CRC). Inflammation may also promote cancer progression and metastasis, and therefore, inflammation might be associated with shorter survival with CRC. This study examined whether CRC that occurs in patients with IBD has a worse prognosis than CRC in patients without IBD.

Adams SV, Ahnen DJ, Baron JA, Campbell PT, Gallinger S, Grady WM, LeMarchand L, Lindor NM, Potter JD, Newcomb PA. Survival after inflammatory bowel disease-associated colorectal cancer in the Colon Cancer Family Registry. *World J Gastroenterol* 2013; 19(21): 3241-3248 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3241.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3241>

## INTRODUCTION

Inflammatory bowel disease (IBD), comprising ulcerative colitis (UC) and Crohn's disease (CD), is an inflammatory disease affecting the gastrointestinal tract<sup>[1]</sup>. IBD is associated with a substantial increase in the risk of colorectal cancer (CRC), especially after 8-10 years of active disease<sup>[2-4]</sup>. Implicated mechanisms include persistent activation of cyclooxygenase-2 and nuclear factor kappa-B pathways and increased oxidative stress<sup>[5,6]</sup>, leading IBD to be considered a model for understanding the role of chronic inflammation in CRC etiology<sup>[2,7]</sup>.

IBD-associated CRCs have distinct molecular and clinical features compared to sporadic CRCs<sup>[6,8]</sup>. Specifically, CRC arising in association with IBD has an initiation and progression sequence that differs from the canonical sequence of adenoma-to-cancer characterizing sporadic CRC. Furthermore, IBD-associated CRC typically affects individuals at an earlier age than sporadic CRC, and the distribution of histologic type and site within the colon may differ<sup>[6,8]</sup>. Despite these differences, the results of many studies support the concept that inflammation at levels more subtle than that seen in IBD is associated with CRC risk<sup>[2]</sup>; in this model, inflammation acts primarily as a tumor-promoter following environmental mutagenic insult. Non-IBD-associated CRC tumors show infiltration by inflammatory immune cells and elevated inflammatory cytokines<sup>[2]</sup>. Epidemiologically, circulating C-reactive protein, a marker of inflammation, has been associated with increased risk<sup>[9,10]</sup>, regular use of anti-inflammatory medication such as aspirin reduces risk of CRC<sup>[11-15]</sup>, and genetic variation in inflammatory path-

way genes may also modulate CRC risk<sup>[16,17]</sup>. Inflammation is also hypothesized to enhance metastasis in both IBD-associated and non-IBD-associated CRC<sup>[18]</sup>.

Recent evidence suggests that the use of non-steroidal anti-inflammatory drugs (NSAIDs) improves survival following CRC diagnosis<sup>[19,20]</sup>. From this it may be inferred that inflammation is involved in disease progression. If this is true, survival of individuals with IBD-associated CRCs would be predicted to be worse than for those with sporadic CRCs. However, the evidence that IBD negatively affects the prognosis of CRC comes primarily from small, hospital-based studies<sup>[21-24]</sup> and is inconsistent. Among the largest previously published studies, one observed an approximately 20% higher risk of mortality among UC-associated IBD patients compared to other CRC patients<sup>[25]</sup> and two others found no difference<sup>[26,27]</sup>.

We accessed data from the large, multi-center Colon Cancer Family Registry (Colon CFR)<sup>[28]</sup> to compare the survival of patients with IBD-associated CRC to those without IBD diagnosed with CRC.

## MATERIALS AND METHODS

### Study population: The Colon CFR

This study includes incident invasive CRC cases diagnosed between 1997 and 2009 registered with the Colon CFR, an international resource comprising seven sites that has been previously described in detail<sup>[28]</sup>. Case ascertainment and recruitment methodology differed between Colon CFR centers and between registry phases (1997-2001 and 2002-2009)<sup>[28]</sup>, and included population-based recruitment in addition to targeted recruitment of CRC cases under age 50 or with a family history of CRC. CRC cases associated with familial adenomatous polyposis ( $n = 89$ ) or Lynch syndrome ( $n = 1438$ ) were excluded. Invasive CRC cases were eligible if age, sex, and self-reported IBD information was available; for the primary analysis both population-based cases and cases recruited through clinics associated with Colon CFR centers were included. Of 7297 eligible cases, 7202 records had complete information on analytical covariates other than CRC stage, and were included in multivariate adjusted analyses.

### Inflammatory bowel disease assessment

IBD prior to CRC diagnosis was assessed through self-report during the Colon CFR baseline epidemiologic and medical history interview. Consecutive questions asked whether a participant had ever been told by doctor that they had CD, UC, irritable bowel syndrome, or diverticulitis. The UC question included a statement that UC is not a stomach ulcer. Each affirmative answer was followed with a question ascertaining the participant's age or calendar year in which the diagnosis was reported to the participant. Participants reporting either UC or CD diagnosis were counted as having IBD in the primary analysis. Participants reporting irritable bowel syndrome or diverticulitis were not considered to have IBD and included with all other CRC cases.

Clinical records at two Colon CFR centers, Ontario and Seattle, were reviewed for references to UC or CD. Of 169 self-reported IBD diagnoses from these centers, at least some medical or pathology records were available for 150 patients.

### Covariate assessment

During the study interview, information was collected on family history of CRC, medical history related to digestive disease including endoscopy and hemoccult testing, height and weight, demographics, and lifestyle (*e.g.*, cigarette smoking, physical activity, and alcohol consumption)<sup>[28]</sup>. A reference date of 2 years prior to CRC diagnosis was used to categorize participants' exposures prior to diagnosis, except for endoscopy and hemoccult testing. Hemoccult testing was categorized as never, 1-4, or 5 or more total tests. Similarly the total lifetime number of reported endoscopies (colonoscopies and sigmoidoscopies) was categorized as none or 1, 2, or 3 or more.

### Tumor characteristics

Cancer stage, histologic type (*i.e.*, adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma), and anatomic site of the tumor were obtained from registry or pathology reports<sup>[28]</sup>. For analysis, tumors in the cecum, ascending colon, hepatic flexure, transverse colon, and splenic flexure (ICD-O-3 codes C180, C182-185) were considered proximal; tumors in the descending colon (C186) and sigmoid colon (C187) were grouped as distal colon tumors; and rectosigmoid junction (C199), rectum (C209), and overlapping lesion of the anus and rectum (C218) were considered rectal tumors. Stage information was obtained by Colon CFR centers from the Surveillance Epidemiology and End Results (SEER) registry (Seattle and Hawaii) or medical records and pathology review (all other centers). Records included American Joint Committee on Cancer staging on tumor extent (T-stage), lymph node positivity (N-stage; number of nodes positive for malignant cells), and the presence of distant metastases (M-stage). From these indices, stage was summarized as local, regional, or distant following SEER guidelines. Microsatellite instability (MSI) status was characterized for 4422 cases with available tumor samples in the Colon CFR, combining the results of genetic analysis of a 10-marker panel and immunohistochemical staining of DNA mismatch repair proteins as previously described<sup>[28-33]</sup>.

### Imputation of stage of CRC

Of 7202 cases eligible for this analysis, 4450 (62%) initially could not be categorized in a SEER summary stage. Of these, 4343 could not be classified in part because of missing M-stage information, primarily because ascertaining distant metastases required medical records not accessible at all Colon CFR centers. However, 2859 of these cases without M-stage information included T- or N-stage information. Missing stage information was imputed in two steps. First, cases without M information but with N information indicating that no lymph nodes tested positive

for malignant cells were coded as having no distant metastases. If T-stage information was also available, SEER summary stage was updated to local or regional. In total 1509 of 4450 cases (34%) originally missing SEER summary stage were completed through this direct imputation.

Following direct imputation, multiple imputation with chained equations was used to complete the remaining cases missing stage information<sup>[34]</sup>. T-stage (T1-3, T4) and N-stage (N0, N > 0) were reduced to dichotomous variables. Fifty datasets were generated with T, N and M imputed using chained logistic regression based on all variables considered in the primary survival analysis (see below), and additional potential predictors of stage: time at risk, vital status, delay between CRC diagnosis and Colon CFR enrollment, histologic type, partial colectomy (ever, never, missing), and tumor site (proximal, distal, rectal, overlapping/NOS). Imputation regression excluded cases with incomplete information in one or more predictors ( $n = 889$ ). In total, 6313 cases were included in multiple imputation regression; T, N or M were imputed for 1677, 1697 and 2438 cases, respectively. Following imputation, SEER summary stage was derived from imputed T, N and M following SEER coding<sup>[35,36]</sup>. Together with cases whose records originally included SEER summary stage or were completed with direct imputation, described above, a total of 6809 cases with SEER summary stage were included in analyses involving stage.

### Vital status

Vital status was updated at each Colon CFR center through linkage to population-based tumor registries and death indices, in addition to active follow-up 4-5 years after initial enrollment and routine contact with enrollees and family members<sup>[28]</sup>.

### Ethical considerations

Each Colon CFR center Institutional Review Board/ethics board approved all study protocols and all participants provided informed consent.

### Statistical analysis

Cox proportional hazards regression was used to estimate adjusted hazard ratios (aHRs) of death from any cause with 95% CIs. Time at risk was measured in days from CRC diagnosis, with participants entering observation on the date of Colon CFR baseline interview. Participants alive at the most recent time of confirmed contact were censored at that date. Parallel analyses were completed for the full follow-up time, and with administrative censoring of all participants 5 years after CRC diagnosis. The proportional hazard assumption was examined graphically and by allowing the variable representing IBD status to interact with the logarithm of failure time, and testing the significance of the time-varying factor<sup>[37]</sup>.

To adjust for age at CRC diagnosis, participants were divided into approximate quartiles of age (cut-points at 47, 56 and 65 years) and a piecewise linear model of hazard of death was constructed. Additional *a priori* can-

**Table 1 Participant characteristics of colorectal cancer cases at enrollment in the Colon Cancer Family Registry according to self-reported diagnosis of inflammatory bowel disease *n* (%)**

| Characteristics                              | Inflammatory bowel disease <i>P</i> value |                          |         |
|--|---|--------------------------|---------|
|  | No<br>( <i>n</i> = 6952)                  | Yes<br>( <i>n</i> = 250) |         |
| Age at CRC diagnosis (yr)                    |   |                          | < 0.001 |
| < 47   | 1731 (25)                                 | 98 (39)                  |         |
| 47-55  | 1791 (26)                                 | 68 (27)                  |         |
| 56-65  | 1803 (26)                                 | 42 (17)                  |         |
| > 65   | 1627 (23)                                 | 42 (17)                  |         |
| Female                                       | 3515 (51)                                 | 133 (53)                 | 0.4     |
| Lifetime endoscopies                         |   |                          | < 0.001 |
| None or one                                  | 2697 (39)                                 | 40 (16)                  |         |
| Two  | 2004 (29)                                 | 51 (20)                  |         |
| Three or more                                | 2251 (32)                                 | 159 (64)                 |         |
| Colon CFR recruitment phase                  |   |                          | 0.4     |
| I (1997-2001)                                | 4310 (62)                                 | 161 (64)                 |         |
| II (2002-2009)                               | 2642 (38)                                 | 89 (36)                  |         |
| Colon CFR center                             |   |                          | < 0.01  |
| Ontario, Canada                              | 1775 (26)                                 | 89 (36)                  |         |
| Southern California, United States           | 1551 (22)                                 | 39 (16)                  |         |
| Melbourne, Victoria, Australia               | 718 (10)                                  | 20 (8)                   |         |
| Hawaii, United States                        | 348 (5)                                   | 6 (2)                    |         |
| Mayo Clinic, MI, United States               | 499 (7)                                   | 16 (6)                   |         |
| Seattle, WA, United States                   | 2061 (30)                                 | 80 (32)                  |         |
| Education <sup>1</sup>                       |   |                          | 0.8     |
| Secondary or less                            | 3367 (49)                                 | 123 (50)                 |         |
| More than secondary                          | 3518 (51)                                 | 124 (50)                 |         |
| Cigarette smoking history <sup>1,2</sup>     |   |                          | 0.02    |
| Never  | 3022 (44)                                 | 130 (52)                 |         |
| Current                                      | 1259 (18)                                 | 44 (18)                  |         |
| Former                                       | 2650 (38)                                 | 75 (30)                  |         |
| Race <sup>1</sup>                            |   |                          | < 0.01  |
| White  | 5033 (74)                                 | 200 (83)                 |         |
| Other  | 1769 (26)                                 | 42 (17)                  |         |
| BMI (kg/m <sup>2</sup> ) <sup>1,2</sup>      |   |                          | < 0.01  |
| ≤ 23.6                                       | 1685 (25)                                 | 85 (34)                  |         |
| 23.7-26.5                                    | 1710 (25)                                 | 59 (24)                  |         |
| 26.6-30.3                                    | 1726 (25)                                 | 51 (21)                  |         |
| ≥ 30.4                                       | 1739 (25)                                 | 53 (21)                  |         |
| Regular NSAID user <sup>1,2</sup>            |   |                          | 0.7     |
| Yes  | 2719 (40)                                 | 95 (39)                  |         |
| No   | 4159 (60)                                 | 151 (61)                 |         |
| 1 <sup>st</sup> degree family history of CRC | 1495 (22)                                 | 35 (14)                  | < 0.01  |
| Tumor site                                   |   |                          | < 0.01  |
| Proximal colon                               | 2346 (34)                                 | 109 (44)                 |         |
| Distal colon or rectum                       | 4276 (62)                                 | 136 (54)                 |         |
| Overlapping/not specified                    | 328 (5)                                   | 5 (2)                    |         |
| Microsatellite instability <sup>1</sup>      |   |                          | 0.1     |
| None or low                                  | 3743 (88)                                 | 150 (87)                 |         |
| High   | 507 (12)                                  | 22 (12)                  |         |
| CRC stage at diagnosis <sup>1,3</sup>        |   |                          | 0.4     |
| Local  | 3088 (47)                                 | 110 (46)                 |         |
| Regional                                     | 2441 (37)                                 | 98 (40)                  |         |
| Distant                                      | 1080 (16)                                 | 34 (14)                  |         |

<sup>1</sup>Numbers do not sum to total due to missing values; <sup>2</sup>2 years prior to colorectal cancer (CRC) diagnosis; <sup>3</sup>Mean of 50 imputed datasets. Colon CFR: Colon Cancer Family Registry; NSAID: Non-steroidal anti-inflammatory drugs; BMI: Body mass index.

didate adjustment variables were Colon CFR phase of enrollment (1997-2001 or 2002-2009), Colon CFR center, sex, cigarette smoking history, use of NSAIDs, education, race, body mass index (BMI), first degree family history of CRC, and utilization of hemoccult tests or endos-

copy. Colon CFR center, NSAID use, BMI, education, race, smoking, hemoccult tests, and family history were omitted from final statistical models because inclusion did not appreciably change aHRs associated with IBD. CRC stage, when included, was parameterized as SEER summary stage (localized, regional, or distant disease).

## RESULTS

Of 7202 eligible CRC patients, 250 (3.5%) reported a physician's diagnosis of IBD (Table 1). IBD-associated CRC patients were younger at CRC diagnosis than non-IBD cases. In addition, IBD-associated CRC patients were less likely to have ever regularly smoked cigarettes, and were leaner on average than other patients with CRC. IBD-associated CRC patients were much more likely than sporadic CRC patients to be white, report a family history of CRC, or have had multiple endoscopies.

Compared to non-IBD cases, a larger proportion of IBD-associated CRC patients were diagnosed with tumors in the proximal colon (Table 1). Among tumors assayed for MSI, no difference in the distribution of MSI between IBD-associated and other CRC was observed. The distribution of SEER summary stage differed only slightly between IBD-associated CRC and other CRC following completion of missing stage information with multiple imputation. However, a more pronounced, but not statistically significant, difference in stage distribution between IBD-associated and sporadic CRC was noted among patients diagnosed before age 50 year; in this age group, 24% of non-IBD associated CRC cases were diagnosed after distant metastases has occurred, compared to 18% of IBD-associated CRC cases (not shown;  $\chi^2$  test  $P > 0.15$ ).

Over a total follow-up of 12.2 years (median of 4.6 years), 2013 and 74 deaths were reported among non-IBD-associated and IBD-associated CRC cases, respectively. Results of Cox proportional hazards regression using the full follow-up period indicated no association between IBD and risk of mortality from any cause (Figure 1 and Table 2).

Graphical inspection of the Kaplan-Meier survival curve (Figure 1) and formal testing of time-varying coefficients (not shown), indicated that the proportional hazards assumption was violated by the variable representing IBD status. Ending follow-up at no later than 5 years following diagnosis for all participants resolved the proportional hazards violation based on time-varying models (not shown).

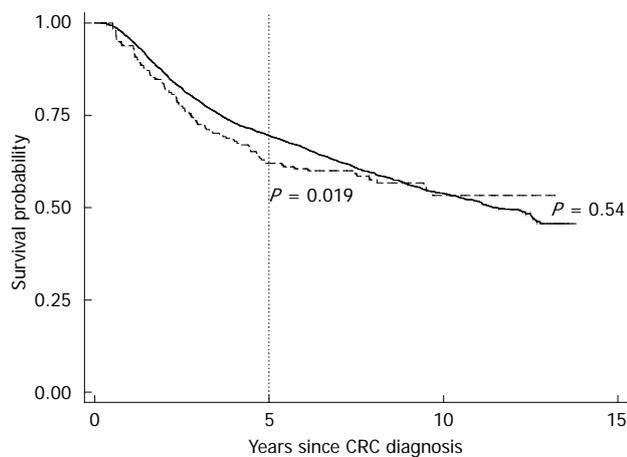
Results from analyses including only the 5 years following diagnosis, but otherwise identical to analysis across the entire follow-up time, showed that IBD-associated CRC patients were at 36% (95%CI: 5%-76%) higher risk of death (Figure 1 and Table 2). Following imputation of CRC stage, additional adjustment for stage left resulting aHR estimates largely unchanged.

Medical and pathology records of 169 eligible cases enrolled at the Seattle and Ontario CFR centers were reviewed; for 14 IBD-associated CRC cases no records

**Table 2** Adjusted hazard ratios and 95%CI for death, either with complete available time-at-risk or with follow-up ended 5 years after colorectal cancer diagnosis

|                    | <i>n</i> | Deaths | aHR <sup>1</sup> (95%CI) | <i>n</i> | Deaths | aHR <sup>2</sup> (95%CI) | <i>n</i> | Deaths | aHR <sup>1,3</sup> (95%CI) |
|--------------------|----------|--------|--------------------------|----------|--------|--------------------------|----------|--------|----------------------------|
| Complete follow-up |          |        |                          |          |        |                          |          |        |                            |
| No IBD             | 6952     | 2013   | Ref.                     | 6567     | 1926   | Ref.                     | 3836     | 1322   | Ref.                       |
| IBD                | 250      | 74     | 1.08 (0.85-1.36)         | 242      | 71     | 1.09 (0.85-1.40)         | 81       | 26     | 1.20 (0.81-1.79)           |
| <i>P</i> value     |          |        | 0.54                     |          |        | 0.48                     |          |        | 0.36                       |
| 5-yr follow-up     |          |        |                          |          |        |                          |          |        |                            |
| No IBD             | 6903     | 1372   | Ref.                     | 6526     | 1320   | Ref.                     | 3801     | 871    | Ref.                       |
| IBD                | 249      | 63     | 1.36 (1.05-1.76)         | 241      | 60     | 1.34 (1.02-1.77)         | 81       | 24     | 1.52 (1.00-2.30)           |
| <i>P</i> value     |          |        | 0.018                    |          |        | 0.037                    |          |        | 0.048                      |

<sup>1</sup>Adjusted for age, recruitment phase, sex, and endoscopy; <sup>2</sup>Additional adjustment for surveillance epidemiology and end results summary stage (local, regional, distant); <sup>3</sup>Inflammatory bowel disease (IBD) confirmed from medical and pathology records at Seattle and Ontario centers only, non-IBD associated cases restricted to these centers. aHR: Adjusted hazard ratio.



**Figure 1** Adjusted survival probability for non-inflammatory bowel disease-associated (solid line) and inflammatory bowel disease-associated colorectal cancer (dashed line). *P* values are from Wald test of coefficient of inflammatory bowel disease from Cox regression. CRC: Colorectal cancer.

were available for review, for 43 only pathology reports or death certificates were available, and for 107 pathology and at least partial medical records were available. During review, 81 of these 169 self-reported IBD diagnoses were confirmed. When proportional hazards regression analysis was repeated including confirmed IBD diagnoses and non-IBD-associated CRC only from the Seattle and Ontario Colon CFR centers, and thus excluding unconfirmed self-reported IBD from analyses, results suggested that IBD was associated with a 52% (95%CI: 0%-130%) higher risk of mortality following CRC diagnosis (Table 2).

In exploratory sub-group analyses of survival through 5 years post-CRC diagnosis, the association between IBD and risk of death did not vary by MSI status of the tumor (not shown). However, IBD was more strongly associated with risk of death among patients with distal or rectal CRC tumors (aHR = 1.62, 95%CI: 1.17-2.26) than among patients with tumors in the proximal colon (aHR = 0.98; 95%CI: 0.64-1.50), although the interaction between IBD and tumor site was not statistically significant (*P* = 0.06). Survival also did not differ by duration of IBD prior to CRC diagnosis (< 8 years IBD, aHR = 1.38, 95%CI: 0.96-1.99; ≥ 8 years IBD, aHR = 1.38, 95%CI:

0.96-2.00). Patients reporting only CD were apparently at higher risk of mortality than those with UC only, although the difference was not statistically significant (CD, aHR = 1.74, 95%CI: 1.09-2.79; UC, aHR = 1.18, 95%CI: 0.85-1.63, *P* = 0.16).

Restriction of analysis to 4144 population-based cases enrolled during phase I did not appreciably alter the association between IBD and hazard of mortality within 5 years of CRC diagnosis (aHR = 1.47, 95%CI: 1.08-2.01). Incorporation of probability weights based on the sampling scheme of the Colon CFR<sup>[28]</sup> also did not change results of death, IBD compared to non-IBD-associated CRC (aHR = 1.62, 95%CI: 1.04-2.54).

## DISCUSSION

We observed a higher risk of death from any cause among patients with IBD-associated CRC compared to those with non-IBD-associated CRC when attention was restricted to the first five years following CRC diagnosis. The increase in risk was of borderline statistical significance. However, no increase in risk of death was seen when all available follow-up data were analyzed. The violation of the proportional hazard assumption made the analysis using all available follow-up time difficult to interpret; in contrast, the period within 5 years following CRC diagnosis showed no violations of the proportional hazards assumption.

CRC stage at diagnosis is a strong predictor of survival, and IBD patients may undergo surveillance designed to detect CRC at early stages. Therefore, we compared CRC stage distribution between cases with and without IBD, and conducted stage-adjusted survival analyses. Our results suggest that IBD-associated CRC is only slightly more frequently diagnosed at local or regional stage than non-IBD-associated CRC. This may be due to active endoscopic surveillance for CRC among IBD patients, consistent with earlier reports<sup>[38]</sup>. We observed that the shift in stage distribution was larger, but not statistically significant, among CRC patients diagnosed prior to age 50 years; this group would generally not receive screening endoscopy in the absence of gastrointestinal symptoms or a strong family history of CRC.

When we repeated survival analysis adjusted for stage, our results still suggested increased risk of mortality among IBD-associated CRC patients. Further exploration revealed that after adjustment for the lifetime number of endoscopic procedures, adjustment for SEER summary stage did not substantially alter the aHR associated with IBD, as would be expected if endoscopy utilization was an important determinant of CRC stage at diagnosis. Examination of our multiple imputation model for CRC stage confirmed that endoscopy was among the strongest predictors of CRC stage. Thus, our results indicate that survival was poorer for IBD-associated CRC patients than for other CRC patients diagnosed at the same SEER stage.

Our results are consistent with reports from several previous studies<sup>[24,39]</sup> including the largest previous population-based studies of this topic<sup>[25,40]</sup>, which included a similar number of UC- and CD-associated CRC cases. These studies reported about 20% or 50% higher mortality among UC-associated CRC patients<sup>[25]</sup> and CD-associated CRC patients<sup>[40]</sup>, respectively, compared to other CRC patients, adjusted for CRC stage. A large Japanese study observed worse prognosis for UC-associated CRC but the relationship was restricted to stage III CRC<sup>[27]</sup>. In contrast with our results, a relatively large ( $n = 290$  IBD-associated CRC cases) clinic-based study did not observe differential risk of death between IBD-associated and other CRC cases, possibly because of the clinical basis of the study<sup>[26]</sup>. Thus, our overall results, based on a set of relatively unselected cases and confirmed in the population-based portion of the Colon CFR, agree with the population-based results<sup>[25,40]</sup>, and strengthen the evidence that the prognosis for IBD-associated CRC is worse than for non-IBD-associated CRC.

Our study has additional important methodological strengths. Approximately 57% of participants in this study were drawn from Colon CFR centers that utilize population-based case ascertainment strategies. Therefore, our results are likely to provide more general insight into the survival of IBD-associated CRC than clinic-based case series. Although our sample size was limited by the rarity of both IBD and CRC, the large, multi-site Colon CFR included a broad spectrum of CRC. Furthermore, because of the size of the Colon CFR, the cases in this study were recruited over 12 years, in comparison to previous reports that required 20 or more years to recruit a comparable number<sup>[25,27]</sup>.

An important limitation of this study was our reliance on participant report of either UC or CD. We believe that our inability to confirm about 50% of self-reported UC or CD in patients from Ontario and Seattle resulted mainly from lack of access to complete medical records prior to CRC diagnosis. Although we expected IBD to be noted on pathology or medical records related to CRC diagnosis, we cannot be sure why information might be missing; the self-report of UC or CD might be incorrect, or the limited records available for review may be incomplete. Nonetheless, when we restricted analysis to confirmed IBD cases, removing unconfirmed IBD from analysis, the association between IBD-associated CRC and

risk of mortality was strengthened. This could be because we successfully reduced misclassification of IBD. Alternatively, IBD confirmed in medical records may have been, on average, more severe than unconfirmed IBD, perhaps because available medical records were those related to CRC diagnosis.

Because we lacked detailed IBD-specific medical records, we were also unable to determine how medications such as 5-aminosalicylic acid (5-ASA) used to treat IBD may have affected our results. In some studies, 5-ASA use has been associated with a reduced risk of CRC in IBD patients<sup>[41,42]</sup>, and IBD patients who are never diagnosed with CRC are not included in our analysis. Like other anti-inflammatory drugs<sup>[19,20]</sup>, 5-ASA treatment prior to CRC may reduce the risk of CRC mortality, and this may account in part for the relatively small difference in survival we observed between IBD-associated and sporadic CRC.

We also lacked specific cause of death information for deaths recorded outside of the Seattle, Ontario, and Mayo Clinic Colon CFR centers. However, at these centers, CRC was the underlying cause of death for 24 of 31 (77%) IBD-associated CRC patients and 499 of 651 (77%) of non-IBD CRC patients who died within 5 years of CRC diagnosis. Therefore, it is likely that most of the deaths included in the 5-year analysis across all Colon CFR centers were due to CRC, and differences in cause of death between IBD-associated and other CRC is unlikely to have influenced our results.

Finally, our sample size was limited to fully evaluate this association. IBD is associated with a strong increase in CRC risk<sup>[2,3,43]</sup>, and we noted a higher prevalence of IBD, approximately 3.5%, among CRC cases than would be expected in the underlying population in which these CRC cases arose. For example, the prevalence of IBD in the United States is less than 1%<sup>[44,45]</sup>. However, the number of IBD-associated CRC cases in our study is approximately equal to the largest previous study comparing survival with non-IBD-associated CRC<sup>[25]</sup>. Nonetheless, sparse numbers certainly hindered our analysis and suggest that cautious interpretation is warranted. Future progress may perhaps be achieved by combining multiple populations through pooled or meta-analyses.

Our observation that IBD-associated CRC appears to have a worse prognosis than non-IBD-associated CRC, even when stage at diagnosis is taken into account, is consistent with evidence that inflammation negatively impacts CRC survival<sup>[19,20]</sup>. The mechanisms through which this may occur remain to be determined.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) - more generally, inflammation - increases risk of colorectal cancer (CRC). Inflammation may also be associated with a poorer prognosis in patients with CRC, perhaps because inflammation may promote tumor progression (growth) and metastasis.

### Research frontiers

The prevalence of IBD is growing worldwide. It remains unclear whether individuals diagnosed with CRC in the context of IBD are at higher risk of death from CRC, compared to CRC patients without IBD.

### Innovations and breakthroughs

The rarity of both IBD and CRC make studying IBD-associated CRC difficult. This study includes a relatively large number of IBD-associated CRC cases, recruited from multiple populations rather than a single clinic case-series. The results suggest that survival with IBD-associated CRC is shorter than survival with CRC arising outside of IBD. This is observed even when comparing CRC cases diagnosed at the same stage, suggesting that IBD-associated CRC progresses more rapidly. However, it may also be due to other factors such as differences in CRC treatment, which could not be measured well in this study.

### Applications

This study suggests that more effort is needed to understand why patients with IBD who are later diagnosed with CRC do worse than patients with CRC.

### Peer review

The study lacked information on how IBD patients were treated for IBD prior to CRC, and whether they received surveillance for CRC incidence (as is commonly prescribed for this group under certain conditions). Surveillance is intended to catch CRC at early stage in IBD patients and therefore could improve survival with CRC in this group. This is a weakness of the paper. However, the study attempted to account for this by adjusting for stage of CRC at diagnosis (comparing cases diagnosed at the same stage) and noted that there was very little overall stage difference between IBD-associated and other CRC. Also, the difference in survival was noted only for 5 years following CRC diagnosis, but not over a long time (12 years), although most deaths occurred within the first 5 years. The study is interesting and may support the hypothesis that inflammation is worse for CRC survival.

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**P- Reviewers** Barreto S, M'Koma A **S- Editor** Zhai HH  
**L- Editor** A **E- Editor** Li JY



## Early dynamic transcriptomic changes during preoperative radiotherapy in patients with rectal cancer: A feasibility study

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Supported by Ligue Contre le Cancer, Programme Hospitalier de Recherche Clinique (20-R6)

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Received: December 20, 2012 Revised: February 22, 2013

Accepted: March 21, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To develop novel biomarkers of rectal radiotherapy, we measured gene expression profiles on biopsies taken before and during preoperative radiotherapy.

**METHODS:** Six patients presenting with a locally advanced rectal cancer (T>T2, N0/Nx, M0) eligible for preoperative radiotherapy (45 Gy in 25 fractions) were selected in a pilot study. Six tumor and 3 normal tissues biopsies were taken before and during radiotherapy,

after a dose of 7.2 Gy at a median time of 1 h following irradiation (0:27-2:12). Tumor or normal tissue purity was assessed by a pathologist prior to RNA extraction. Mean RNA content was 23 µg/biopsy (14-37) before radiotherapy and 22.7 µg/biopsy (12-35) during radiotherapy. After RNA amplification, biopsies were analysed with 54K HG-U133A Plus 2.0 Affymetrix expression micro-arrays. Data were normalized according to MAS5 algorithm. A gene expression ratio was calculated as: (gene expression during radiotherapy - gene expression before radiotherapy)/gene expression before radiotherapy. Were selected genes that showed a ratio higher than  $\pm 0.5$  in all 6 patients.

**RESULTS:** Microarray analysis showed that preoperative radiotherapy significantly up-regulated 31 genes and down-regulated 6 genes. According to the Gene Ontology project classification, these genes are involved in protein metabolism (*ADAMDEC1; AKAP7; CAPN5; CLIC5; CPE; CREB3L1; NEDD4L; RAB27A*), ion transport (*AKAP7; ATP2A3; CCL28; CLIC5; F2RL2; NEDD4L; SLC6A8*), transcription (*AKAP7; CREB3L1; ISX; PABPC1L; TXNIP*), signal transduction (*CAPN5; F2RL2; RAB27A; TNFRSF11A*), cell adhesion (*ADAMDEC1; PXDN; SPON1; S100A2*), immune response (*CCL28; PXDN; TNFRSF11A*) and apoptosis (*ITM2C; PDCD4; PVT1*). Up-regulation of 3 genes (*CCL28; CLIC5; PDCD4*) was detected by 2 different probes and up-regulation of 2 genes (*RAB27A; TXNIP*) by 3 probes.

**CONCLUSION:** Micro-arrays can efficiently assess early transcriptomic changes during preoperative radiotherapy for rectal cancer, and may help better understand tumor radioresistance.

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**Key words:** *CCL28; CLIC5; PDCD4; RAB27A; TXNIP*; Protein metabolism; Cell adhesion; Cell migration; *SPON1*; Carboxypeptidase E

**Core tip:** To develop novel biomarkers of radiotherapy for rectal cancer, we measured gene expression profiles on biopsies taken before and during preoperative radiotherapy in a pilot study. Microarray analysis showed that preoperative radiotherapy significantly up-regulated 31 genes and down-regulated 6 genes, involved in protein metabolism, ion transport, transcription, signal transduction, cell adhesion, immune response and apoptosis. Micro-arrays could efficiently assess early transcriptomic changes during preoperative radiotherapy for rectal cancer. This may help better understand tumor radioresistance.

Supiot S, Gouraud W, Campion L, Jezéquel P, Buecher B, Charrier J, Heymann MF, Mahé MA, Rio E, Chérel M. Early dynamic transcriptomic changes during preoperative radiotherapy in patients with rectal cancer: A feasibility study. *World J Gastroenterol* 2013; 19(21): 3249-3254 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3249.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3249>

## INTRODUCTION

In patients with rectal adenocarcinoma, preoperative radiotherapy (RT), either alone or combined with chemotherapy, reduces the 5-year rate of local recurrence by 5% to 10%<sup>[1]</sup>. However, owing to high inter-individual variation, 6-18 patients have to be treated in order to avoid one recurrence. Identifying patients likely to benefit is thus essential.

End outcomes after preoperative RT can hardly be predicted by analyzing the expression of known proteins on pre-treatment biopsies<sup>[2]</sup> or clinical parameters<sup>[3]</sup>. However, micro-array gene expression profiling can help define diagnostic, prognostic and predictive factors for response to RT<sup>[4]</sup>. Transcriptomic profiles obtained on pre-RT biopsies can be used to identify patients with rectal adenocarcinoma who are likely to relapse despite appropriate treatment<sup>[5]</sup>. Validation studies on larger cohorts are ongoing.

Sequential biopsies have highlighted changes in tumor dynamics (proliferation, cell cycle, apoptosis) during pelvic RT and identified treatment targets that might modify RT outcomes in patients with cervical cancer, where tumor access is easier than for the rectum<sup>[6,7]</sup>. Although sequential biopsies have also revealed histological changes in rectal mucosa due to radiation toxicity, data are few<sup>[8-11]</sup>. Very recently, biopsy specimens could be obtained 7 d after starting chemoradiotherapy and provided interesting biomarkers of response to treatment in rectal cancer patients<sup>[12]</sup>.

A study of radiation-induced cellular and biochemical changes in rectal tumors might help better understand radiation-induced cell death and identify new targets for enhancing RT efficacy. We postulated that sequential biopsies could be used to detect transcriptional changes

during preoperative RT of rectal cancer and help detect new predictors for response to radiation. A large-scale prospective study is needed to test this hypothesis. To eliminate the risk of increasing toxicity from repeated biopsy, we first conducted a pilot study to assess the safety of pre- and post-RT rectal tumor biopsies, and also the feasibility of detecting gene expression changes on biopsies from irradiated tumors.

## MATERIALS AND METHODS

### Patients

Patients presenting with locally advanced rectal cancer (T>T2, N0/Nx, M0) and eligible for preoperative RT were enrolled into the study. Exclusion criteria were: anti-coagulant therapy, cardiac valvular disease, and pelvic pain from prior biopsies. All patients gave their informed written consent to the study, which was approved by the University of Nantes Institutional Review Board for human studies.

### Biopsies

Patients were delivered 45 Gy (1.8 Gy/fraction, over 5 wk, 5 d per week). Six tumor and 3 normal tissue biopsies were taken from each patient before RT and one hour after a dose of 7.2 Gy (4<sup>th</sup> fraction) during RT. Patients were assessed for biopsy toxicity (infection or bleeding) during RT. Tumor purity was measured on tumor cell smears.

### RNA isolation and microarray procedures

Tissues were frozen immediately in liquid nitrogen and disrupted using a mortar and pestle. Samples were homogenized in lysis buffer using a syringe and needle. Total RNA was prepared using the RNeasy<sup>®</sup> Mini kit (Qiagen, Valencia, CA, United States). The integrity of the RNA was assessed for each sample using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, United States). Double-stranded complementary DNA (cDNA) and labeled complementary RNA were synthesized from the total RNA and hybridized to the Affymetrix Human U133 plus 2 gene chips (Affymetrix, Santa Clara, CA, United States). The chips were further processed and scanned according to the manufacturer's protocol. The arrays were scanned with a laser scanner and the data was visualized and normalized using the MAS 5.0 Affymetrix software (Affymetrix, Santa Clara, CA, United States). Over- and under-expressed genes were classified by Gene Ontology category<sup>[13]</sup>.

### Statistical analysis

Data were normalized according to the MAS5 algorithm. The ratio "gene expression during RT/gene expression before RT" was calculated. Genes with a ratio > 2.5 or < 0.4 in all patients and a false discovery rate (FDR) of < 11%, as estimated by significance analysis of microarrays, were selected.

**Table 1 Patient characteristics**

| Patient | Age (yr) | Disease stage | > G1 radiotherapy toxicity | Surgical toxicity | Surgical stage | Outcome at 2 yr of follow-up | Tumor purity in biopsies | Mean RNA content (µg) |                   |
|---------|----------|---------------|----------------------------|-------------------|----------------|------------------------------|--------------------------|-----------------------|-------------------|
|         |          |               |                            |                   |                |                              |                          | Pre-RT biopsies       | Early-RT biopsies |
| 1       | 66       | uT2Nx         | No                         | No                | ypT2N0         | NED                          | 100%                     | 18.0                  | 19.7              |
| 2       | 55       | uT3N1         | No                         | No                | ypT4N1         | NED                          | 100%                     | 27.0                  | 12.6              |
| 3       | 84       | uT3N1         | No                         | No                | ypT3N1         | M1                           | 100%                     | 21.0                  | 35.8              |
| 4       | 78       | uT3N1         | No                         | No                | ypT2N0         | NED                          | 100%                     | 37.1                  | 28.7              |
| 5       | 60       | uT3N0         | No                         | No                | ypT3N1         | NED                          | 100%                     | 20.0                  | 19.4              |
| 6       | 67       | uT3N0         | No                         | No                | ypT2N1         | NED                          | 100%                     | 14.2                  | 19.9              |

NED: Non evolutive disease, M1: Metastatic disease; RT: Radiotherapy.

**Table 2 Up- or down-regulated genes in rectal cancer biopsies**

| Fold                 | False discovery rate | ID        | Gene title   | Symbol    |
|----------------------|----------------------|-----------|--|-----------|
| Up-regulated genes   |                      |           |  |           |
| 2.649                | 0                    | AI827789  | Anterior gradient homolog 3 (Xenopus)                                  | AGR3      |
| 3.427                | 0                    | AA743462  | -  | -         |
| 2.524                | 0                    | NM_006472 | Thioredoxin interacting protein  | TXNIP     |
| 5.514                | 0                    | AB018305  | Spondin 1, extracellular matrix protein                                | SPON1     |
| 2.95                 | 7.551                | NM_152315 | Family with sequence similarity 55, A                                  | FAM55A    |
| 3.289                | 7.551                | AL536553  | Neural precursor cell expressed, developmentally down-regulated 4-like | NEDD4L    |
| 2.684                | 7.551                | AF055009  | cAMP responsive element binding protein 3-like 1                       | CREB3L1   |
| 3.111                | 7.551                | AL137063  | A kinase (PRKA) anchor protein 7                                       | AKAP7     |
| 2.717                | 7.551                | AF266504  | Chemokine (C-C motif) ligand 28  | CCL28     |
| 2.546                | 8.39                 | BF589413  | FERM domain containing 3   | FRMD3     |
| 2.637                | 8.39                 | AA554045  | Polypeptide N-acetylgalactosaminyltransferase 12                       | GALNT12   |
| 3.047                | 8.39                 | NM_030926 | Integral membrane protein 2C   | ITM2C     |
| 2.775                | 8.39                 | AW026379  | Tumor necrosis factor receptor superfamily, member 11a                 | TNFRSF11A |
| 2.609                | 8.39                 | U38654    | RAB27A, member RAS family  | RAB27A    |
| 2.629                | 8.39                 | NM_003570 | Cytidine monophosphate-N-acetylneuraminic acid hydroxylase pseudogene  | CMAH      |
| 2.521                | 8.58                 | AV728268  | Chromosome 11 open reading frame 32                                    | C11orf32  |
| 2.579                | 8.58                 | NM_016929 | Chloride intracellular channel 5                                       | CLIC5     |
| 2.541                | 10.487               | NM_016548 | Golgi membrane protein 1   | GOLM1     |
| 2.728                | 10.487               | AW162846  | Calcium/calmodulin-dependent protein kinase II inhibitor 1             | CAMK2N1   |
| 2.806                | 10.487               | AI659927  | prostate androgen-regulated mucin-like protein 1                       | PARM1     |
| 2.896                | 10.487               | NM_024709 | Chromosome 1 open reading frame 115                                    | C1orf115  |
| 3.333                | 10.487               | NM_005629 | Solute carrier family 6 member 8                                       | SLC6A8    |
| 2.869                | 10.487               | NM_019062 | Ring finger protein 186  | RNF186    |
| 3.174                | 10.487               | AK025181  | Intestine-specific homeobox  | ISX       |
| 3.968                | 10.487               | AB007899  | Neural precursor cell expressed, developmentally down-regulated 4-like | NEDD4L    |
| 8.2                  | 10.487               | NM_001873 | Carboxypeptidase E   | CPE       |
| 4.845                | 10.487               | NM_014479 | ADAM-like, decysin 1   | ADAMDEC1  |
| 2.56                 | 10.487               | BF195709  | Calpain 5  | CAPN5     |
| 2.601                | 10.487               | NM_014456 | Programmed cell death 4  | PDCD4     |
| 2.931                | 10.787               | AW971415  | cDNA clone IMAGE:5745639   | -         |
| 2.787                | 10.787               | NM_005173 | ATPase, Ca++ transporting, ubiquitous                                  | ATP2A3    |
| Down-regulated genes |                      |           |  |           |
| 0.265                | 8.58                 | AI378647  | Coagulation factor II (thrombin) receptor-like 2                       | F2RL2     |
| 0.356                | 8.58                 | BG200951  | Pvt1 oncogene homolog, MYC activator                                   | PVT1      |
| 0.322                | 10.787               | AL109839  | Poly(A) binding protein, cytoplasmic 1-like                            | PABPC1L   |
| 0.356                | 10.787               | NM_005978 | S100 calcium binding protein A2  | S100A2    |
| 0.373                | 10.787               | AL041761  | Transcribed locus  | -         |
| 0.38                 | 10.787               | D86983    | Peroxidasin homolog (Drosophila)                                       | PXDN      |

**RESULTS**

Seven patients with rectal cancer (median age: 66 years, range 55-84 years) were included in the study but one later refused to participate (Table 1). Six patients underwent biopsy before and during RT. The median time of the biopsy was 1 h after the 4<sup>th</sup> RT session (0:27-2:12). No grade 2 biopsy-induced toxicity was reported. Surgery was performed at a median time of 6 wk after RT. No

grade 2 intra-operative toxicity was reported.

Mean RNA content was 23 µg/biopsy (range 14-37 µg/biopsy) before RT and 22.7 µg/biopsy (range 12-35 µg/biopsy) during RT (Table 1). Microarray analysis showed that preoperative RT significantly up-regulated 31 genes and down-regulated 6 genes (the full names of the genes are given in Table 2). According to the Gene Ontology project classification, these genes are involved in protein metabolism (*ADAMDEC1*; *AKAP7*; *CAPN5*;

*CLIC5*; *CPE*; *CREB3L1*; *NEDD4L*; *RAB27A*), ion transport (*AKAP7*; *ATP2A3*; *CCL28*; *CLIC5*; *F2RL2*; *NEDD4L*; *SLC6A8*), transcription (*AKAP7*; *CREB3L1*; *ISX*; *PABPC1L*; *TXNIP*), signal transduction (*CAPN5*; *F2RL2*; *RAB27A*; *TNFRSF11A*), cell adhesion (*ADAMDEC1*; *PXDN*; *SPON1*; *S100A2*), immune response (*CCL28*; *PXDN*; *TNFRSF11A*) and apoptosis (*ITM2C*; *PDCD4*; *PVT1*)<sup>[13]</sup>. Up-regulation of 3 genes (*CCL28*; *CLIC5*; *PDCD4*) was detected by 2 different probes and up-regulation of 2 genes (*RAB27A*; *TXNIP*) by 3 probes.

## DISCUSSION

Biopsies taken during preoperative RT for rectal cancer were not associated with enhanced toxicity (infection or bleeding). cDNA micro-array analysis on tumor biopsies uncontaminated by normal tissue was possible provided that the extracted RNA was amplified.

Analysis of gene transcription pre- and post-RT detected many up-regulated genes involved in tumor development such as *GOLM1* (prostate cancer)<sup>[14]</sup>, *CAMK2N1* (a tumor suppressor gene in colon cancer)<sup>[15]</sup>, *AGR3* (breast cancer)<sup>[16]</sup> and *PDCD4* (lung and ovarian cancer)<sup>[17]</sup>. However and contrarily to *in vitro* studies<sup>[18]</sup>, it did not detect genes thought to be involved in cell repair after radiation-induced damage.

On the other hand, we detected several early response genes mostly involved in stress such as *CPE* which protects against oxidative stress-induced cell death and ROS-induced cell apoptosis<sup>[19]</sup>, hypoxia-induced *TXNIP* which is regulated by hypoxia-induction factor 1<sup>[20,21]</sup>, *CREB3L1* which codes for a protein that is cleaved in response to stress on the endoplasmic reticulum<sup>[22]</sup>, and *ITM2C* which is over-expressed after alpha radiation but whose role is not known<sup>[23]</sup>.

Many genes implicated in ion channel regulation were up-regulated during radiotherapy, such as *NEDD4L* which regulates sodium channels *via* the Wnt/beta-catenin signaling pathway during colon carcinogenesis<sup>[24]</sup>, *SLC6A8* which codes for a Na<sup>+</sup>Cl<sup>-</sup> dependent creatine transporter<sup>[25]</sup>, *ATP2A3* which encodes an intracellular pump participating in Ca<sup>2+</sup> sequestration, and *CLIC5*, a member of the chloride intracellular channel gene family, structurally homologous to the glutathione-S-transferase superfamily<sup>[26]</sup>.

Among up-regulated genes, we were surprised to find many that are implicated in the immune response, such as *ADAMDEC1* (decysin) which plays a key role in the interaction between dendritic cells and germinal center T-helper cells<sup>[27]</sup>, *ITM2C* which is involved in TNF-induced cell death<sup>[28]</sup>, *TNFRSF11A* which codes for a protein of the TNF receptor superfamily, *CCL28*, a member of the small cytokine CC gene subfamily<sup>[29]</sup>, *RAB27* which regulates exocytosis of neutrophil granules<sup>[30]</sup>, and *PDCD4* (programmed cell death 4) which is regulated by several interleukins (IL-2, IL-15 and IL-12) in natural killer and T cells<sup>[31]</sup>. This was the only gene of our list that has been reported to be a predictor of response to

preoperative radiochemotherapy in pre-treatment biopsies of patients with rectal cancer<sup>[32]</sup>. It codes for a tumor suppressor protein that inhibits translation initiation factor eIF4A which lies downstream of the AKT/mTOR pathway and plays a role in response to DNA damage<sup>[33-35]</sup>. *PDCD4* mRNA levels during RT may thus be an interesting surrogate marker in studies of mTOR inhibitors plus RT for rectal cancer.

Three of the up-regulated genes we detected (*GALNT12*, *CMAH* and *SPON1*) are involved in metabolic processes and interactions with glycans, and would seem to occupy a key role in triggering an immune response<sup>[36]</sup>. *SPON1* protein belongs the thrombospondin type 1 repeat superfamily of proteins that bind transforming growth factor- $\beta$ <sup>[57]</sup>. By analogy with *SPON2*, it might be involved in mechanisms of activation of innate and adaptive immune responses<sup>[38]</sup>.

Apart from *PDCD4* and *SPON1*, we identified at least two other genes that might constitute novel therapeutic targets in preoperative RT for rectal cancer. *TXNIP* (the gene coding for thioredoxin interacting protein) is a key regulator of redox status. Thioredoxin is released from cells in response to oxidative stress and its plasma or serum level is a good marker of cancer-related oxidative stress. Because thioredoxin mediates redox-induced cell death in colon cancer cells, it is an attractive target for anti-tumor therapy<sup>[39]</sup>. Thioredoxin inhibitors are currently under investigation<sup>[40]</sup>. *Rab27A* gene expression was also up-regulated during RT. *Rab27A* is a small G protein which regulates secretory activity in colon cancer cells and promotes invasiveness and metastasis in breast cancer cells<sup>[41,42]</sup>. Novel inhibitors of Rab geranylgeranyl-transferase might thus prove to be inhibitors of rectal tumor cell proliferation and RT enhancers<sup>[43]</sup>. Interestingly, the highly up-regulated clone 5745639 was found by Blast analysis 2022 bp from the 3' end of the Ras-related protein Rab-27A.

A limitation of our study is the small number of tumors analyzed. Our results are thus essentially hypothesis generating. Future attention should focus on the genes that have yielded the most robust results (low FDR and detected by several probes).

In conclusion, biopsies taken during early RT sessions may be used for *in vivo* measurement of tumor sensitivity and have low morbidity. Many genes involved in triggering immune response seem to be expressed during RT. We hypothesize that the changes in gene profiles observed early during RT may help predict rectal tumor response to preoperative RT, whether alone or combined with chemotherapy. Gene profiling may help: (1) identify predictors of resistance to RT that will enable exclusion of patients likely to be cured by surgery alone; (2) assess the validity of surrogate markers during phase I testing of new radiosensitizing drugs which are used together with RT to treat selected resistant rectal tumors; and (3) define new targets for improving the efficacy of preoperative RT of rectal cancer. To test our hypothesis, larger cohorts of patients are needed and the best time for gene

profiling during and after RT needs to be determined.

## COMMENTS

### Background

Because 6-18 patients have to be treated in order to avoid one recurrence, identifying patients with rectal adenocarcinoma likely to benefit from preoperative radiotherapy is essential. End outcomes after preoperative radiotherapy (RT) can hardly be predicted by analyzing pre-treatment clinical parameter, the expression of known proteins or transcriptomic profiles.

### Research frontiers

Sequential biopsies have highlighted changes in tumor dynamics during pelvic RT and identified treatment targets that might modify RT outcomes. The authors postulated that sequential biopsies could be used to detect transcriptional changes during preoperative RT of rectal cancer and help detect new predictors for response to radiation.

### Innovations and breakthroughs

Analysis of gene transcription pre- and post-RT detected many up-regulated genes involved in tumor development. However and contrarily to *in vitro* studies, it did not detect genes involved in DNA repair. Several early response genes mostly involved in stress response and genes involved in ion channel regulation were up-regulated during radiotherapy. Surprisingly, many genes that are implicated in the immune response were found to be up-regulated.

### Applications

Biopsies taken during early RT sessions can be used for *in vivo* measurement of tumor sensitivity and have low morbidity. Many genes involved in triggering immune response seem to be expressed during RT. The changes in gene profiles observed early during RT may help predict rectal tumor response to preoperative RT, whether alone or combined with chemotherapy.

### Terminology

DNA microarray is a collection of microscopic DNA spots attached to a solid surface used to measure the expression levels of large numbers of genes in a tumor sample.

### Peer review

In this study, the authors examined the transcriptomic changes during preoperative radiotherapy in French patients with rectal cancer. In general, this is a good attempt to seek transcriptomic biomarkers for patients with rectal cancer and can provide important information for clinicians.

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P-Reviewer Cui G S-Editor Zhai HH L-Editor A  
E-Editor Li JY



## Treatment of hepatitis C in compensated cirrhotic patients is equally effective before and after liver transplantation

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Received: January 21, 2013 Revised: March 29, 2013

Accepted: April 27, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate differences in tolerability and response to treatment in compensated cirrhotic patients affected by hepatitis C virus (HCV) infection before and after liver transplantation.

**METHODS:** Forty-three HCV non-liver transplanted (LT) cirrhotics (mean age  $55 \pm 8$  years, 65.1% male, Child-Pugh-A, genotype 1-4: 65.1%, 2-3: 34.9%) and 17 LT recipients with recurrent HCV-related cirrhosis (mean age  $57 \pm 9$  years, 88.2% male, Child-Pugh-A, genotype 1-4: 76.5%, 2-3: 23.5%) were included in the analysis from retrospective series. All patients received recombinant or pegylated interferon plus ribavirin at a standard dose and duration. Adverse events were recorded and classified according to the Common Terminology Criteria for Adverse Events. The mean duration of follow-up was of  $4.3 \pm 1.8$  years after the end of the treatment.

**RESULTS:** An early virological response (EVR) was

achieved in 30/43 (69.8%) non-LT and in 8/17 (47.1%) LT cirrhotics, a sustained virological response (SVR) in 18/43 (41.9%) and 5/17 (29.4%), respectively. No statistical difference was observed in EVR and SVR rates between the two groups. Among HCV non-LT cirrhotics, 6/43 (13.9%) discontinued the treatment prematurely, 11.6% of them receiving  $\leq 80\%$  of treatment; 8/17 (47%) LT cirrhotics withdrew the treatment, 35.2% of them receiving  $\leq 80\%$  of treatment. If compared with LT-ones ( $P = 0.015$ ), an higher risk of treatment discontinuation could affect LT cirrhotics, who undergo more frequently  $\leq 80\%$  of treatment ( $P = 0.05$ ). None of the non-LT cirrhotics died after the end of the treatment. With no regards to the achievement of SVR, LT cirrhotic patients showed a reduced survival in respect to non-LT ones (87% at 1 year, 76% at 3 and 5 years after the end of treatment).

**CONCLUSION:** HCV antiviral treatment is equally effective in compensated cirrhotics both before and after LT, which patients show a higher risk of premature treatment withdrawal and a reduced survival, independently of the achievement of SVR.

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**Key words:** Hepatitis C virus; Hepatitis C virus antiviral treatment; Liver cirrhosis; Liver transplantation; Sustained virological response, Efficacy; Safety

**Core tip:** In patients with hepatitis C virus compensated liver cirrhosis antiviral treatment should be considered in order to prevent short to mid-term complications. However, the results of treatment with pegylated interferon plus ribavirin are worse than in non-cirrhotic patients. Furthermore, in non-liver transplanted (LT) cirrhotics, a sustained virological response to antiviral treatment is associated to improved survival, while, at present, no data regarding LT cirrhotic is available in literature. The present study highlights that cirrhotic

patients who undergo antiviral treatment after liver transplantation have a worse prognosis, compared to non-LT ones, independently of the achievement of sustained virological response.

Ponziani FR, Annicchiarico EB, Siciliano M, D'Aversa F, Pompili M, Gasbarrini A. Treatment of hepatitis C in compensated cirrhotic patients is equally effective before and after liver transplantation. *World J Gastroenterol* 2013; 19(21): 3255-3262 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3255.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3255>

## INTRODUCTION

Hepatitis C virus (HCV)-related hepatitis is one of the leading cause of end-stage liver disease worldwide, accounting for half of transplantations in many centers<sup>[1]</sup>. The natural history of HCV-related liver cirrhosis is characterized by several complications that could affect patients' survival<sup>[2-6]</sup>; therefore, a good response to combined antiviral treatment with pegylated interferon (PEG-IFN) and ribavirin (RBV) may produce a benefit on patients quality of life and survival.

According to the recent European Association for the Study of the Liver guidelines<sup>[4]</sup>, in absence of contraindications, the antiviral treatment with PEG-IFN and RBV should be considered for patients with compensated cirrhosis in order to prevent short to mid-term complications. However, results are worse than in non-cirrhotics (30.6%), with a more frequent need for treatment discontinuation and dose reduction<sup>[7-11]</sup>. However, patients who experience a favorable response to antiviral treatment show an improved survival rate in respect to non-responders [98% *vs* 86% at 5 years, respectively, patients with sustained virological response (SVR)] and a lower risk of decompensation<sup>[7,8]</sup>.

In liver transplanted (LT) patients, the perspective is quite different. Although several data regarding treatment of post-liver transplant recurrent hepatitis C are already available, data about the treatment of LT cirrhotic patients are scarce. In particular, in different series of LT patients with recurrent hepatitis C, SVR rates range between 30% (genotype 1 HCV) and 60% (genotype 2 and 3 HCV) and the achievement of SVR has resulted in reducing the progression of liver disease and the incidence of decompensation, alongside patients' survival<sup>[12-17]</sup>. However, these beneficial effects of antiviral treatment have not been proven yet in LT HCV patients with HCV-related liver cirrhosis.

The aim of this retrospective study is to analyze the safety (incidence of adverse events) and efficacy (rate of SVR, and impact on patients survival) of combined antiviral treatment with PEG-IFN and RBV in a series of LT compensated cirrhotic patients, in comparison with non-LT ones.

## MATERIALS AND METHODS

Patients with HCV-related compensated liver cirrhosis, either LT or not, treated with combined PEG-IFN and RBV treatment have been included in this retrospective study.

The Agostino Gemelli Hospital database, Division of Internal Medicine and Gastroenterology has been the source for the cirrhotic non-LT patients' information; further, data about LT cirrhotic patients have been collected by the AISF group for the study of hepatitis C recurrence after liver transplantation (RECOLT-C).

Chronic HCV infection has been diagnosed both on the basis of serum positivity for both anti-HCV antibodies and quantitative HCV-RNA, and the presence of complete or incomplete cirrhosis by histological evaluation of liver tissue specimens performed within 6 mo before the beginning of the treatment (staging of 5 or 6 according to the Ishak scoring system<sup>[18]</sup>). The population of non-LT cirrhotic patients have received combined treatment with PEG-IFN a2a or a2b, at the dose of 180 mcg/wk or 1.5 mcg/kg per week respectively, and ribavirin 800-1200 mg, based on body weight and HCV genotype, for a whole duration of 24 wk (genotypes 2 and 3 HCV) or 48 wk (genotypes 1 and 4 HCV) according to the international guidelines<sup>[4]</sup>. On the other hand, the group of LT cirrhotic patients have undergone combined treatment with PEG-IFN a2a or a2b, at the dose of 180 mcg/wk or 1.5 mcg/kg per week respectively, and ribavirin 800-1200 mg based on body weight and HCV genotype, for a whole duration of 48 wk independently of HCV genotype, according to the international guidelines<sup>[4]</sup>. Patients treated before 2003 (17%) have been subjected to standard IFN alpha2b (3 million units thrice weekly) plus ribavirin.

Quantitative serum HCV-RNA determinations have been made available for all patients at week 12 after the beginning of treatment, at the end of treatment and 6 mo after the end of it. Early virological response (EVR) has been defined as undetectable or more than 2 log drop but detectable HCV RNA level at week 12, end of treatment virological response (EOT-VR) as negative HCV RNA at the end of treatment and SVR as negative HCV RNA 6 mo after the end of treatment.

Safety of antiviral treatment has been evaluated by the occurrence of adverse events, either treatment-related (fatigue, flu-like symptoms and depression, defined as "intolerance"; anemia and neutropenia, defined as "hematological toxicity") or related to liver disease decompensation (ascites, encephalopathy and gastrointestinal bleeding, defined as "decompensation"). The severity of adverse events has been described according to the Common Terminology Criteria for Adverse Events (CTCAE) v3.0<sup>[19]</sup> and is shown in Table 1. Grade 1 have been defined as transient, with mild discomfort, no limitation in activity and no need of medical intervention/therapy; grade 2 as a mild to moderate impairment in daily activity, no or minimal medical intervention/therapy required;

**Table 1 Adverse events classification according to Common Terminology Criteria for Adverse Events v3.0**

| Adverse event     | Grade 1  | Grade 2  | Grade 3                                     | Grade 4                                     | Grade 5 |
|-------------------|--|--|---|---|---------|
| Anemia            | Hb < LLN-10.0 g/dL                                 | Hb 10.0-8.0 g/dL   | Hb < 8.0-6.5 g/dL                           | Hb < 6.5 g/dL                               | Death   |
| Neutropenia       | PMN<br>< LLN-1.5 × 10 <sup>9</sup> /L              | PMN<br>< 1.5-1.0 × 10 <sup>9</sup> /L  | PMN<br>< 1.0-0.5 × 10 <sup>9</sup> /L       | PMN<br>< 0.5 × 10 <sup>9</sup> /L           | Death   |
| Intolerance:      |  |  |   |   |         |
| Fatigue           | Mild fatigue over baseline                         | Moderate or causing difficulty performing some ADL   | Severe fatigue interfering with ADL         | Disabling                                   |         |
| Flu-like symptoms | Symptoms present but not interfering with function | Moderate or causing difficulty performing some ADL   | Severe symptoms interfering with ADL        | Disabling                                   | Death   |
| Depression        | Mild mood alteration not interfering with function | Moderate mood alteration interfering with function, but not interfering with ADL; medication indicated | Severe mood alteration interfering with ADL | Suicidal ideation; danger to self or others | Death   |

ADL: Activities of daily living; PMN: Polymorphonuclear neutrophil.

**Table 2 Patients population characteristics *n* (%)**

| Variable               | Non-liver transplanted cirrhotic group frequency ( <i>n</i> = 43) | Liver transplanted cirrhotic group frequency ( <i>n</i> = 17) | <i>P</i> |
|------------------------|---|---|----------|
| Age, yr (mean ± SD)    | 55 ± 8 (39-69)  | 57 ± 9 (38-73)  | 0.753    |
| Sex                    |   |   | 0.073    |
| Male                   | 28 (65.1)   | 15 (88.2)   |          |
| Female                 | 15 (34.9)   | 2 (11.8)  |          |
| Genotype               |   |   | 0.750    |
| 1                      | 2 (58.1)  | 11 (64.7)   |          |
| 2                      | 13 (30.2)   | 3 (17.6)  |          |
| 3                      | 2 (4.7)   | 31 (5.9)  |          |
| 4                      | 3 (7)   | 2 (11.8)  |          |
| Previous treatment     | 16 (37.2)   | 4 (23.5)  | 0.069    |
| HCV RNA 800000 (UI/mL) | 15  | 9   | 0.198    |
| EVR                    | 30 (69.8)   | 8 (47.1)  | 0.100    |
| SVR                    | 18 (41.9)   | 5 (29.4)  | 0.371    |

HCV: Hepatitis C virus; EVR: Early virological response; SVR: Sustained virological response.

grade 3 as a markedly reduction in daily activity, requiring medical intervention/therapy and possible hospitalization or hospice care; grade 4 as extreme limitation to daily activity, significant medical intervention/therapy required and hospitalization or hospice care very common; finally, grade 5 of adverse events always causes death.

### Statistical analysis

Statistical analysis has been performed by reporting continuous variables as mean and standard deviation and categorical ones as absolute and relative frequencies.

Evaluated end-points were antiviral therapy outcome, overall survival and the occurrence of adverse events. Logistic regression has been used to assess the correlation between each variable and antiviral treatment outcome, and the Fisher's exact test was applied to categorical variables to assess the differences between the two groups of cirrhotic LT and non-LT patients. The Kaplan-Meier curve has been employed to analyze patients' survival and the differences between groups have been assessed using log-rank tests.

All tests were 2-sided and  $P \leq 0.05$  has been considered to be statistically significant.

## RESULTS

Baseline patients' characteristics are shown in Table 2. Forty-three patients belonged to the group of non-LT cirrhotics, 17 to the group of LT ones. The majority of patients were male and 9 of them were 65-year-old or more (7 in LT and 2 in non-LT group). All patients were classified in Child-Pugh class A.

Among non-LT cirrhotics, 16/43 (47.1%) experienced a previous unsuccessful antiviral treatment while the rate was 4/12 (33.3%) among the LT ones, for whom the data was available. Most of patients presented with genotype 1 HCV related chronic hepatitis and a high viral replication; HCV RNA levels  $\geq 600000$  UI/mL were indeed detected in 20/43 (46.5%) of non-LT cirrhotics and in 11/17 (64.7%) of LT cirrhotics.

An EVR has been reported respectively in 30/43 (69.8%) non-LT cirrhotic patients and in 8/17 (47.1%) LT cirrhotic ones, a SVR in 18/43 (41.9%) non-LT cirrhotic patients and 5/17 (29.4%) in LT cirrhotic ones. Among the investigated factors (age, sex, HCV genotype, viral load, previous treatment, EVR, treatment discontinuation and less than 80% of treatment) only EVR has seemed to have a positive effect on the achievement of SVR in both groups ( $P = 0.42$ ; OR = 3.3, 95%CI: 0.072-2.287). Statistical analysis has not shown any difference in EVR and SVR rates between the two groups.

Table 3 indicates the general reported adverse events dealing with antiviral treatment safety assessment. No grade 4 or 5 adverse events have been recorded. Six cirrhotic patients (2 non-LT and 4 LT) experienced grade 2 anemia requiring erythropoietin administration, while grade 3 anemia non responsive to RBV dose reduction and erythropoietin administration caused treatment discontinuation in 2 LT cirrhotics. The incidence of grade 3 neutropenia has been reported in 6 non-LT and in 1 LT cirrhotic patients, and has been treated with granulocyte colony stimulating factors (G-CSF), while treatment discontinuation was required in 1 LT cirrhotic patient.

**Table 3** Reported liver-related and treatment-related adverse events

|  | Non-liver transplanted cirrhotics | Liver transplanted cirrhotics |
|--|-----------------------------------|-------------------------------|
| Intolerance (fatigue, flu-like symptoms, depression) |                                   |                               |
| Grade 2  | 1                                 | 0                             |
| Grade 3  | 3                                 | 0                             |
| Anemia   |                                   |                               |
| Grade 1  | 12                                | 0                             |
| Grade 2  | 2                                 | 4                             |
| Grade 3  | 0                                 | 2                             |
| Neutropenia  |                                   |                               |
| Grade 2  | 0                                 | 2                             |
| Grade 3  | 6                                 | 1                             |
| Liver decompensation                                 |                                   |                               |
| Grade 1 (encephalopathy)                             | 0                                 | 1                             |
| Other  |                                   |                               |
| Hepatocellular carcinoma                             | 2                                 | 0                             |
| Polytrauma   | 1                                 | 0                             |

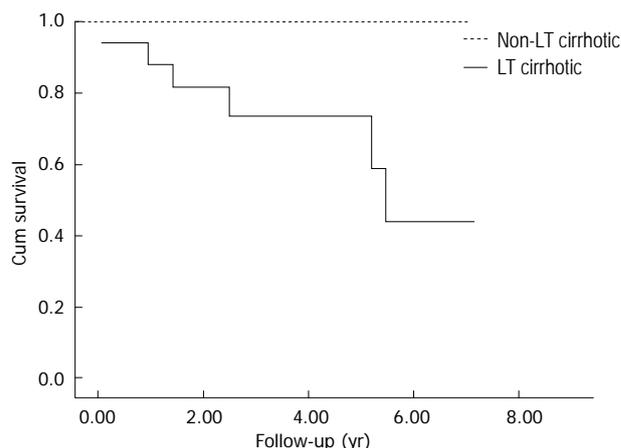
Only 1 liver-related adverse event (encephalopathy) has occurred during the antiviral treatment in LT cirrhotic patients group.

Overall, among HCV non-LT cirrhotics 6/43 (13.9%) patients prematurely discontinued the treatment: 2 due to the development/recurrence of hepatocellular carcinoma, 3 for grade 3 fatigue, 1 for polytrauma. Then, 5 of them (11.6%) received less than 80% of the intended treatment. None of the 6 patients who discontinued the treatment achieved a SVR, except of 2 of them who received 41 and 35 wk of treatment.

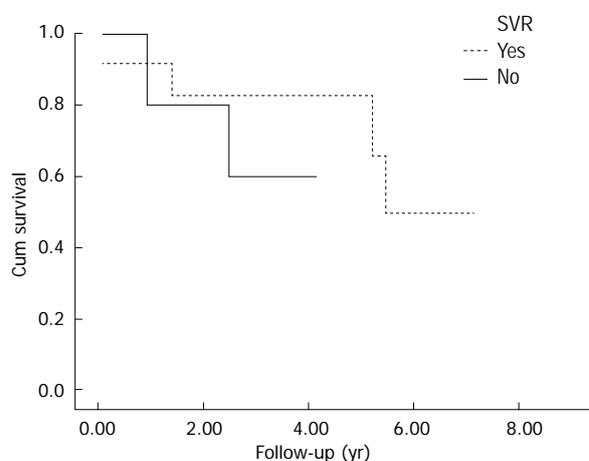
Among HCV LT cirrhotics 8/17 (47%) withdrew the treatment (4 for lack of response and 1 for early relapse, 2 for anemia and 1 for severe neutropenia), 6 of them (35.2%) receiving less than 80% of the intended treatment. None of the 6 patients who discontinued the treatment achieved a SVR, except 2 of them who received 40 and 43 wk of treatment. As a result, we found that LT cirrhotic patients present a higher risk of treatment discontinuation when compared with non-LT ones ( $P = 0.015$ , Fisher's exact test) and undergo more frequently less than 80% of the intended treatment ( $P = 0.05$ , Fisher's exact test).

Patients were followed-up until December 2009. None of the non-LT cirrhotics died and the mean follow-up was of  $4.3 \pm 1.8$  years after the end of the treatment; among LT cirrhotics, 6 deaths occurred (one due to extrahepatic causes, 1 due to sepsis, and 4 due to liver insufficiency), with survival rates of 87% at 1 year and of 76% at 3 and 5 years after the end of treatment (mean follow-up of  $3.3 \pm 2.2$  years, Figure 1).

With no regards to the achievement of SVR, LT cirrhotic patients (Figure 1) showed a reduced survival in respect to non-LT ones. Moreover, SVR didn't show to have a beneficial effect in terms of survival: Kaplan-Meier curve didn't show any difference between LT-cirrhotic patients who achieved SVR in respect to those who did not ( $P < 0.463$ , log-rank test, Figure 2).



**Figure 1** Cumulative survival of non-liver transplantation and liver transplantation cirrhotic patients. LT: Liver transplanted.



**Figure 2** Cumulative survival of liver transplanted patients according to sustained virological response stratification. SVR: Sustained virological response.

## DISCUSSION

### Efficacy and impact on survival

International guidelines recommend to treat HCV hepatitis in non-LT cirrhotic patients, especially those with an early stage liver disease (Child-Turcotte-Pugh, CTP, score A or B7), with genotype 2 HCV infection and in absence of contraindications, since the achievement of SVR is possible in about one third of them<sup>[4,7-11,20]</sup>.

Data previously published about the efficacy of HCV treatment in this subgroup of patients have been confirmed by our series, which includes patients affected by compensated liver cirrhosis (CTP A). In non-LT cirrhotic patients, the achievement of a SVR seems related to an improvement of overall survival<sup>[8]</sup> and 5-year "event-free" survival, also reducing the risk of liver decompensation<sup>[7,8]</sup>. Moreover, an improvement in liver function has been reported in SVR patients (CTP 7.8 *vs* 6.4) in respect to non-responders (CTP 8.0 *vs* 8.7) and to controls (CTP 8.3 *vs* 9.4)<sup>[7]</sup>. Finally, a SVR to combined HCV antiviral treatment preempts liver graft re-infection for those pa-

tients who will undergo LT<sup>[4,9,10,21,22]</sup>.

However, at present, data regarding the impact of SVR on survival of cirrhotic patients undergoing HCV antiviral treatment after liver transplantation, are scarce. As it can be shown by our analysis, independently of the achievement of SVR, LT cirrhotic patients have a worse survival if compared to non-LT ones (Figure 1,  $P < 0.0001$ ). In except of 1 case of an infection, which could be easily explained in immunosuppressed patients such as LT ones, the most common reported cause of death in our series was liver insufficiency, in agreement with literature data<sup>[14,23]</sup>.

Furthermore, no difference in survival has been observed between LT cirrhotic patients who achieved a SVR and those who did not. More to the point, it could be interesting to compare our results in terms of survival with the existing data regarding the natural history of post-liver transplant HCV cirrhosis recurrence, in patients who did not receive an antiviral treatment. Berenguer *et al*<sup>[1]</sup> followed-up 49 patients with genotype 1b HCV recurrent cirrhosis after liver transplant. The 1-year survival rate of those patients with clinically compensated HCV-graft cirrhosis (39/49) was of 74%, lower than that reported by our study (87%, Figure 1). Firpi *et al*<sup>[23]</sup> reported the natural history of 88 HCV LT cirrhotic patients, the major part clinically compensated and of genotype 1; the cumulative probability of survival was 83% at 1 year and 41% at 5 years from the time of diagnosis of compensated cirrhosis. Nevertheless, for those patients who remained free from decompensation, the reported survival was of 90% at 1 year and 70% at 5 years, which is comparable to that reported by our study. Finally, in a series of 55 HCV LT recipients with recurrent cirrhosis, 70 with genotype 1 and 28 with genotype 2-3, Kalambokis *et al*<sup>[24]</sup> reported 90% and 74% rates of survival at 1 and 5 years in those subjects who did not developed liver decompensation.

Then, in the subgroup of LT patients with recurrent HCV-related compensated liver cirrhosis, the achievement of SVR to the antiviral treatment is not able to modify the natural course of the liver disease and, therefore, to improve patients' survival. Probably, the fact that in the liver transplant setting the course of liver cirrhosis has a more rapid and unavoidable progression, difficult to be stopped even with the eradication of HCV infection, is the main reason to support this evidence. Berenguer *et al*<sup>[25]</sup> assessed that the rate of post-transplantation fibrosis progression was significantly higher than pre-transplantation [0.2/year (0.09-0.8)]. The suppression of immune system, on one hand, confirmed by the reported histologic improvements due to immunosuppression weaning-off<sup>[26]</sup>, and the tendency to develop a mild, persistent chronic damage due to rejection, on the other hand, may be the main factors affecting the natural course of post-liver transplant HCV-related cirrhosis. However, the impact of immunosuppression weaning-off on liver histology decreases during the time<sup>[27]</sup>. Moreover, systematic reviews didn't show any significant re-

lationship between IFN treatment and acute or chronic rejection, with incidence rates comparable to the whole population of LT recipients<sup>[28,29]</sup>. Pooling the results of 6 studies<sup>[17,30-33]</sup>, rejection rates seem higher (11%-21% acute rejection; 9%-17% chronic rejection), and a recent review by Selzner *et al*<sup>[34]</sup> reported acute and chronic rejection rates of 0%-35% and 4%-8%, respectively.

It is also well-known that HCV infection itself could be associated with autoimmune diseases (cryoglobulinemia, Sjogren like syndrome, anti-nucleus or anti-liver kidney microsomes antibodies positivity)<sup>[34]</sup>. It has been hypothesized that this kind of liver damage could be a variant of rejection rather than the manifestation of a *de novo* autoimmune hepatitis<sup>[35]</sup>. Several cases following HCV antiviral treatment have been reported<sup>[36,37]</sup>, probably related to the various IFN and RBV immunomodulating effects and often developing after HCV clearance<sup>[34]</sup>.

In conclusion, HCV infection control in liver allografts is certainly linked to the stimulation of immune system, dealing with the risk of developing acute or chronic rejection or *de novo* autoimmune liver damage. These kind of damage may impact negatively on the progression of liver cirrhosis, already faster in LT recipients, even in case of SVR to antiviral treatment. We are, at present, still far from a complete knowledge of the mechanism underlying allograft acceptance or HCV clearance.

### Safety

Among non-LT cirrhotic patients, the reported combined antiviral treatment discontinuation rates are widely variable. In a large series of 568 non-LT cirrhotic patients by Fernández-Rodríguez *et al*<sup>[8]</sup> a 29.6% rate of treatment discontinuation due to adverse events was reported, while in 19.2% and 17.4% of them, respectively, a PEG-IFN or RBV dose reduction was necessary. The overall reported drop-out rate was 66%. In decompensated cirrhotic patients, rates of treatment withdrawal or dose reduction due to adverse events seems almost comparable: 59% by Iacobellis *et al*<sup>[7]</sup>, 65% by Everson *et al*<sup>[22]</sup>, 87% by Crippin *et al*<sup>[11]</sup>, 30% by Forns *et al*<sup>[10]</sup>.

With regards to LT cirrhotics undergoing combined antiviral treatment, Berenguer *et al*<sup>[16]</sup> reported respectively a 37% rate of discontinuation, and a 45% and a 36% rates of PEG-IFN or RBV dose reduction. In the study by Carrión *et al*<sup>[14]</sup>, treatment discontinuation rate was of 39% while 67% and 24% of patients experienced RBV or PEG-IFN dose reduction. Angelico *et al*<sup>[17]</sup> reported a 33% drop-out rate, and a 45% rate of RBV and a 38% rate of PEG-IFN dose reduction.

In non-cirrhotic LT patients, the most frequently reported causes of treatment withdrawal are cytopenia, in particular anemia, neuropsychiatric symptoms, thyroid dysfunction, intolerance and rejection<sup>[12,14,16]</sup>. However, these studies included LT patients with HCV hepatitis recurrence after liver transplant but without liver cirrhosis; data about LT cirrhotic patients are scarce, but it is

likely to expect a higher incidence of adverse events and a consequent higher rate of treatment dose reduction or discontinuation.

In our study, we did not observe the occurrence of any severe or fatal adverse event (grade 4 or 5). Thirteen patients have received supportive therapy with growth factors due to hematological adverse events, such as anemia and leucopenia, and 3 LT cirrhotics withdrew the treatment. None among LT cirrhotics experienced fatigue, flue-like symptoms and depression, while one grade 2 and three grade 3 adverse events (requiring treatment discontinuation) occurred among non-LT ones.

Overall, as a consequence of reduced tolerance to treatment, LT cirrhotics included in the present analysis reported a higher rate of discontinuation than non-LT ones (47% LT *vs* 13.9% non-LT;  $P = 0.015$ ), thus receiving less than 80% of the treatment in a larger proportion of cases (35.2% LT *vs* 11.6% non-LT;  $P = 0.05$ ). It is also interesting that one half of non-LT cirrhotic patients discontinued the treatment due to adverse events unrelated to the treatment itself, such as polytrauma or hepatocellular carcinoma recurrence, while after LT treatment discontinuation was almost related to treatment side effects.

Therefore, LT cirrhotic patients have a higher risk to receive an overall treatment dose is not adequate to achieve a SVR. Indeed, none of the 8 LT cirrhotic patients who discontinued the treatment achieved a SVR, in except of two of them who received respectively 40 and 43 wk of treatment; similarly, among non-LT cirrhotics who discontinued the treatment, only the two patients who underwent 41 and 35 wk of treatment achieved a SVR.

### Concluding remarks

Our series shows that the survival of LT compensated HCV cirrhotic patients is lower if compared to non LT ones; nevertheless, the achievement of SVR seems to have no effect on the natural history of these patients and to not improve survival. Moreover, LT HCV cirrhotic patients have a worse tolerance to antiviral treatment than non-LT ones and experience more frequently adverse events. In this era of important changes in the paradigms and drugs employed in HCV antiviral treatment<sup>[38,39]</sup>, some issues about the new incoming drugs could be raised. Indeed, the real gain in risk/benefit ratio of any antiviral treatment should be carefully evaluated. However, the retrospective nature of the present study, and the discrepancy between the sample size of non-LT and LT cirrhotic patients, make possible to provide speculative considerations only. Future prospective studies evaluating the effects of SVR on the long-term outcome of LT HCV cirrhotic patients, and assessing the efficacy and safety of the new therapeutic regimens in LT HCV cirrhotic patients are necessary to “bring light into the dark”, and to develop specific guidelines for clinical practice.

### ACKNOWLEDGMENTS

We would like thank AISF RECOLT-C Group: Raffa-

ella Viganò Niguarda Ca' Granda Hospital-Milan, Rosa Maria Iemmo University of Modena -Modena, Maria Francesca Donato IRCCS Foundation Ca' Granda Maggiore Hospital-Milan, Maria Rendina University of Bari-Bari, Pierluigi Toniutto University of Udine-Udine, Luisa Pasulo, Stefano Faggioli Ospedali Riuniti-Bergamo, Matteo Cescon Sant'Orsola Malpighi Hospital-Bologna, Patrizia Burra University of Padua-Padua, Eleonora De Martin University of Padua-Padua, Lucia Miglioresi San Camillo Spallanzani Hospital-Rome, Manuela Merli Sapienza University-Rome, Valerio Giannelli Sapienza University-Rome, Daniele Di Paolo University of Torvergata-Rome.

## COMMENTS

### Background

Hepatitis C virus (HCV)-related hepatitis is one of the leading causes of end-stage liver disease worldwide, accounting for half of liver transplanted (LT) in many centers. Antiviral treatment should be considered for patients with compensated cirrhosis in order to prevent short to mid-term complications. The results of antiviral treatment with pegylated interferon plus ribavirin in patients presenting with compensated liver cirrhosis are worse than in non-cirrhotic ones. However, cirrhotics who experience a favorable response to antiviral treatment show an improved survival rate in respect to non-responders (98% *vs* 86% at 5 years, respectively, for sustained virological response (SVR) and a lower risk of decompensation.

### Research frontiers

The research hotspot is to compare the efficacy and safety of antiviral treatment in LT and non-LT cirrhotic patients, and to understand how it can affect patients' survival, especially after liver transplantation.

### Innovations and breakthroughs

At present, no data regarding the impact of SVR on survival of cirrhotic patients undergoing HCV antiviral treatment after LT are available in literature. The natural history of the underlying liver disease may heavily affect cirrhotic patients' survival, independently of the achievement of a sustained virological response to antiviral therapy. HCV infection control in liver allografts is certainly linked to the stimulation of immune system, dealing with the risk of developing acute or chronic rejection or *de novo* autoimmune liver damage. These kind of damage may impact negatively on the progression of liver cirrhosis, already faster in LT recipients, even in case of sustained virological response to antiviral treatment. The present study highlights that cirrhotic patients who undergo antiviral treatment after LT have a worse prognosis, compared to non-LT ones, independently of the achievement of SVR.

### Applications

The present study suggests that cirrhotic patients have a worse response to antiviral treatment, especially after LT. Probably, a SVR to antiviral treatment could not be able to protect patients with such an advanced liver damage and a complex alteration of the immune response from an unfavorable evolution of the disease.

### Terminology

SVR is the negativization of HCV RNA 6 mo after the end of antiviral treatment.

### Peer review

This is an interesting paper comparing the safety and efficacy of antiviral treatment in patients with HCV infection and cirrhosis before and after transplantation.

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**P- Reviewers** Grattagliano I, Mudawi HMY, Tziomalos K  
**S- Editor** Zhai HH **L- Editor** A **E- Editor** Zhang DN



## Stage and size using magnetic resonance imaging and endosonography in neoadjuvantly-treated rectal cancer

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Supported by The Gothenburg Medical Association, the Lions Cancerfond Väst and the Björnsson Foundation

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Received: December 3, 2012 Revised: April 12, 2013

Accepted: April 28, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To assess the stage and size of rectal tumours using 1.5 Tesla (1.5T) magnetic resonance imaging (MRI) and three-dimensional (3D) endosonography (ERUS).

**METHODS:** In this study, patients were recruited in a phase I/II trial of neoadjuvant chemotherapy for biopsy-proven rectal cancer planned for surgical resection with or without preoperative radiotherapy. The feasibility and accuracy of 1.5T MRI and 3D ERUS were compared with the histopathology of the fixed surgical specimen (pathology) to determine the stage and size of the rectal cancer before and after neoadjuvant chemotherapy. A Philips Intera 1.5T with a cardiac 5-channel synergy surface coil was used for the MRI, and a B-K Medical Falcon 2101 EXL 3D-Probe was used

at 13 MHz for the ERUS. Our hypothesis was that the staging accuracy would be the same when using MRI, ERUS and a combination of MRI and ERUS. For the combination, MRI was chosen for the assessment of the lymph nodes, and ERUS was chosen for the assessment of perirectal tissue penetration. The stage was dichotomised into stage I and stage II or greater. The size was measured as the supero-inferior length and the maximal transaxial area of the tumour.

**RESULTS:** The staging feasibility was 37 of 37 for the MRI and 29 of 36 for the ERUS, with stenosis as a limiting factor. Complete sets of investigations were available in 18 patients for size and 23 patients for stage. The stage accuracy by MRI, ERUS and the combination of MRI and ERUS was 0.65, 0.70 and 0.74, respectively, before chemotherapy and 0.65, 0.78 and 0.83, respectively, after chemotherapy. The improvement of the post-chemotherapy staging using the combination of MRI and ERUS compared with the staging using MRI alone was significant ( $P = 0.046$ ). The post-chemotherapy understaging frequency by MRI, ERUS and the combination of MRI and ERUS was 0.18, 0.14 and 0.045, respectively, and these differences were non-significant. The measurements of the supero-inferior length by ERUS compared with MRI were within 1.96 standard deviations of the difference between the methods (18 mm) for tumours smaller than 50 mm. The agreement with pathology was within 1.96 standard deviations of the difference between imaging and pathology for all tumours with MRI (15 mm) and for tumours that did not exceed 50 mm with ERUS (22 mm). Tumours exceeding 50 mm in length could not be reliably measured by ERUS due to the limit in the length of each recording.

**CONCLUSION:** MRI is preferable to use when assessing the size of large or stenotic rectal tumours. However, staging accuracy is improved by combining MRI with ERUS.

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**Key words:** Rectal cancer; Magnetic resonance imaging; Endosonography; Predictive value of tests; Neoadjuvant treatment

**Core tip:** To the best of our knowledge, the possibility of increasing the accuracy of the staging of rectal cancer by combining the strengths of magnetic resonance imaging (MRI) and endosonography (ERUS) in the same patient has not been reported. In neoadjuvantly treated rectal cancer, a combination of lymph node assessment by MRI and assessment of perirectal tissue penetration by ERUS improved the staging accuracy, with stage II as the cut-off. Furthermore, this study showed that ERUS could replace MRI in the measurement of the transaxial area of all non-stenotic tumours and in the measurement of the length of non-stenotic tumours up to 50 mm.

Swartling T, Kälebo P, Derwinger K, Gustavsson B, Kurlberg G. Stage and size using magnetic resonance imaging and endosonography in neoadjuvantly-treated rectal cancer. *World J Gastroenterol* 2013; 19(21): 3263-3271 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3263.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3263>

## INTRODUCTION

Radical surgery is the curative treatment option for rectal cancer. The preoperative work-up generally includes an assessment of the stage and size of the rectal tumour to optimise the treatment plan. This work-up ranges from early tumours possibly eligible for local excision to advanced cancers for which long-term radiochemotherapy is indicated prior to full-scale resection. To decrease recurrence, preoperative short-course radiotherapy is generally indicated in stages II-III, and long-course radiochemotherapy is usually indicated in “ugly” tumours with threatened surgical margins<sup>[1]</sup>. New strategies have been tested, including the use of preoperative chemotherapy, regardless of any radiotherapy, to potentially downstage and downsize the tumour. This approach has been tried, and further studies are on-going<sup>[2,3]</sup>. Furthermore, local excision is normally only indicated in T1 tumours with a diameter of less than 30 mm, and Zlobec *et al*<sup>[4]</sup> found that a diameter greater than 34 mm predicts late T-stage. These preoperative treatment strategies demand improved staging and enhanced responses for evaluating neoadjuvant therapy using accurate and accessible size measurement methods.

The main instruments for the preoperative assessment of rectal tumours are magnetic resonance imaging (MRI) and endorectal ultrasound (ERUS). Many groups have concluded that MRI is superior to ERUS in the staging of rectal tumours<sup>[5-7]</sup>. However, Bipat *et al*<sup>[8]</sup> found that ERUS was more accurate than MRI for perirectal tissue invasion and that the assessment of lymph nodes and invasion of adjacent organs was comparable in a meta-analysis that

included all stages. Some studies have shown that MRI is preferable in advanced and stricturing tumours and in the assessment of lymph node involvement, whereas ERUS is advantageous in assessing the wall penetration of the early stages of rectal cancer<sup>[9-14]</sup>. Many of these studies used 1 Tesla (1T) MRI and two-dimensional (2D) ERUS<sup>[15,16]</sup>, and the results may be improved in newer models of the MRI and ERUS equipment. Increased specificity has been reported with 3T MRI machines compared with 1T or 1.5T<sup>[17]</sup>. However, Maas *et al*<sup>[18]</sup> did not find that 3T MRI improved the accuracy compared with 1.5T in borderline T2-T3 rectal cancer. Diffusion-weighted MRI appears promising, but the specificity in lymph node detection is still limited<sup>[19]</sup>. Compared with 2D ERUS, the development of 3D ERUS has resulted in increased accuracy, but a problem remains regarding the detection of lymph nodes and the margins of the mesorectal fascia<sup>[20]</sup>. Earlier studies of the measurement of the rectal tumour size have primarily involved MRI<sup>[21]</sup>. Torkzad *et al*<sup>[22]</sup> showed that rectal tumours can be measured on MRI images, with results that correlated well with the histopathology results, and Nougaret *et al*<sup>[23]</sup> showed that MRI volumetry may predict early responders to neoadjuvant therapy. Murad-Regadas *et al*<sup>[24]</sup> found that size measurement by ERUS was useful in the selection of possible sphincter-saving surgery after neoadjuvant therapy. In many parts of Western Europe, MRI is preferred for the assessment of rectal cancer, except in the disease’s early stage, but in other parts of the world, ERUS is still the method of choice for the initial evaluation of rectal cancer at all stages<sup>[16,20,25]</sup>. Even in parts of Western Europe, this issue was not settled in 2012<sup>[7,26]</sup>. Many authors regard the methods as complementary<sup>[12,14,27,28]</sup>. Some authors have argued that a combination of both MRI and ERUS may increase the accuracy of the preoperative staging<sup>[13,14,27]</sup>. The restaging of advanced rectal cancer after neoadjuvant therapy has been studied for MRI<sup>[15,29,30]</sup> and ERUS<sup>[15,31,32]</sup>. In this context, MRI appears more promising, but there is a problem with accuracy for both methods.

In this study, 1.5T MRI and 3D ERUS were compared with histopathology of the fixed surgical specimen (pathology) to determine the stage and size of the rectal cancer before and after neoadjuvant chemotherapy. Our hypothesis was that the staging accuracy would be the same when using MRI, ERUS and a combination of MRI and ERUS. For the combination, MRI was chosen for the assessment of the lymph nodes, and ERUS was chosen for the assessment of perirectal tissue penetration because studies have reported that these are the respective strengths of the two methods<sup>[11,27,28,33]</sup>. Furthermore, we hypothesised that the MRI and ERUS results would be in agreement with regard to the tumour size measurement. The aims of the study were to evaluate the feasibility and accuracy of MRI and ERUS in staging and to measure the supero-inferior length and maximal transaxial area before and after neoadjuvant chemotherapy compared with postoperative histopathology.

**Table 1 Demographic and histopathological data of 21 patients assessed for both stage and size before chemotherapy**

|                                   |                             |
|-----------------------------------|-----------------------------|
| Age, median (IQ range)            | 60 (60-66)                  |
| Gender (male/female)              | 17/4                        |
| Surgery (AR/APR/Hartman)          | 13/8/0                      |
| Laparoscopy (yes/no)              | 10/11                       |
| Differentiation grade (G1/2/3/4)  | 0/19/0/0 (2 not classified) |
| T-stage (T1/2/3/4)                | 2/8/9/2                     |
| N-stage (N0/1/2)                  | 12/6/3                      |
| Assessed nodes, median (IQ range) | 15 (14-18)                  |

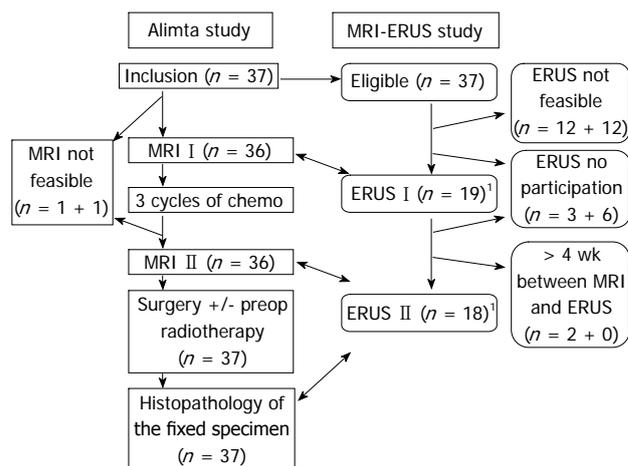
## MATERIALS AND METHODS

The present study was conducted in cooperation with a prospective phase I /phase II study of neoadjuvant treatment with pemetrexed in rectal cancer set at a university hospital in Sweden (Alimta trial)<sup>[2]</sup>. Briefly, the study included 37 patients with rectal cancer who all received three cycles of chemotherapy prior to surgery. They underwent MRI scans before and after chemotherapy. Radiotherapy followed by resection surgery was conducted as established by national guidelines. The study concluded that the treatment concept was feasible, with limited risks of side-effects and a majority of the patients experiencing symptom relief and downsizing of the tumour.

All patients who participated in the Alimta trial were asked to participate in the associated MRI-ERUS study. The demographic data of the included patients are shown in Table 1. The intention was to match the scheduled investigations using MRI with ERUS before and after chemotherapy to enable a comparison of the staging and size measurements, including paired analyses. All surgically removed specimens were routinely sent for histopathological examination, which also provided a comparison between MRI, ERUS and the fixed specimen in terms of both stage and size. The supero-inferior length was compared between MRI, ERUS and the fixed specimen. The maximal transaxial area in a dimension perpendicular to the long axis of the rectum at the site of the cancer was compared between MRI and ERUS. The MRI and ERUS were performed as detailed below. When the reports included more than one stage, the more advanced stage was chosen in the analysis. The order in which the MRI and ERUS were conducted was not controlled. Investigations with a time lapse between the MRI and ERUS greater than four weeks were not included in the analysis. The study is schematically presented in Figure 1. The protocol was approved by the local hospital ethics committee. Patients provided written consent to participate in the investigation.

### MRI

Philips Intera 1.5T was used together with a cardiac 5-channel synergy surface coil (Philips) for an optimal signal. Antispasmodic drugs, 40 mg of buscopan and 1 mg of glucagon, were administered before the start of the examination. There was no bowel preparation of the patient.

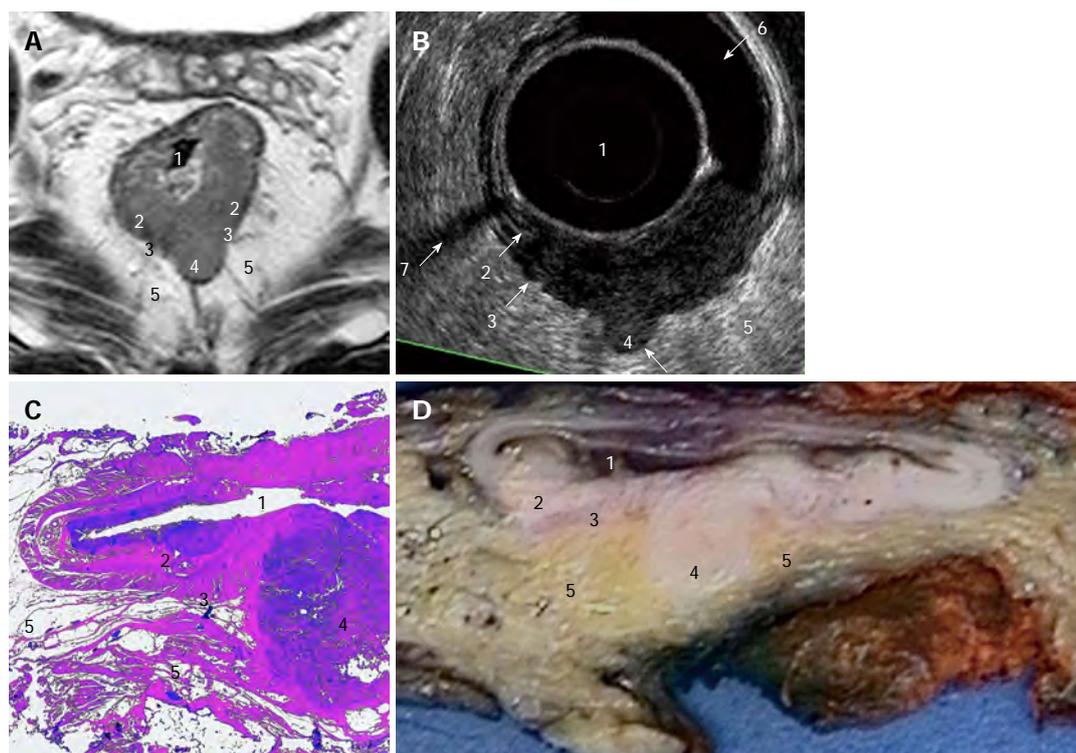


**Figure 1 Study algorithm of the treatment and the examinations.** The stages and sizes using magnetic resonance imaging (MRI) were compared with the corresponding stages and sizes using endosonography (ERUS) before and after chemotherapy and with postoperative histopathology. The figure shows the assessments for size. <sup>1</sup>Another 10 patients before chemo and 5 after chemo had complete pairs of MRI and ERUS assessments for stage.

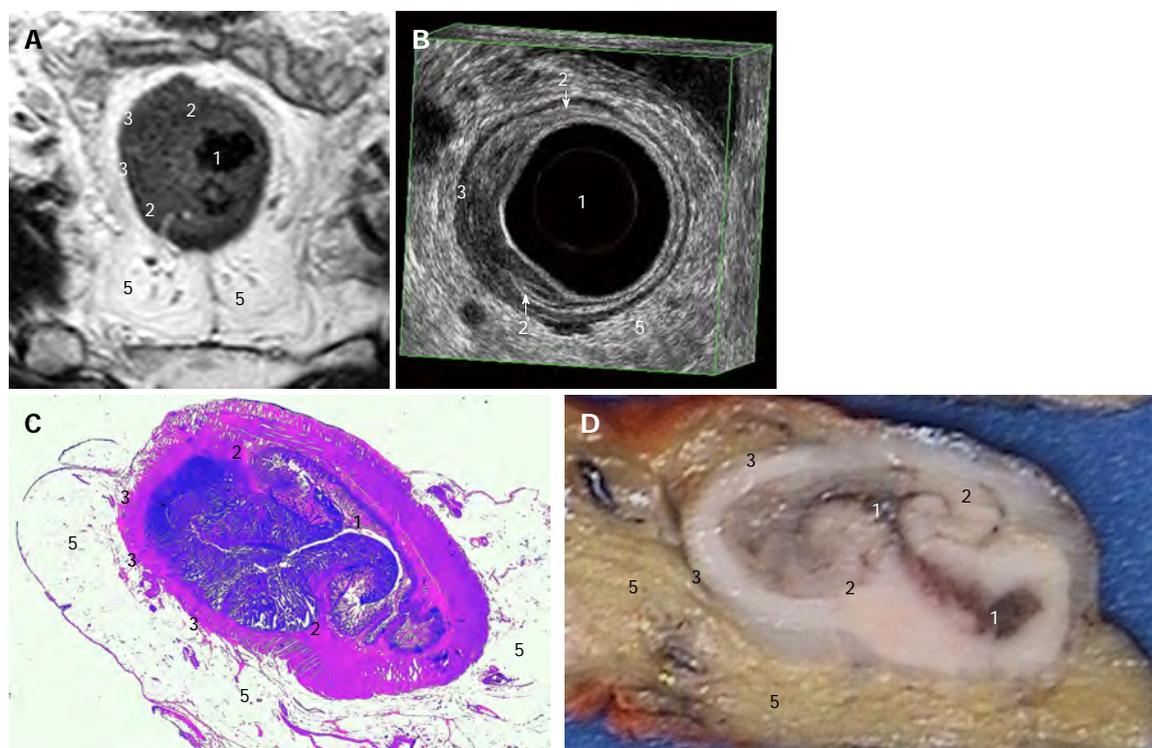
The protocol included three T2-weighted turbo spin-echo sequences in the sagittal, oblique axial and oblique coronal planes. The following conditions were used: slice thickness 3 mm, gap 0.3 mm, turbofactor between 20-22, field-of-view (FOV) 20-22 cm, 30-36 slices and in-plane resolution 0.67-0.7 mm. The echo time (TE) was 90 ms, and the repetition time (TR) of 2800-3400 ms was dependent on the sequence and number of signals acquired (NSA) 4. The scan time varied between 4-6 min per sequence. At the end, an additional oblique axial contrast-enhanced 3D T1-weighted gradient echo sequence with fat sat in the venous phase (Thrive, Philips, Eindhoven, The Netherlands) was used with the following conditions: 2 mm slices, FOV 26 cm, NSA 2, TR 9.1 ms, TE 4.5 ms, flip 10 degrees, scan time 1.49 min, and voxel size 0.81 mm. On average, each investigation lasted 40 min, during which the patients were required to lie still. The MRI images were interpreted by one radiologist (Kålebo P). The measurements were performed using a software function and recorded in millimeter. The TN staging (Figures 2A and 3A), supero-inferior length and maximal transaxial area in a dimension perpendicular to the long axis of the rectum at the site of the cancer were registered.

### ERUS

A B-K Medical Falcon 2101 EXL, 3D-Probe, 13 MHz was used for the ERUS. The ERUS was performed by one of two investigators who had at least four years of experience. The preparation included an enema. The investigation was conducted in the left lateral position using a probe covered by a condom filled with water and introduced through a rigid proctoscope. The maximum length of one recording was 60 mm. The measurements were recorded in mm. The recordings were saved in the 3D-mode (Figures 2B and 3B).



**Figure 2** Images of a stage II (T3N0) rectal tumour. The numbers denote the bowel lumen (1), the submucosal layer (2) and the interface (3) between the muscularis layer and the perirectal tissue (5) and a definite protrusion (4) through the muscularis layer. A: Magnetic resonance imaging gives a good view of the surrounding structures; B: Endosonography (ERUS) shows the bowel lumen (1) expanded by a condom filled with water and details of the bowel wall with an interruption of the submucosal layer (2). The artefacts from an air pocket (6) and some bowel remnants (7) can be a problem in ERUS; C: The histologic slice (hematoxylin and eosin staining); D: The specimen after the first part of fixation and cutting.



**Figure 3** Images of a stage I (T2N0) rectal tumour. The numbers denote the bowel lumen (1), the submucosal layer at its interruptions (2) and the smooth interface (3) between the muscularis layer and the perirectal tissue (5). A: Magnetic resonance imaging gives a good view of the surrounding structures; B: Endosonography shows the bowel lumen (1) expanded by the probe (the black space inside the innermost white ring) and the condom filled with water (the black space between the probe and the bowel wall). The arrows show the middle white ring corresponding to the submucosal layer (2), in this case interrupted by tumour penetration beyond the submucosa into the muscularis propria. The interface (3) between the muscularis layer and the perirectal tissue was smooth as a sign of no penetration beyond the muscularis layer; C: The histologic slice (hematoxylin and eosin staining); D: The specimen after the first part of fixation and cutting.

**Table 2** Feasibility and accuracy of the tumour node metastasis staging, with stage II as the cut-off, using magnetic resonance imaging, endosonography and a combination (combo) of assessments of lymph nodes using magnetic resonance imaging and assessments of perirectal tissue penetration using endosonography compared with the histopathology of resected specimens in 23 patients before and after chemotherapy

|   | MRI-pre | ERUS-pre | Combo-pre | MRI-post | ERUS-post | Combo-post |
|---|---------|----------|-----------|----------|-----------|------------|
| Feasibility of stage                          | 1.00    | 0.81     |           |          |           |            |
| Feasibility of size                           | 0.97    | 0.74     |           |          |           |            |
| Accuracy of differentiating stages I / II     | 0.65    | 0.70     | 0.74      | 0.65     | 0.78      | 0.83       |
| Sensitivity of differentiating stages I / II  | 0.73    | 0.87     | 0.87      | 0.73     | 0.80      | 0.93       |
| Specificity of differentiating stages I / II  | 0.5     | 0.38     | 0.50      | 0.50     | 0.75      | 0.62       |
| Kappa value of differentiating stages I / II  | 0.23    | 0.26     | 0.40      | 0.23     | 0.53      | 0.58       |
| Overstaging of differentiating stages I / II  | 0.17    | 0.22     | 0.17      | 0.17     | 0.087     | 0.13       |
| Understaging of differentiating stages I / II | 0.17    | 0.087    | 0.087     | 0.17     | 0.13      | 0.048      |
| Accuracy of perirectal penetration            | 0.48    | 0.70     |           | 0.52     | 0.78      |            |
| Accuracy of lymph node detection              | 0.74    | 0.56     |           | 0.74     | 0.74      |            |

The last two columns present the accuracy of the prediction of perirectal tissue penetration and lymph nodes using magnetic resonance imaging (MRI) and endosonography (ERUS).

**Table 3** Tumour length (median and range in mm) and the area (median and range in mm) using magnetic resonance imaging and endosonography before and after chemotherapy in 18 patients

| Modality       | Before chemotherapy |                | After chemotherapy |                | Specimen Length |
|----------------|---------------------|----------------|--------------------|----------------|-----------------|
|                | Length              | Area           | Length             | Area           |                 |
| MRI            | 47 (36-68)          | 641 (283-1345) | 44 (19-68)         | 616 (156-1431) | Not applicable  |
| ERUS           | 46 (29-59)          | 680 (290-2110) | 41 (15-56)         | 595 (100-1380) | Not applicable  |
| Histopathology | Not applicable      | Not applicable | Not applicable     | Not applicable | 34 (12-70)      |

MRI: Magnetic resonance imaging; ERUS: Endosonography.

### Histopathology

The histopathological assessments of the resected surgical specimens were performed after fixation. The specimens were immersed in 4% formaldehyde for 48-72 h. The specimens were then cut (Figures 2D and 3D) and again immersed in formaldehyde for another 12 h before dehydration overnight. Then, the specimens were embedded in paraffin and sectioned. Routine staining was used (Figures 2C and 3C). The slides were analysed by specialist pathologists according to the everyday protocol utilized in the pathology department. The diameter in three directions and the TN staging were recorded. The histology report did not include an area measurement.

### Statistical analysis

The SPSS 18.0 statistics software was used for the analyses. The tumour stage (I-IV) was dichotomised into stage I and stages II-IV because this was a cut-off for the indication for radiotherapy at the time of the study. Accuracy was defined as the number of correct positive and negative predictions divided by the total. Sensitivity was defined as the number of correct predictions of stage II or higher divided by the number of stage II or higher based on pathology. Specificity was defined as the number of correct predictions of stage I or lower divided by the number of stage I or lower based on pathology. Understaging was defined as the number of incorrect predictions, given as stages that were too low (or too early), divided by the total. Staging with MRI and ERUS

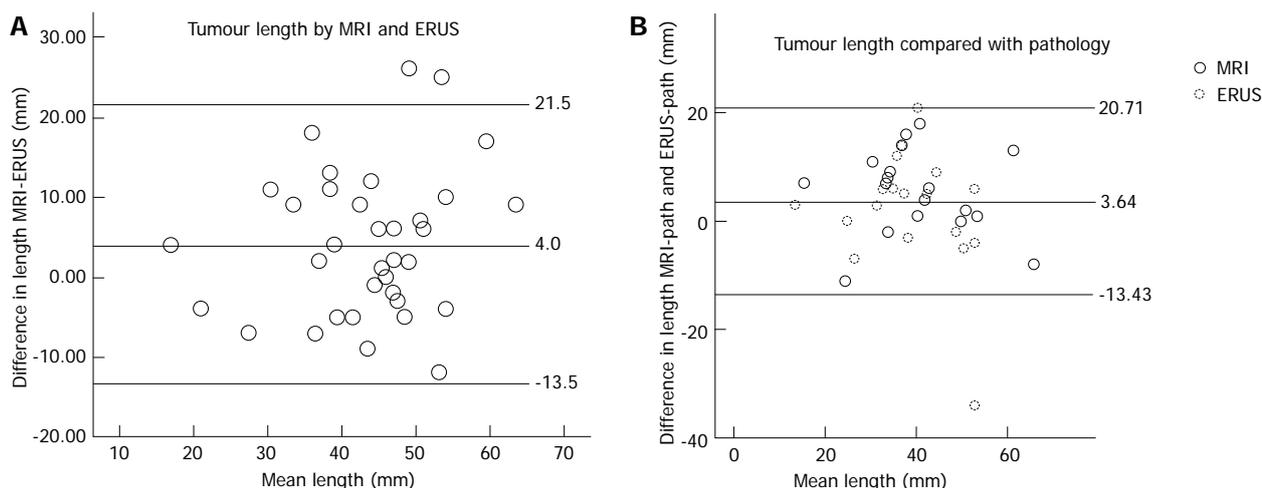
was compared with histopathology, which was regarded as the gold standard. The measurement correlations among MRI, ERUS and histopathology were shown in Bland-Altman plots, and a related samples comparison was applied to the correct stage predictions, lengths and areas by MRI, ERUS and histopathology using the non-parametric related samples Wilcoxon Signed Rank Test.

## RESULTS

Of the 37 eligible patients, 19 were assessed for size before chemotherapy, and 18 were assessed for size after chemotherapy using both MRI and ERUS within a period of four weeks (Figure 1). Another 10 patients were assessed for stage before chemotherapy, and five were assessed for stage after chemotherapy using both MRI and ERUS. The stage estimates are summarised in Table 2. A comparison of the supero-inferior length of the resected specimen by histopathology was conducted with the 18 post-chemotherapy measurements using MRI and ERUS. The size estimates are summarised in Table 3.

### Feasibility

The staging feasibility was 37 of 37 for MRI and 29 of 36 for ERUS. The feasibility of the size assessment was 36 of 37 for MRI and 25 of 34 for ERUS. The inability to lie still for approximately 40 min was the reason for noncompliance in the MRI and stenosis or pain on examination was the reason for noncompliance in the ERUS.



**Figure 4** Measurements of supero-inferior length using magnetic resonance imaging and endosonography. A: 37 pairs of measurements using both methods in the same patients before (19) and after (18) chemotherapy. The Bland-Altman plot illustrates the agreement between the methods. The reference lines are set at the mean difference and plus and minus 1.96 standard deviation from the mean difference; B: Measurements in 18 patients after chemotherapy compared with the resected specimens after fixation. The middle reference line shows the mean of differences in length between magnetic resonance imaging (MRI) and endosonography (ERUS) and pathology. The outer lines show plus and minus 1.96 the mean standard deviation from the mean difference.

In a number of patients, air pockets within the bowel lumen and remnants of bowel contents compromised the quality of ERUS but not of MRI (Figure 2A and B). The assessment of size demanded higher quality examinations than the assessment of stage for both MRI and ERUS. For example, relative stenosis could allow a sufficient view to assess a tumour as stage II or higher but did not allow for a precise assessment of size.

### Stage

The staging accuracy of the perirectal tissue penetration was 0.48 and 0.52 by MRI and 0.70 and 0.78 by ERUS before and after chemotherapy, respectively. The staging accuracy of the lymph node metastases was 0.74 and 0.74 by MRI and 0.56 and 0.74 by ERUS before and after chemotherapy, respectively.

The accuracy of the TNM staging, with stage II as the cut-off, by MRI, ERUS and the combined MRI and ERUS examinations was 0.65, 0.70 and 0.74, respectively, before chemotherapy and 0.65, 0.78 and 0.83, respectively, after chemotherapy. Thus, the post-chemotherapy staging by MRI alone was improved by a combination of MRI assessment of the lymph nodes and ERUS assessment of the perirectal tissue penetration ( $P = 0.046$ , Wilcoxon signed rank test). The post-chemotherapy understaging frequency by MRI, ERUS and the combined MRI and ERUS exams was 0.18, 0.14 and 0.045, respectively, but the differences were not significant.

### Size

For tumours smaller than 50 mm, measurements of the supero-inferior length by ERUS compared with MRI were within 1.96 standard deviations of the difference between the methods (18 mm). For all tumours, agreement with the histopathology of the resected specimen after fixation was within 1.96 standard deviations of the difference

between imaging and pathology for MRI (15 mm), and agreement with the histopathology of the resected specimen after fixation was within 1.96 standard deviations of the difference between imaging and pathology for ERUS (22 mm) for tumours that did not exceed 50 mm. There was an overestimation of the supero-inferior length by MRI ( $P = 0.016$ ) compared with histopathology and ERUS/histopathology ( $P = 0.021$ ). The ERUS measurement of tumour downsizing did concur ( $P < 0.05$ ) with the MRI size assessments. The measurements of length are shown by Bland-Altman plots in Figure 4.

The measurements of the maximal transaxial area by ERUS compared with MRI were within 1.96 standard deviations of the difference between the methods (684 mm<sup>2</sup>) for all tumours. An area measurement was not obtained by histopathology.

## DISCUSSION

The aim of this study was to assess the feasibility and accuracy of 1.5T MRI and 3D-ERUS compared with histopathology to determine the stage and size of rectal tumours. The staging feasibility was 37 of 37 for MRI but only 29 of 36 for ERUS. The accuracy of the staging with stage II as the cut-off was improved by combining the results of the lymph node assessments by MRI with the results of the perirectal tissue penetration by ERUS. Overstaging and understaging were observed in 17% of the patients by MRI both before and after chemotherapy. Overstaging could be explained by downstaging due to neoadjuvant therapy, but this does not explain understaging. Understaging with stage II as the cut-off means that the patients are likely to be incorrectly denied preoperative radiotherapy. Overstaging, in contrast, means that the patients will likely be given unnecessary radiotherapy. If a combination of MRI and ERUS could increase

the accuracy in patients on the cut-off edge between stages I and II, performing both examinations might be worthwhile, even if this approach increases the number of investigations.

The staging accuracy of both modalities was lower in this study than those reported in many other studies<sup>[33]</sup>. Still, only a fair agreement between MRI and histopathology was reported by Tytherleigh *et al.*<sup>[34]</sup>, and Harewood<sup>[35]</sup> showed that the ERUS staging was lower outside the initial studies. Ashraf reported that ERUS staging in “real world” practice in the United Kingdom is much lower than that shown in many studies<sup>[36]</sup>. These authors have concluded that the accuracy of MRI or ERUS can be expected to be lower in clinical practice than often reported from dedicated institutions. Another possible explanation for the low accuracy could be that the neoadjuvant chemotherapy treatment caused a desmoplastic reaction, making correct staging more difficult. A reduction in accuracy has been noted after neoadjuvant radiochemotherapy<sup>[13,32]</sup>. Another reason may be the overrepresentation of T2-T3 tumours, which are difficult to distinguish from each other using both MRI and ERUS<sup>[14,18]</sup>.

One central issue in preoperative staging is the possible presence of tumour-affected lymph nodes. This issue is important in standard resection surgery because the risk of node involvement affects the use of short-course preoperative radiotherapy before surgery<sup>[1]</sup>. Preoperative staging of lymph node involvement is certainly central when considering local resections and could be of great value when assessing patients who could benefit from a neoadjuvant chemotherapy regime. Several groups have found that MRI is the preferred method for lymph node involvement<sup>[37]</sup>, whereas ERUS is the preferred method for wall and perirectal tissue penetration<sup>[8,33]</sup>. However, Halefoglou *et al.*<sup>[12]</sup> reported that MRI can be better in more advanced stages, even in cases of wall penetration. Some authors have suggested that the two methods complement each other, which concurs with the findings of the present study<sup>[12,14,27,28]</sup>. As previously reported, MRI tends to be superior in the assessment of the lymph nodes, whereas ERUS tends to be superior in the assessment of the penetration of the bowel wall and perirectal tissue<sup>[11,27,28,33]</sup>; however, the possibility of increasing accuracy by combining the strengths of each modality in the same patient has not yet been reported to the best of our knowledge.

MRI has several advantages for use in the measurement of tumour size. Its feasibility in stenosing tumours and in patients who are sensitive to pain on introduction of the probe was important as in approximately 1/4 of the cases, these factors made ERUS measurements impossible. Furthermore, the probe used for ERUS provided a view that was 60 mm in supero-inferior length at most. Tumours larger than this could not be reliably measured with ERUS because of the necessity for multiple recordings. Thus, MRI had a better agreement with histopathology in the tumours with a supero-inferior length greater than 50 mm. The measurements of the supero-inferior tumour length by ERUS compared with MRI agreed

within 1.96 standard deviations (18 mm) for tumours up to 50 mm. Accepting, at most, an 18 mm deviation, ERUS can only replace MRI in the measurement of supero-inferior length for tumours up to approximately 50 mm in length. A deviation by 18 mm seems large, but even MRI deviated up to 15 mm compared with pathology. The measurement of the maximum transaxial area agreed within 1.96 standard deviations of the difference between the two methods (684 mm<sup>2</sup>) for all tumours. For ERUS, the transaxial area appears more reproducible than the supero-inferior length for large tumours.

Both MRI and ERUS overestimated the tumour length compared with histopathology. This difference is not explained by specimen, shrinkage as studies have indicated that the specimen slightly increases in size (up to 8%) due to fixation<sup>[38]</sup>. A more likely explanation could be that both MRI and ERUS overestimate length due to desmoplastic reactions, which may not be distinguished from the tumour itself<sup>[28]</sup>. Another explanation could be the short-course preoperative radiotherapy; however, this explanation is less likely because Brown *et al.*<sup>[38]</sup> found no stage effect. A theoretical cause of size difference could also be a further anti-tumoural effect from the chemotherapy administered in the Alimta study.

This study focused on the accuracy of the staging and size measurements. There were limitations to this study, including the low number of patients. However, this study does involve a special group of patients and includes information on the examinations before and after treatment, as well as histopathological data. Its other strengths are that all of the MRI measurements were performed by one radiologist and that the ERUS was performed by one of two senior surgeons. The measurement of the size of the rectal tumours by MRI compared with ERUS has not been extensively described previously. The increase in the staging accuracy using a combination of MRI and ERUS is interesting and could warrant further studies.

For staging rectal tumours, 1.5T MRI was more feasible than 3D ERUS because of a higher yield in stenotic tumours. The level of accuracy did not differ in the tumours assessed using both methods. A combination of lymph node assessment by MRI and assessment of perirectal tissue penetration by ERUS improved the staging accuracy, with stage II as the cut-off. For the measurement of tumour size, MRI is more feasible and accurate when large and stenotic tumours are included. However, in non-stenotic tumours, ERUS could replace MRI in the measurement of the transaxial area of all tumours and in the measurement of the length of tumours up to 50 mm.

## ACKNOWLEDGMENTS

We thank the Gothenburg Medical Association, the Lions Cancerfond Väst and the Björnsson Foundation for financial support. We would also like to express our gratitude to study nurse Hillevi Björkqvist for assisting in the collection and registration of data and samples and to

biomedical scientist Karin Blomqvist for helping in the preparation of the pathology images.

## COMMENTS

### Background

Magnetic resonance imaging (MRI) and endorectal ultrasound (ERUS) are the most widely used methods to assess the stage and size of rectal cancer to select the patients who benefit from neoadjuvant therapy. Lymph node metastasis, which is an indication for neoadjuvant therapy, is difficult to detect in early stages. Therefore, all patients with T3-tumours are often given neoadjuvant radiotherapy because a high rate of non-detectable lymph node metastases can be anticipated. Accurate preoperative assessment of both lymph node involvement and wall penetration is vital in this context. Likewise, reliable measurements of tumour size are important to monitor the effects of neoadjuvant therapy.

### Research frontiers

Several studies have shown that MRI is more accurate in staging of lymph nodes and the relation of the tumour margins to the surrounding structures, while ERUS is more accurate in staging of early wall penetration. Measurement of tumour size using either MRI or ERUS has been described but not in comparison to each other.

### Innovations and breakthroughs

In neoadjuvantly treated rectal cancer, a combination of lymph node assessment by MRI and assessment of perirectal tissue penetration by ERUS improved the staging accuracy, with stage II as the cut-off. Furthermore, this study showed that ERUS could replace MRI in the measurement of the trans-axial area of all non-stenotic tumours and in the measurement of the length of non-stenotic tumours up to 50 mm.

### Applications

To increase staging accuracy, MRI and ERUS may be combined in the same patient, paying more attention to the strengths of each method. A combined examination may especially be considered in tumours at the cut-off between stage I and II for more correct use of preoperative radiotherapy. To measure tumour size, MRI is better suited in large and stenotic tumours, while ERUS is a sufficient alternative in small tumours passable by proctoscopy.

### Terminology

MRI with 1.5 Tesla strength of the magnetic field is widely used in assessment of rectal cancer. Three-dimensional (3D) ERUS saves a 3D image, which may be assessed in several planes. 2D-versions allow assessment in one plane only. Neoadjuvant chemo- and/or radio-therapy is given before surgery to decrease the risk of tumour recurrence.

### Peer review

The paper compares imaging of neoadjuvantly treated rectal cancer using MRI and ERUS. Among weaknesses are a relatively small number of patients with many assessments lost. The strength of the paper is the application of everyday clinical practice in imaging of rectal cancer.

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E- Editor Li JY



## Low trypsinogen-1 expression in pediatric ulcerative colitis patients who undergo surgery

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Supported by Päivikki and Sakari Sohlberg Foundation, to Piekkala M; Helsinki University Central Hospital Grant, to Kolho KL; and the Finnish Pediatric Research Foundation, to Kolho KL  
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Received: November 20, 2012 Revised: April 15, 2013

Accepted: April 18, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate whether matrix metalloproteinases-9 (MMP-9) or trypsinogens could serve as histological markers for an aggressive disease course in pediatric ulcerative colitis (UC).

**METHODS:** We identified 24 patients with pediatric onset ( $\leq 16$  years) UC who had undergone surgery during childhood/adolescence a median of 2.1 years (range 0.1-7.4 years) after the diagnosis (between 1990 and 2008) in Children's Hospital, Helsinki, Finland. We also identified 27 conservatively treated UC patients and matched them based on their age at the time of diagnosis and follow-up at a median of 6 years (range 3-11 years) to serve as disease controls. Twenty children for whom inflammatory bowel disease (IBD)

had been excluded as a result of endoscopy served as non-IBD controls. Colon biopsies taken by diagnostic endoscopy before the onset of therapy were stained using immunohistochemistry to study the expression of MMP-9, trypsinogen-1 (Tryp-1), Tryp-2, and a trypsin inhibitor (TATI). The profiles of these proteases and inhibitor at diagnosis were compared between the surgery group, the conservatively treated UC patients and the non-IBD controls.

**RESULTS:** The proportions of Tryp-1 and Tryp-2 positive samples in the colon epithelium and in the inflammatory cells of the colon stroma were comparable between the studied groups at diagnosis. Interestingly, the immunopositivity of Tryp-1 (median 1; range 0-3) was significantly lower in the epithelium of the colon in the pediatric UC patients undergoing surgery when compared to that of the conservatively treated UC patients (median 2; range 0-3;  $P = 0.03$ ) and non-IBD controls (median 2; range 0-3;  $P = 0.04$ ). For Tryp-2, there was no such difference. In the inflammatory cells of the colon stroma, the immunopositivities of Tryp-1 and Tryp-2 were comparable between the studied groups at diagnosis. Also, the proportion of samples positive for TATI, as well as the immunopositivity, was comparable between the studied groups in the colon epithelium. In the stromal inflammatory cells of the colon, TATI was not detected. In UC patients, there were significantly more MMP-9 positive samples and a higher immunopositivity in the stromal inflammatory cells of the colon when compared to the samples from the non-IBD patients ( $P = 0.006$  and  $P = 0.002$ , respectively); the immunopositivity correlated with the histological grade of inflammation (95%CI: 0.22-0.62;  $P = 0.0002$ ), but not with the other markers of active disease. There were no differences in the immunopositivity or in the proportions of MMP-9 positive samples when examined by epithelial staining. The staining profiles in the ileal biopsies were comparable between the studied groups for all of the studied markers.

**CONCLUSION:** For pediatric UC patients who require surgery, the immunopositivity of Tryp-1 at diagnosis is lower when compared to that of patients with a more benign disease course.

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**Key words:** Children; Ulcerative colitis; Inflammatory bowel disease; Matrix metalloproteinase-9 resection; Trypsin inhibitor

**Core tip:** The risk factors for aggressive pediatric ulcerative colitis (UC) were studied in 24 patients who had undergone surgery by staining diagnostic tissue samples for matrix metalloproteinase-9 and trypsinogen-1 (Tryp-1) and Tryp-2, as well as a trypsin inhibitor. In the UC group, there were significantly more samples that were matrix metalloproteinase-9 positive in comparison to the samples from non-inflammatory bowel disease patients. UC patients undergoing colectomy showed lower immunopositivity of Tryp-1 in the colon epithelium in their diagnostic biopsies when compared to that of conservatively treated patients and non-inflammatory bowel disease patients. The discovery of a low trypsinogen level at diagnosis warrants further study.

Piekkala M, Hagström J, Tanskanen M, Rintala R, Haglund C, Kolho KL. Low trypsinogen-1 expression in pediatric ulcerative colitis patients who undergo surgery. *World J Gastroenterol* 2013; 19(21): 3272-3280 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i21/3272.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3272>

## INTRODUCTION

Inflammatory bowel disease (IBD) is a common name for Crohn's disease (CD), ulcerative colitis (UC), and unclassified colitis. Some patients may have only one episode of active disease and remain in remission for the rest of their lives, while others will suffer from a relapsing or continuously active disease and require colectomy<sup>[1-3]</sup>. The studied risk factors for an aggressive disease course (steroid dependency<sup>[4]</sup>, pancolitis<sup>[4,5]</sup>, extraintestinal manifestations<sup>[5]</sup>, severe disease at diagnosis<sup>[6]</sup>, or disease extension from the primary site of the active disease) are related to the clinical presentation and thus it is impossible to foresee the phenotype of the disease at diagnosis.

Since many pediatric UC patients present with aggressive disease during their disease course, it would be beneficial to find biomarkers that at the onset of disease could distinguish patients with complicated disease behavior and a high risk for surgery from those with a more benign course. The current opinion, however, is that immunosuppressive treatment is needed for a patient with a complicated disease. If it was possible to recognize an aggressive disease with biomarkers in the early disease phase, then

such patients could be introduced to immunosuppressive treatment aiming to improve the disease outcome. Previous pediatric data show the benefits of immunosuppressive treatment<sup>[7-9]</sup>, although biological treatment with a tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) antagonist has, disappointingly, not reduced the surgery rates<sup>[10]</sup>.

Matrix metalloproteinases (MMPs), a family of 24 zinc-dependent enzymes, comprise a group of proteinases<sup>[11]</sup> that degrade the extracellular matrix and basement membrane proteins in tissue remodeling processes both in normal and in pathological conditions<sup>[11]</sup>. In IBD the most abundantly expressed MMP is MMP-9<sup>[12]</sup>. Previously, we have shown that the immunopositivity of MMP-9 in the colon decreases after TNF- $\alpha$  antagonist treatment in adult CD<sup>[13]</sup>. Trypsinogens are serine proteases that are capable of degrading extracellular matrix proteins and the pro-forms of acute phase reaction proteins<sup>[14]</sup>, such as TNF- $\alpha$ , which cause damage to the mucosal barrier and UC-like inflammation<sup>[15]</sup>. The exact role of trypsinogens in IBD for the most part remains unknown, but it is known that trypsins activate promatrix metalloproteinases (proMMPs), especially the IBD-related proMMP-9<sup>[16-19]</sup>. Tumor associated trypsin inhibitor (TATI), also called pancreatic secretory trypsin inhibitor<sup>[20]</sup>, inhibits trypsin in a 1:1 molar ratio<sup>[21]</sup>. Extrapancreatically secreted TATI is assumed to play additional roles in ulcer healing and tissue regeneration<sup>[22]</sup>. TATI also takes part in preventing the excessive digestion of gastrointestinal (GI) mucus<sup>[22,23]</sup>. As with trypsinogens, the role of TATI in IBD-related inflammation is mostly unknown.

Matrix and serine proteases may be regarded as "regulators" of the barrier and inflammation cascade of the gut<sup>[14]</sup>. Accordingly, we hypothesized that the presence of expression of such proteases would be associated with the severity of the course of IBD. The hypothesis was tested by comparing the results of immunohistochemical stainings with MMP-9, trypsinogen-1 (Tryp-1), Tryp-2, and TATI on the biopsy material of pediatric patients who underwent surgery *vs* conservatively treated patients and subjects without IBD.

## MATERIALS AND METHODS

### Patients and controls

We reviewed all pediatric onset ( $\leq 16$  years old) UC patients from the IBD patient registry of Children's Hospital, Helsinki University Central Hospital who had been diagnosed between 1990 and 2008. From this database, we identified 24 UC patients who had undergone surgery (time from diagnosis to surgery, maximum 7 years) and 27 conservatively treated disease controls. The latter group had been diagnosed at the same age as the operated patients and the follow-up took place within the period of time that had elapsed between the diagnosis and the surgery in the index case. None of the disease controls underwent an operation during follow-up (median 6 years). All of the patients had undergone diagnostic ileocolonoscopy and upper gastrointestinal endoscopy,

**Table 1** Clinical data of the pediatric study groups at the time of diagnostic endoscopy

| Patient groups                                      | Surgery          | Disease controls | Non-IBD controls |
|---|------------------|------------------|------------------|
| No. of patients (male)                              | 24 (11)          | 27 (15)          | 20 (10)          |
| Age at diagnosis (yr), median (range)               | 13.1 (3.1-16.0)  | 12.1 (2.8-16.6)  | 13.5 (2.7-16.8)  |
| Time from diagnosis to surgery (yr), median (range) | 2.1 (0.1-7.4)    | -                | -                |
| Follow-up (yr), median (range)                      | -                | 6.3 (2.8-10.7)   | 1.0 (0.0-4.0)    |
| Endoscopic disease extension                        |                  |                  |                  |
| Proctitis   | 0                | 1                | -                |
| Left-sided colitis                                  | 9                | 6                | -                |
| Pancolitis  | 15               | 20               | -                |
| No inflammation                                     | -                | -                | 20               |
| Histological inflammation <sup>1</sup>              |                  |                  |                  |
| No inflammation                                     | -                | -                | 18               |
| Mild  | 6                | 10               | 1                |
| Moderate to severe                                  | 18               | 17               | -                |
| Laboratory markers, median (range)                  |                  |                  |                  |
| Hb (g/L)  | 112 (86-135)     | 116 (86-140)     | 131.0 (93-153)   |
| ESR (mm/h)  | 25 (7-61)        | 22 (7-63)        | 8 (2-23)         |
| CRP (mg/L)  | 20 (< 3-92)      | 9 (< 5-36)       | < 3 (< 3-< 5)    |
| WBC (E9/L)  | 9.6 (6.7-26.3)   | 7.5 (4.1-15.9)   | 5.1 (3.0-16.3)   |
| Alb (g/L)   | 34.6 (28.0-40.0) | 36.2 (23.6-42.6) | 42.3 (39.8-47.2) |

<sup>1</sup>For the non-inflammatory bowel disease (IBD) controls, data were missing in one case. Alb: Albumin; CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; Hb: Haemoglobin; WBC: White blood cell count.

and during follow-up the diagnosis remained consistent for the UC patients. Table 1 presents the background data of the study groups. The data on the indications and type of surgical therapy of the patients is shown in Table 2. Twenty children who had undergone ileocolonoscopy with biopsies and who did not suffer from IBD served as non-IBD controls. The indications for endoscopy in these children were as follows: suspected IBD ( $n = 12$ ), abdominal pain ( $n = 4$ ), colorectal bleeding ( $n = 3$ ), and pancreatic insufficiency ( $n = 1$ ).

For all of the patients and controls, tissue samples from the diagnostic ileocolonoscopy (colonic and ileal biopsies) were stained with immunohistochemistry to test for MMP-9, Tryp-1, Tryp-2, and TATI antibodies (see below). Based on the patient records, we reviewed the laboratory values of albumin, C-reactive protein (CRP), the erythrocyte sedimentation rate (ESR), the white blood cell count (WBC), and the haemoglobin at the time of the ileocolonoscopy.

### Inflammatory score and disease severity

The disease was considered to be acute and severe if the patient required hospitalization at diagnosis and the PU-CAI score was over 65<sup>[24]</sup>. The endoscopic distribution of the disease at the time of the diagnostic colonoscopy was scored as 0, indicating no inflammation, as 1 for proctitis, as 2 for left-sided colitis, and as 3 for pancolitis. The degree of histological inflammation was recorded based on the primary diagnostic histological examination reports provided by experienced pathologists. The samples

**Table 2** Surgical data for 24 pediatric patients with ulcerative colitis

|   |                 |
|---|-----------------|
| Age at first surgery (yr), median (range)           | 15.3 (5.5-19.8) |
| Time from diagnosis to surgery (yr), median (range) | 2.1 (0.1-6.6)   |
| Indication for primary operation                    |                 |
| Stricture   | 1 (perianal)    |
| Refractory disease to medication                    | 20              |
| Steroid-dependency                                  | 18              |
| No response to TNF-alpha antagonist                 | 2               |
| Fulminant colitis                                   | 3               |
| Primary operation                                   |                 |
| Colectomy (IRA)                                     | 1               |
| Proctocolectomy (IPAA)                              | 23              |

IPAA: Ileal pouch anal anastomosis; IRA: Ileorectal anastomosis; TNF: Tumor necrosis factor.

were graded according to the presence of ulceration, the frequency of acute and chronic inflammatory cells, crypt distortion, and goblet cell depletion recorded on a scale of 0 to 2 (0 no inflammation, 1 mild inflammation, 2 moderate to severe inflammation) (modified from Beattie *et al*<sup>[25]</sup>). The presence of backwash ileitis in the diagnostic ileocolonoscopy was also recorded.

### Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (4- $\mu$ m) were deparaffinized in xylene and rehydrated in graded concentrations of ethanol and water. To block endogenous peroxidase activity, the sections were treated with 0.3% Dako REAL Peroxidase-Blocking Solution (DAKO, Glostrup, Denmark) for 5 min. For the antigen retrieval tissue, the sections were treated in the pretreatment module of the Autostainer (LabVision UK Ltd) in a Tris-HCl buffer (pH 8.5) for 20 min at 98 °C (Tryp-1 and MMP), a 0.1% pepsin solution for 15 min at 37 °C (Tryp-2), or a 0.01% trypsin solution for 30 min at 37 °C (TATI). After pretreatment, a Lab Vision Autostainer TM 480 (LabVision, Fremont, CA, United States) was used for immunohistochemistry. The sections were incubated with antibodies against Tryp-1 at a dilution of 1:500 (MAB 1482, Chemicon, Temecula, CA, United States), against MMP-9 (RB-1539-PO, LabVision Fremont, CA) at a dilution of 1:1000, and against Tryp-2 (8F7)<sup>[26]</sup> and TATI (6E8)<sup>[27]</sup> at dilutions of 1:200 and 1:500, respectively. The incubation times were one hour for Tryp-1, MMP-9, and TATI and overnight for Tryp-2. Subsequently, the sections were incubated with a peroxidase-conjugated Dako REAL EnVision/HRP, Rabbit/Mouse (ENV) reagent for 30 min. The final visualization was performed by incubating the sections in DAKO REAL DAB+ Chromogen for 10 min. Between the staining steps, the slides were washed with 0.04% PBS. Mayer's hematoxylin was used for counterstaining. Finally, the sections were rinsed in tap water for 10 min and mounted in an aqueous mounting medium (Aguamount, BDH, Poole, United Kingdom). As positive controls, we used formalin-fixed, paraffin-embedded sections of pancreatic papillary carcinoma (Tryp-1), pancreatic adenocarcinoma (Tryp-2 and TATI), and ventricular adenocarcinoma (MMP-9). A

**Table 3** Semi-quantitative immunopositivity (score 0 to 3) and number of positive samples in the diagnostic colonic tissue samples of pediatric ulcerative colitis patients and non-inflammatory bowel disease patients

|   | Surgery<br>( <i>n</i> = 24) | Conservative treatment<br>controls ( <i>n</i> = 27) | Non-IBD controls<br>( <i>n</i> = 20) | <i>P</i> value<br>Surgery <i>vs</i> conservative treatment controls | <i>P</i> value<br>Surgery <i>vs</i> non-IBD controls |
|---|-----------------------------|---|--------------------------------------|---|--|
| Number of positive samples <i>n</i> (%) |                             |   |                                      |   |  |
| Tryp-1                                  |                             |   |                                      |   |  |
| Epithelium                              | 13 (57)                     | 19 (76)   | 15 (79)                              | 0.22  | 0.19   |
| Stroma; inflammatory cells              | 11 (48)                     | 8 (32)  | 5 (26)                               | 0.38  | 0.21   |
| Tryp-2                                  |                             |   |                                      |   |  |
| Epithelium                              | 21 (95)                     | 21 (81)   | 18 (95)                              | 0.20  | 0.22   |
| TATI                                    |                             |   |                                      |   |  |
| Epithelium                              | 18 (78)                     | 24 (92)   | 15 (75)                              | 0.23  | 1.00   |
| MMP-9                                   |                             |   |                                      |   |  |
| Epithelium                              | 4 (18)                      | 2 (8)   | 0 (0)                                | 0.40  | 0.17   |
| Stroma; inflammatory cells              | 11 (50)                     | 12 (48)   | 1 (5)                                | 1.00  | 0.006 <sup>1</sup>                                   |
| Median immunopositivity                 |                             |   |                                      |   |  |
| Tryp-1                                  |                             |   |                                      |   |  |
| Epithelium                              | 1.0                         | 2.0   | 2.0                                  | 0.03 <sup>2</sup>   | 0.04 <sup>2</sup>                                    |
| Stroma; inflammatory cells              | 0.0                         | 0.0   | 0.0                                  | 0.16  | 0.10   |
| Tryp-2                                  |                             |   |                                      |   |  |
| Epithelium                              | 1.0                         | 1.0   | 1.0                                  | 0.56  | 0.38   |
| TATI                                    |                             |   |                                      |   |  |
| Epithelium                              | 1.0                         | 1.0   | 1.0                                  | 0.81  | 0.97   |
| MMP-9                                   |                             |   |                                      |   |  |
| Epithelium                              | 0.0                         | 0.0   | 0.0                                  | 0.33  | 0.17   |
| Stroma; inflammatory cells              | 0.5                         | 0.0   | 0.0                                  | 0.89  | 0.003 <sup>3</sup>                                   |

<sup>1</sup>Both UC patient groups had significantly more positively stained inflammatory cells in their colonic epithelium when compared to the non-IBD controls;

<sup>2</sup>The patients who had undergone surgery had significantly lower immunopositivity when compared to the conservatively treated and non-IBD controls;

<sup>3</sup>Both UC patient groups had significantly higher immunopositivity when compared to the non-IBD controls. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; TATI: Trypsin inhibitor; Tryp-2: Trypsinogen-2; MMP-9: Matrix metalloproteinase-9.

tissue sample without a primary antibody served as a negative control during each staining.

### Interpretation of staining results

The immunohistochemical specimens were scored independently by two investigators (Piekkala M and Hagström J) in a semi-quantitative fashion under a light-field microscope at  $\times 100$  magnification using a marking and staining immunopositivity scale. This was done as follows: negative immunoreactivity was scored as 0, a diffuse weak positivity was scored as 1, a moderately positive or focally strongly positive immunopositivity was scored as 2, and a homogeneously strong immunopositivity was scored as 3 (modified from Böckelman *et al.*<sup>[28]</sup>). An experienced pathologist (Hagström J) confirmed the identity of the cell types producing each Tryp-1, Tryp-2, TATI, or MMP-9.

### Ethics

The National Supervisory Authority for Welfare and Health gave its permission for use of the tissue samples. In accordance with Finnish regulations, no informed consent is required for this kind of study.

### Statistical analysis

Because of the small number of patients, the non-parametric Mann-Whitney's test and Kruskal-Wallis test were used to compare the significance of the differences in the level of immunopositivities between the studied groups. An independent samples *t* test (Spearman), and Fisher's

exact test were performed to investigate the significance of the association between the level of immunopositivities and the clinical markers. A  $P < 0.05$  was considered significant.

## RESULTS

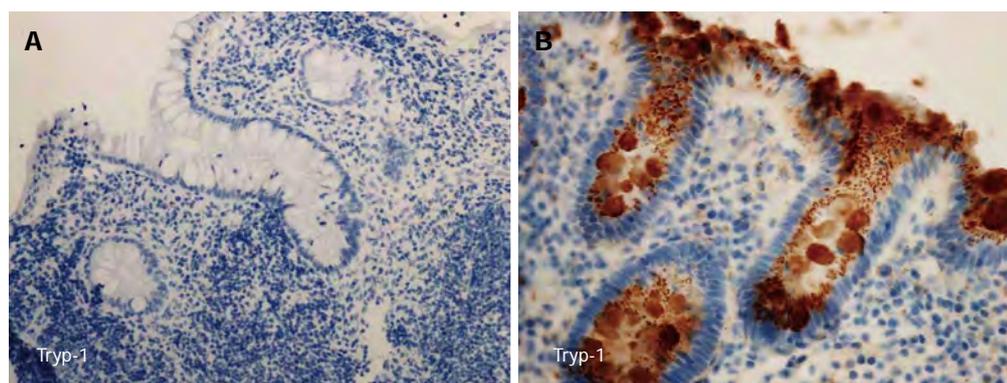
### Disease severity and distribution and medication

The endoscopic distribution of inflammation was comparable between the UC groups ( $P = 0.7$ ). During the diagnostic biopsies, we detected mild inflammation in 16 of 51 (31%) and moderate to severe inflammation in 35 of 51 UC patients (69%), without major differences between surgical and conservative groups ( $P = 0.2$ ). Of the 24 patients requiring surgery (median two years after diagnosis), 46% ( $n = 11$ ) had an acute severe disease at diagnosis. This was significantly more than in the disease controls (15%,  $n = 4$ ) ( $P = 0.03$ ). In total, 15 of 51 UC patients (30%) had an acute severe disease at diagnosis (73% of them underwent surgery).

### Staining results in the colon

Table 3 shows the proportions of Tryp-1, Tryp-2, TATI, and MMP-9 positive samples, the median levels of immunopositivity, and the *P* values when comparing the proportions of positive samples and the level of immunopositivity between the surgery group and the conservatively treated or non-IBD groups.

**Tryp-1:** Tryp-1 was stained in the colon epithelium and



**Figure 1** Immunohistochemistry for trypsinogen-1. A: Lack of immunopositivity for trypsinogen-1 (Tryp-1) in a colonic sample at the diagnosis of a pediatric patient with ulcerative colitis who was operated on two years after diagnosis ( $\times 200$ ); B: Immunopositivity for Tryp-1 in the colonic epithelial cells of a diagnostic tissue sample from a conservatively treated patient with seven years follow-up from the diagnosis ( $\times 400$ ).

in the inflammatory cells of the stroma. The staining of Tryp-1 was granular. The immunopositivity in the colon epithelium was significantly lower in the samples from the surgery group (Figure 1A) when compared to that of the conservatively treated (Figure 1B) and non-IBD controls (Table 3). No difference in the immunopositivity of the colon epithelium was found between the conservatively treated and non-IBD controls. The proportion of positive samples and the level of immunopositivity in the inflammatory cells of the stroma were similar in the studied groups (Table 3).

**Tryp-2:** Tryp-2 was stained in the epithelium. All of the samples, regardless of the study group, had the same level of Tryp-2 immunopositivity in the inflammatory cells of the colon stromal tissue (Figure 2A and B). The proportion of positive samples and the level of immunopositivity in the epithelium were comparable between the surgery group, the conservatively treated, and non-IBD controls (Table 3). In total, 83% (39 of 47) of the samples that were Tryp-1 positive in the colon epithelium were also Tryp-2 positive.

**TATI:** TATI was only detected in the colon epithelium, the inflammatory cells of the colon stroma being negative for TATI (Figure 2C and D). The proportion of TATI positive samples and the level of immunopositivity were comparable between the surgery group and the conservative and non-IBD groups (Table 3). The immunopositivity of TATI did not relate to the expression of Tryp-1 or Tryp-2 (data not shown).

**MMP-9:** In the colon, MMP-9 was mainly found in the stromal inflammatory cells and positive staining was detected in the epithelium (Figure 2E and F). The proportion of MMP-9 immunopositive samples and the level of immunopositivity in both the inflammatory cells and the epithelium were similar for the surgery group and the conservative group (Table 3). When compared to non-IBD patients, the proportion and immunopositivity of MMP-9 in the inflammatory cells was higher in the

UC patients, but we detected no differences when considering the epithelial positivity (Table 3). There was no association between the expression of MMP-9 and the expression of Tryp-1, Tryp-2, or TATI (data not shown).

#### Staining results in the ileal samples

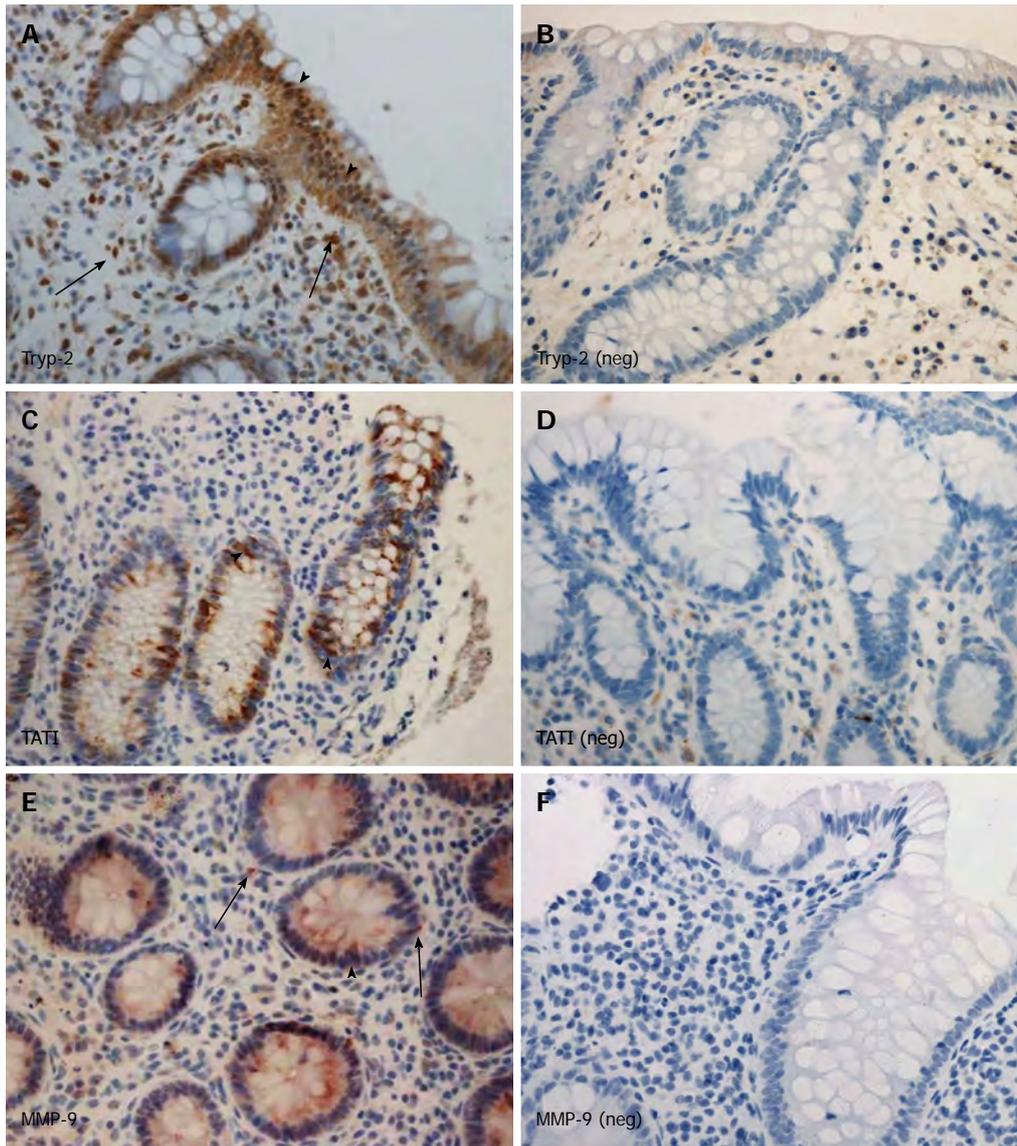
In 59% of the ileal samples (available for 36 UC patients and 14 non-IBD controls), the epithelium was Tryp-1 positive. Of the inflammatory cells in the stroma, 33% were Tryp-1 positive. The comparative staining proportions were 100% for Tryp-2 (positivity of the epithelium and inflammatory cells), 82% for TATI (positivity of the epithelium, inflammatory cells negative) and 28% and 26% for MMP-9 (positivity of the epithelium and of the inflammatory cells of the stroma, respectively). The proportions of positively stained samples for Tryp-1, Tryp-2, and TATI were comparable between the studied groups. All UC patients, regardless of treatment outcome, had significantly more MMP-9 positivity in the ileal epithelium than non-IBD patients ( $P = 0.005$ ). The staining intensities of the studied markers were similar in all of the study groups (data not shown).

#### Association between disease activity and immunohistochemistry

The immunopositivity of MMP-9 in the inflammatory cells was positively associated with the grade of inflammation in the colon (95%CI: 0.22-0.62;  $P = 0.0002$ ). However, there was no association with MMP-9 immunopositivity and the other markers of active disease. The staining intensities of the other markers in the epithelium of the colon or in the inflammatory cells of the stroma did not associate with the initial disease severity at diagnosis, the grade of inflammation at diagnosis, the endoscopic distribution of the disease at diagnosis, or the presence of backwash ileitis.

#### Laboratory markers

All of the primary laboratory values in the surgery group and disease controls were comparable. The blood inflammatory markers (CRP, ESR, WBC) were significantly



**Figure 2** Trypsinogen-2 was stained in the epithelium. A: Immunopositivity for trypsinogen-2 (Tryp-2); B: Immunonegativity for Tryp-2; C: Immunopositivity for tumor associated trypsin inhibitor (TATI); D: Immunonegativity for TATI; E: Immunopositivity for matrix metalloproteinase-9 (MMP-9); F: Immunonegativity for MMP-9. Arrows (A, E): Immunopositivity in the stromal inflammatory cells; Arrowheads (A, C, E): Immunopositivity in the epithelial cells. A-F,  $\times 400$ .

higher and the albumin level lower in UC patients compared to the non-IBD controls (data not shown).

## DISCUSSION

The exact roles of the active inflammation markers trypsinogens, TATI, and MMP-9 have not been studied in pediatric UC patients in relation to the disease outcome. Therefore, we compared the immunohistochemical positivity of Tryp-1, Tryp-2, TATI, and MMP-9 in diagnostic colonoscopic tissue samples from pediatric UC patients who required surgery with those of conservatively treated patients and non-IBD controls.

For the first time serine proteases Tryp-1 and Tryp-2 were demonstrated in the epithelium and in the inflammatory cells of the colon and ileum and TATI was found in the epithelium of the colon and ileum in pediatric UC

patients. At the time of the diagnosis, the immunopositivity of Tryp-1 in the colon epithelium is lower when comparing patients that have aggressive disease requiring surgery with those who have conservative treatment. We could also confirm that MMP-9 expression correlated with the grade of inflammation of the colon similarly, as reported earlier in adult and pediatric IBD patients<sup>[12]</sup>.

### Trypsinogens in pediatric UC

It is known that serine proteases regulate the gut barrier and inflammation cascade<sup>[14]</sup>, and that they also take part in protease-activated receptor signaling, which has been shown to activate intestinal inflammation<sup>[29]</sup>. Thus, our finding of a low Tryp-1 level in the colon epithelium of the surgery group is important because as many as 75% of the patients had more than mild inflammation and nearly half had acute severe disease at the time of diag-

nosis. Acute inflammation is, however, not the only background factor explaining the low expression of Tryp-1 in patients undergoing surgery; in addition, the immunopositivity did not associate with the degree of inflammation at diagnosis. The expression of Tryp-2 was also low in all of the studied groups. These findings, when taken together, indicate that trypsinogens are weakly expressed in the colon and not upregulated by inflammation in UC patients. Since they are also expressed in the healthy colon, their role might be mainly physiological. It may be speculated that severe inflammation suppresses the physiological secretion rate of Tryp-1 but not Tryp-2 and TATI. At the moment, however, the exact role of trypsins Tryp-1 and Tryp-2 in intestinal inflammation remains unknown.

### **Trypsin inhibitor TATI**

In healthy subjects proteases are expressed in the GI tract at low concentrations. An increase in concentrations is regarded as destructive; thus the balance between the proteases and their inhibitors is considered important<sup>[14]</sup>. It is known that TATI inhibits both Tryp-1 and Tryp-2 equally<sup>[21]</sup>. We found no correlation between the expression of the major trypsin inhibitor TATI and trypsinogens. Since the expression of TATI was low, the weak expression of trypsinogens could not be explained by the upregulation of TATI. The low expression of TATI in pediatric UC patients is in line with earlier findings showing reduced intestinal TATI in IBD and other GI conditions associated with abnormalities in the internal mucous layer<sup>[30]</sup>. However, as TATI and trypsinogens were also expressed in healthy colon, their low expression might be physiological.

### **MMP-9 association with trypsinogens**

This study reports for the first time together the expression of MMP-9 and trypsinogens in pediatric UC patients. However, the immunopositivity of MMP-9 had no association with that of Tryp-1 or Tryp-2. However, the small sample size in the present study has to be considered, and this finding does not either contradict or support the earlier report that trypsins associate with proMMP-9<sup>[16-19]</sup>. MMP-9 is expressed in the neutrophils but not in the colon epithelium in pediatric IBD<sup>[31]</sup>. In the present study we showed, in addition to the upregulated MMP-9 expression in the inflammatory cells, epithelial immunopositivity for MMP-9 as well. This may be a novel finding as we validated our staining with care to avoid any unspecific staining reactivity. Of the MMP inhibitors (TIMPs), TIMP-3 is expressed in pediatric IBD<sup>[14,30,32,33]</sup>, and it correlates with the degree of inflammation<sup>[30]</sup>. Unfortunately, the TIMP levels could not be assessed in this study.

### **Strengths and limitations of the study**

The aim of the study was to compare two pediatric UC groups: those who would undergo surgery during childhood or adolescence and patients whose disease course is more benign. Although the patient population in this study is quite small, one strength of the study was that the

patient age and disease extension at diagnosis were comparable. Also, the duration of disease of patients who had undergone surgery and the disease controls was matched. Restorative proctocolectomy was performed at a median of 2 years after diagnosis (median age 15 years), and all the patients were followed-up at least until that time. In total, the disease controls were followed up for a median of 6 years and none of them had undergone surgery during this time period. We also had control samples from non-IBD children, which demonstrated the natural expression of studied markers in non-inflammatory bowel disease.

In terms of the limitations of the results, we could only use one method for assessing trypsinogens, TATI, and MMP-9 profiles because the biopsy samples were very small and did not allow further analysis. Using molecular biological methods such as reverse transcription-polymerase chain reaction or Western blotting to analyze the expression of the studied markers would have given more strength to the study. However, we had the unique possibility to study the diagnostic samples and relate the data to the disease course of the patients. Furthermore, two investigators blinded to the patient data assessed the samples. The discovery of a low Tryp-1 level related to an aggressive disease course is preliminary and was not related to the altered expression of its major inhibitor, TATI. Thus, the physiological mechanisms underlying these findings require further studies.

In conclusion, significant proportions of pediatric UC patients suffer from aggressive disease and require surgery. It is estimated that approximately one in four pediatric patients undergo colectomy during the first decade after the diagnosis<sup>[2,3,34]</sup>. No change in the surgery rate has been seen between the years 1994 and 2007<sup>[55]</sup>. By current diagnostic modalities, it is impossible to foresee the disease outcome at the time of the diagnosis of UC, although an initial aggressive disease increases the risk of colectomy<sup>[6]</sup>. We found the expression of Tryp-1 to be weaker in the epithelial cells of patients with aggressive disease but the mechanism and significance of that finding remained unclear. Therefore, there is a need for further investigations on Tryp-1 and other biomarkers to identify patients with aggressively proceeding UC.

## **ACKNOWLEDGMENTS**

The authors thank Ms Anne Nikkonen for her excellent help in collecting the patient data and Ms Päivi Peltokangas for her skilful technical assistance.

## **COMMENTS**

### **Background**

Significant proportions of pediatric patients with ulcerative colitis (UC) suffer from aggressive disease and require surgery. It is estimated that approximately one out of four pediatric patients undergo colectomy during the first decade after the diagnosis. No change in the surgery rate was observed between the years 1994 and 2007.

### **Research frontiers**

By current diagnostic modalities, it is impossible to foresee the disease out-

come at the time of the diagnosis of UC, even though an aggressive initial disease presentation increases the risk of colectomy. If it was possible to recognize an aggressive disease course by biomarkers in the early disease phase, such patients could be introduced to early immunosuppressive treatment aimed at improving the disease outcome. The role of matrix metalloproteinase-9 (MMP-9) is scarcely reported in pediatric inflammatory bowel disease (IBD). Trypsinogens and a trypsin inhibitor (TATI) have not been studied at all in pediatric UC patients. Accordingly, the authors hypothesized that the expression of such proteases would be associated with the severity of the course of the UC. The hypothesis was tested by comparing the results of immunohistochemical staining on the diagnostic biopsy material of pediatric patients who underwent surgery vs conservatively treated patients and subjects without inflammatory bowel disease.

### Innovations and breakthroughs

For the first time, the presence of the serine proteases trypsinogen-1 (Tryp-1) and Tryp-2 was demonstrated in the epithelium and in the inflammatory cells of the colon and ileum in pediatric UC. Accordingly, this is the first study to describe the expression of their inhibitor, TATI, in the epithelium of the colon and in the ileum in pediatric UC patients. The authors found that at the time of the diagnosis, the immunopositivity of Tryp-1 in the colon epithelium is lower when comparing pediatric UC patients who have an aggressive disease course requiring surgery to those managed by non-operative treatment. The authors could also confirm that the expression of MMP-9 correlated with the grade of inflammation of the colon similarly, as reported earlier in adult and pediatric IBD patients.

### Applications

By demonstrating the protease profile of the colon mucosa of pediatric patients who will suffer from aggressive UC, this study may aid experts in characterizing the molecular environment of aggressive UC. The final goal is to find clinically applicable biomarkers for an aggressive disease.

### Terminology

UC is a chronic inflammatory disease of the colon and the rectum that is of unknown origin; it is characterized by bloody diarrhea, abdominal pain, and weight loss; MMP, a family of 24 zinc-dependent enzymes, comprise a group of proteinases that degrade the extracellular matrix and basement membrane proteins in tissue remodeling processes both in normal and in pathological conditions; Tryps are serine proteases that are capable of degrading extracellular matrix proteins and the pro-forms of acute phase reaction proteins, such as tumor necrosis factor, which cause damage to the mucosal barrier and UC-like inflammation. Tumor associated TATI inhibits trypsin in a 1:1 molar ratio.

### Peer review

This is an interesting study delineating the colonic expression of key proteases in the setting of paediatric UC.

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P-Reviewer Day AS S-Editor Huang XZ L-Editor O'Neill M  
E-Editor Li JY



## Possible ameliorative effect of breastfeeding and the uptake of human colostrum against coeliac disease in autistic rats

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Supported by The King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia; the NPST Health Research and Studies program at King Saud University

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Received: December 28, 2012 Revised: March 28, 2013

Accepted: April 27, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To examine the possible ameliorative effect of breastfeeding and the uptake of human colostrum against coeliac disease in autistic rats.

**METHODS:** Female rats were fed a standard diet and received a single intraperitoneal injection of 600 mg/kg sodium valproate on day 12.5 after conception. In study 1, neonatal rats were randomly subjected to blood tests to investigate autism. In study 2, the 1<sup>st</sup> group was fed by the mother after an injection of interferon- $\gamma$  (IFN- $\gamma$ ) and administration of gliadin. The pups in the 2<sup>nd</sup> group were prevented from accessing maternal milk, injected IFN- $\gamma$ , administered gliadin, and hand-fed human colostrum. The normal littermates fed by the table mothers were injected with physiological saline and served as normal controls in this study.

**RESULTS:** The protein concentration was higher in group 2 than in group 1 in the duodenum ( $161.6 \pm 9$  and  $135.4 \pm 7$  mg/g of tissue, respectively,  $P < 0.01$ ). A significant increase ( $P < 0.001$ ) in body weight was detected in human colostrum-treated pups on post natal day (PND) 7 and 21 *vs* suckling pups in group 1. A delay in eye opening was noticed in the treated rats in group 1 on PND 13 compared with the control group and group 2. Administration of a single intraperitoneal injection of 600 mg/kg sodium valproate on day 12.5 after conception resulted in significantly reduced calcium and vitamin D levels in study 1 compared with the control groups ( $P < 0.001$ ). However, human colostrum uptake inhibited increases in the level of transglutaminase antibody in autistic pups with coeliac disease.

**CONCLUSION:** The effects of early-life nutrition and human colostrum on the functional maturation of the duodenal villi in autistic rats with coeliac disease that might limit or prevent the coeliac risk with autism.

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**Key words:** Autism; Breastfeeding; Coeliac disease; Human colostrum

**Core tip:** Research examining the potential benefits of using breastfeeding and/or human colostrum for a wide range of gastroenterologic conditions of coeliac disease in autistic rats which is never studied before. Early results are encouraging and we envisage the standard use of human colostrum in the clinical management of gastrointestinal diseases within the next decade.

Selim ME, Al-Ayadhi LY. Possible ameliorative effect of breastfeeding and the uptake of human colostrum against coeliac disease in autistic rats. *World J Gastroenterol* 2013; 19(21): 3281-3290 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Two retrospective studies, which analysed representative populations of children with autism, have reported gastrointestinal tract (GI) symptoms in 20% of young children previously diagnosed with autism<sup>[1]</sup>. In contrast, prospective reports from paediatric gastroenterology and general autism clinics have described GI symptoms in 46%-84% of patients with autism spectrum disorder (ASD)<sup>[2]</sup>. However, there are few prevalence estimates from population-based epidemiologic studies. Reported GI abnormalities include low activities of disaccharidase enzymes, defective sulphation of ingested phenolic amines (Tylenol), bacterial overgrowth with a greater diversity and number of clostridial species, more numerous Paneth cells, increased intestinal permeability, and positive effects on behavioural cognition following dietary intervention<sup>[3]</sup>. Coeliac disease (CD) exhibits a multifactorial aetiology, and both genetic and environmental factors contribute to disease development. CD is a human leukocyte antigen (HLA)-associated disorder, and the majority of CD patients express HLA-DQ2 and HLA-DQ8 to a lesser extent<sup>[4]</sup>. Disease development is a consequence of the ingestion of immunogenic fragments in gluten by genetically predisposed subjects<sup>[5]</sup>. The treatment of CD involves a lifelong diet, which may cause difficulties, because avoiding gluten completely is almost impossible. However, over the last few years, new studies have suggested that prevention of CD may be possible<sup>[6]</sup>. The statement "prevention is better than a cure" is desirable for every disease, but is especially true for CD. Primary prevention avoids the development of a disease. In CD, primary prevention aims to avoid CD by intervening before the onset of the initial disease processes. In this context, we will outline the rationale for primary prevention in CD, which is based on factors contributing to CD and the possibility to improve tolerance to (food) allergens. Breastfeeding and colostrum provide immunological integration between the mother and neonate. Breastfeeding has immunological advantages, which allow it to prevent infections. Moreover, there is evidence that breastfeeding protects against cardiovascular disorders, obesity, Crohn's disease, colitis ulcerosa, allergies, Diabetes mellitus type I and other (autoimmune) disorders such as CD<sup>[7]</sup>. The risk of these disorders could increase if the duration of breastfeeding is less than 3-6 mo<sup>[8]</sup>. During the lactation period, breast milk undergoes three different phases with different milk compositions: colostrum, first milk and mature milk<sup>[9]</sup>. Colostrum is the first milk produced after birth and is particularly rich in immunoglobulins, antimicrobial peptides (*e.g.*, lactoferrin and lactoperoxidase), and other bioactive molecules, including growth factors. Colostrum is important for the nutrition, growth, and development of new-born infants

and contributes to the immunologic defence of neonates. The composition of mammary secretions changes continuously throughout the suckling period; however, for the purposes of this research, we define colostrum as the milk produced in the first 48 h after birth. Breast milk contains all of the immunoglobulins (IgA, IgE, IgG, IgD and IgM)<sup>[10]</sup>. However, for new-borns, the most important immunoglobulins are IgA and IgG. IgA, specifically secretory IgA, provides mucosal defence, and IgG antibodies support tissue defence. Several studies describe the detection of wheat gliadins and other gluten peptides in breast milk along with specific IgA-antibodies against gliadin<sup>[11]</sup>.

The low levels of gluten in breast milk could potentially be involved in the induction of oral tolerance to gluten in breastfed infants. The concentration of IgA anti-gliadin is highest in colostrum and decreases after one month<sup>[11]</sup>. Many studies have been published regarding the preventive effect of breastfeeding in CD. An important systematic review and meta-analysis of observational studies on breastfeeding and CD by Akobeng *et al.*<sup>[12]</sup> concludes that breastfeeding offers protection against the development of CD. However, it is unclear whether breastfeeding permanently protects against the development of CD or whether it only delays the onset of symptoms<sup>[11]</sup>. The mechanism of protection against CD by breast milk is not well understood. Hanson *et al.*<sup>[9]</sup> suggested that breastfeeding modulates the early exposure of the neonate's intestinal mucosa to microbes and limits bacterial translocation through the gut mucosa. In addition, by preventing inflammation in the gut, breastfeeding also diminishes the passage of gluten peptides into the lamina propria and prevents the development of CD<sup>[10]</sup>. Another possible preventive mechanism is that human colostrum may decrease tissue transglutaminase expression in the gut and diminish the generation of deaminated gluten peptides<sup>[13]</sup>. The immune-modulating properties of human colostrum may also be exerted through a T-cell-specific suppressive effect, which has been shown in experiments on peripheral lymphocytes stimulated with phytohaemagglutinin, OKT3 and allo-antigens<sup>[11]</sup>. Thus, human colostrum may be an ideal antimicrobial for selective targeting in the gastrointestinal tract in autistic rats with coeliac disease.

## MATERIALS AND METHODS

### *Rat model of autism and CD*

Female Wistar rats with controlled fertility cycles were mated overnight. The morning when spermatozoa were found was designated as the first day of gestation. The females were fed a standard diet and received a single intraperitoneal injection of 600 mg/kg of sodium valproate on day 12.5 after conception. The control females were fed a normal standard diet and injected with physiological saline. Sodium valproate (Sigma) was dissolved in saline at a concentration of 250 mg/mL. Administration of this dose of sodium valproate to rats during

embryogenesis has been shown to result in a maximum level of total VPA (900 µg/mL) in maternal plasma in less than 1 h with a mean plasma elimination half-life of 2.3 h<sup>[14]</sup>. The dams were housed individually and were allowed to raise their own litters. To induce coeliac disease, autistic male neonatal rats from valproate-treated females were divided into 2 experimental groups (twenty pups/group) and treated with interferon-γ (IFN-γ) (1000 U per animal administered intraperitoneally) after birth. Gliadin (0.5 and 3 mg) was intragastrically administered to the pups of both groups on day 0 and 3, and a 30 mg challenge dose was administered on day 20 (24 h before the termination of the experiment)<sup>[15]</sup>. The control pups from control females received physiological saline until the experiment was terminated.

### Animals

In study 1, male Wistar neonatal rats were randomly selected from the above experimental groups before the treatment and were subjected to blood tests to investigate autism. Study 2 consisted of two experimental groups of new-born male autistic rats. The pups in group 1 were randomly assigned to be mother-fed after injection of IFN-γ and administration of gliadin. The pups in group 2 were collected from the mothers immediately after birth to prevent suckling of maternal milk. No foster nursing took place because the major objective was to study pup viability. The animals were injected with IFN-γ, administered gliadin, placed in an infant incubator to control body temperature and hand-fed human colostrum every 3-4 h using a silicone rubber tube. The method was both time and labour intensive; however, the method was essential because gastrotomy of new-born rats is associated with a very high surgery-related death rate. Normal littermates fed by control mothers were injected with physiological saline and served as normal non-treated controls in this study.

### DNA and protein measurements on post natal day 21

The total DNA content in tissue was assayed using the diphenylamine method of Burton<sup>[16]</sup> and determined by spectrophotometry. Assays for total protein content<sup>[17]</sup> were performed on homogenates and supernatants; the concentrations were determined using spectrophotometry (SpectraMAX Plus, Molecular Devices, Sunnyvale, CA, United States).

### Postnatal growth and maturation development

On post natal day (PND) 0, the pup weights were determined, and the pups were examined for malformations. The measurement of the pup weights was repeated on PND 7, 14 and 21 when the offspring were weaned from their mothers in all of the experimental groups. Eye opening was observed once daily from day 12 to 16 and rated as follows: on day 0, both eyes were closed; on day 1, one eye opened; and on day 2, both eyes opened.

### Blood test

**Study 1 (to investigate autism):** In study 1, blood samples from each experimental group (ten pups) from groups 1 and 2 and the control group were pooled for analyses before the administration of IFN-γ and gliadin in groups 1 and 2 or physiological saline in the control group. The samples were obtained from the eye using capillary tubes and were collected in ethylene diamine tetraacetic acid tubes containing aprotinin at 0 °C and centrifuged at 1600 g for 15 min. The plasma was analysed for 1-epinephrine, norepinephrine, catecholamine, and 2-serotonin using high performance liquid chromatography as previously described<sup>[18,19]</sup>. The serum was analysed for 3-calcium and 25-OH vitamin D after collection in microfuge tubes using a chemiluminescent microparticle immunoassay<sup>[20]</sup>.

**IFN-γ:** Recombinant rat IFN-γ (PRP24; Serotec, Oxford, United Kingdom) was used in this study. IFN-γ was lyophilised (0.1 mg), reconstituted in 0.3 mL of distilled water, and stored until use in aliquots of 50 µL (10000 U) at -70 °C. A dose of 1000 U was used for each new-born rat. After application of IFN-γ, the pups were fed either milk from their mother or human colostrum.

**Gliadin administration:** Gliadin (from wheat gluten, G-3375; Sigma, St. Louis, MO, United States) was administered intragastrically using a silicon tube. Young rats were repeatedly administered gliadin in the following doses: day 0, 0.5 mg in one intragastric dose and day 3, 3 mg in one intragastric dose. The pups in each group were euthanised at 21 d of age after receiving a provocative dose of 30 mg of gliadin per animal 24 h before euthanasia (on postnatal day PND 20).

### Blood test study 2 (to investigate coeliac disease):

Study 2 aimed to investigate coeliac disease in autistic pups on PND 21. After the application of interferon-γ in group 1 (suckling pups receiving gliadin) and group 2 (human colostrum and gliadin), serum tissue transglutaminase tTG antibody titres were measured quantitatively by an enzyme-linked immunosorbent assay (QuantaLite tTG, Inova Diagnostics, CA, United States). Additionally, all the chemical analyses for study 1 were repeated on PND 21 for the two experimental groups and the control group.

### Transmission and scanning electron microscopic study

Small samples from the duodenum of each experimental group on PND 21 were immediately fixed in 3% phosphate-buffered glutaraldehyde (pH = 7.4; 4 °C) for 2 h. The tissues were postfixed in 1% aqueous osmium tetroxide in an appropriate buffer for 1 h and embedded in Epon. Ultrathin sections (80-100 nm) were prepared and stained with uranyl acetate and lead citrate. To examine the duodenum using scanning electron microscopy, the tissues were fixed as described previously, dehydrated,

**Table 1 Protein and DNA content in the duodenum of the studied groups (mean ± SE)**

|                       | Control    | Group 1                | Group 2                  |
|-----------------------|------------|------------------------|--------------------------|
| Protein content, mg/g | 135.42 ± 2 | 122.4 ± 7 <sup>b</sup> | 151.6 ± 9 <sup>bd</sup>  |
| DNA content, mg/g     | 6.3 ± 0.03 | 6.1 ± 0.06             | 7.7 ± 0.03 <sup>bd</sup> |

<sup>b</sup>*P* < 0.01 *vs* control rats, <sup>d</sup>*P* < 0.01 *vs* group 1.

**Table 2 Pup weight until weaning (mean ± SD)**

| Groups  | PND 0                  | PND 7                  | PND 14                  | PND 21                    |
|---------|------------------------|------------------------|-------------------------|---------------------------|
| Control | 3.53 ± 0.3             | 10.1 ± 2.0             | 17.5 ± 1.6              | 33.4 ± 2.7                |
| Group 1 | 1.7 ± 0.1 <sup>b</sup> | 5.5 ± 0.1 <sup>b</sup> | 14.2 ± 2.0 <sup>b</sup> | 21.40 ± 2.36 <sup>b</sup> |
| Group 2 | 1.6 ± 0.3 <sup>b</sup> | 7.4 ± 0.5 <sup>d</sup> | 15.9 ± 1.0 <sup>c</sup> | 29.6 ± 1.7 <sup>d</sup>   |

Changes in pup weight. <sup>b</sup>*P* < 0.01 *vs* control group; <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 *vs* group 1. PND: pups on post natal day.

mounted on aluminium stubs with conductive carbon glue, and sputter coated with a 100 Å layer of gold. The control and treated samples were examined using a Joel EX 1200 transmission electron microscope at the central lab of King Saud University.

### Statistical analysis

SPSS 13.0 was used for the statistical analysis. The data were expressed as the mean ± SD and were analysed using one-way analysis of variance followed with a post-hoc test for multiple comparisons. The differences were considered significant at the *P* < 0.05 level.

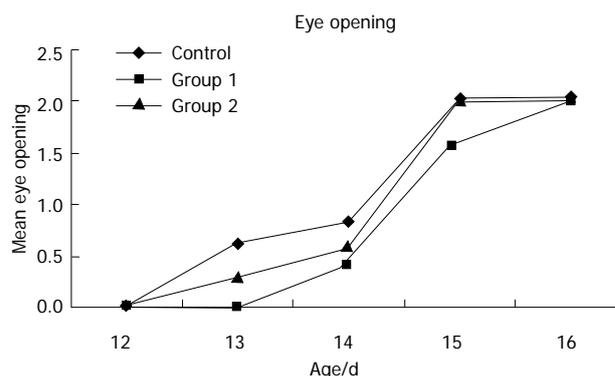
## RESULTS

### Changes in the protein and DNA content in the duodenum of the different experimental groups on PND 21

As shown in Table 1, the duodenal DNA and protein concentrations were significantly different between group 1 and group 2 and significantly higher in group 2 compared with the control animals. The protein concentration in the duodenum was significantly higher in group 2 compared with group 1 (151.6 ± 9 mg/g *vs* 122.4 ± 7 mg/g of tissue, respectively). However, no significant difference in the duodenal DNA concentration was observed between group 1 and the control group *P* > 0.05 (6.1 ± 0.06 *vs* 6.3 ± 0.03, respectively).

### Changes in body weight and postnatal growth

As shown in Table 2, the pup weight decreased significantly (*P* < 0.001) in the autistic neonatal pups on PND 0 (study 1) compared with the normal control pups. The animals from group 1 (suckling pups administered gliadin) exhibited a statistically significantly (*P* < 0.001) reduced increase in body weight until weaning compared with the control group. (21.40 ± 2.36 g *vs* 33.4 ± 2.7 g, respectively). A significant increase (*P* < 0.001) in body weight was detected in human colostrum-treated pups on PND 7 and 21 compared with suckling pups in group 1.



**Figure 1 Mean eye opening age in the studied groups.**

However, no significant difference was noticed between the two groups on PND 0 (*P* > 0.05). The stools were sticky, and the animals showed long bone malformations in autistic rats (results not shown).

### Eye opening

As shown in Figure 1, there was a delay in eye opening in the treated rats in group 1 on PND 13 compared with the control group and group 2. No difference was observed on PND 12 and 16 between group 1 and group 2 (*P* > 0.05) *vs* the control group.

### Changes in biochemical parameters in the control group and study 1 on PND 0

Table 3 shows the changes in biochemical parameters of control and autistic neonatal pups on PND 0. The mean concentrations of epinephrine, norepinephrine and serotonin were greatly increased in study 1 compared with the control group (*P* < 0.001). Administration of a single intraperitoneal injection of 600 mg/kg sodium valproate on day 12.5 after conception resulted in significantly reduced calcium and vitamin D levels in study 1 *vs* the control group (*P* < 0.001).

**Mean vitamin D:** As shown in Table 4, a significant difference in the vitamin D level was observed between group 2 and the control group (33.62 ± 0.581 ng/mL *vs* 44.36 ± 0.302 ng/mL; *P* < 0.05). Furthermore, on PND 21, the pups in group 1 exhibited a significantly lower mean vitamin D level *vs* the control group and group 2 (9.4 ± 0.225 ng/mL *vs* 44.36 ± 0.302 ng/mL and 33.62 ± 0.581 ng/mL; *P* < 0.01). Treatment with human colostrum inhibited decreases in the level of vitamin D in autistic pups with coeliac disease (*P* < 0.01).

**Mean serotonin:** As shown in Table 4, a significant increase in the mean serotonin levels was detected in group 1 *vs* the control pups (30.5 ± 0.565 ng/L *vs* 9.12 ± 0.126 ng/L; *P* < 0.001). A significant decrease in the serotonin level was detected in group 2, which was treated with human colostrum, *vs* group 1 (15.32 ± 0.367 ng/L *vs* 30.5 ± 0.565 ng/L; *P* < 0.001).

**Table 3** Changes in biochemical parameters in the control group and study 1 on post natal day 0 (mean  $\pm$  SD)

| Parameters | Vitamin D (ng/mL)            | Serotonin ( $\mu$ g/L)       | Epinephrine (ng/L)            | Norepinephrine (ng/L)          | Calcium (mg/dL)              |
|------------|------------------------------|------------------------------|-------------------------------|--------------------------------|------------------------------|
| Method     | CMIA                         | HPLC                         | HPLC                          | HPLC                           | Calorimetric                 |
| Control    | 34.26 $\pm$ 0.514            | 7.26 $\pm$ 0.604             | 16.54 $\pm$ 0.185             | 101.32 $\pm$ 1.683             | 10.01 $\pm$ 0.211            |
| Study 1    | 6.21 $\pm$ 0.13 <sup>b</sup> | 33.3 $\pm$ 0.24 <sup>b</sup> | 84.25 $\pm$ 5.95 <sup>b</sup> | 184.29 $\pm$ 8.44 <sup>a</sup> | 6.52 $\pm$ 0.16 <sup>b</sup> |

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  *vs* control group. CMIA: Chemiluminescent microparticle immunoassay; HPLC: High performance liquid chromatography.

**Table 4** Changes in the biochemical parameters in the control group and study 2 groups on post natal day 21 (mean  $\pm$  SD)

| Parameters | Vitamin D (ng/mL)               | Serotonin ( $\mu$ g/L)          | Epinephrine (ng/L)             | Norepinephrine (ng/L)           | Transglutaminase (g/L)        | Calcium (mg/dL)                |
|------------|---------------------------------|---------------------------------|--------------------------------|---------------------------------|-------------------------------|--------------------------------|
| Method     | CMIA                            | HPLC                            | HPLC                           | HPLC                            | ELISA                         | Calorimetric                   |
| Control    | 44.36 $\pm$ 0.302               | 9.12 $\pm$ 0.126                | 21.73 $\pm$ 0.29               | 110.46 $\pm$ 0.99               | 0.9 $\pm$ 0.066               | 10.91 $\pm$ 0.241              |
| Group 1    | 9.4 $\pm$ 0.225 <sup>b</sup>    | 30.5 $\pm$ 0.565 <sup>b</sup>   | 76.22 $\pm$ 1.15 <sup>b</sup>  | 204.87 $\pm$ 1.83 <sup>b</sup>  | 3.1 $\pm$ 0.149 <sup>b</sup>  | 7.31 $\pm$ 0.411 <sup>a</sup>  |
| Group 2    | 33.62 $\pm$ 0.581 <sup>bd</sup> | 15.32 $\pm$ 0.367 <sup>bd</sup> | 56.15 $\pm$ 0.07 <sup>bc</sup> | 155.42 $\pm$ 0.08 <sup>bc</sup> | 1.6 $\pm$ 1.021 <sup>ad</sup> | 10.12 $\pm$ 0.819 <sup>e</sup> |

<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* group 1. CMIA: Chemiluminescent microparticle immunoassay; HPLC: High performance liquid chromatography; ELISA: Enzyme-linked immunosorbent assay.

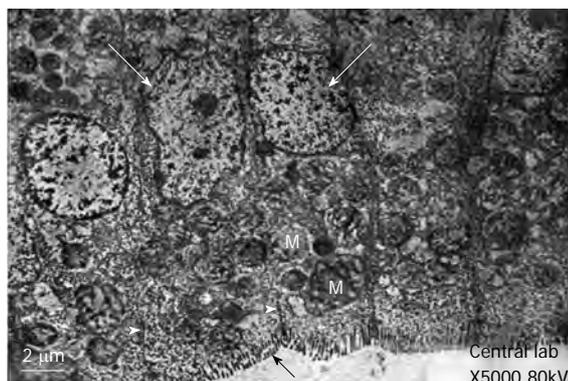


Figure 2 Transmission electron-micrograph of duodenum of rat of control group showing regularly arranged enterocytes with vesicular nucleus (white arrows) and continuous microvillus border (black arrow). Notice the cell junction (white arrowhead) between the enterocytes and abundant mitochondria (M).

**Mean epinephrine:** As shown in Table 4, the mean epinephrine concentration was significantly increased ( $P < 0.001$ ) in all of the treated groups including group 1 and group 2 on PND 21 *vs* the control group (76.22  $\pm$  1.15 ng/L and 56.15  $\pm$  0.071 ng/L *vs* 21.73  $\pm$  290 ng/L, respectively). However, the epinephrine level was significantly ( $P < 0.01$ ) decreased in group 2 pups *vs* group 1 pups (56.15  $\pm$  0.071 ng/L *vs* 76.22  $\pm$  1.15 ng/L, respectively).

**Mean norepinephrine:** As shown in Table 4, the mean norepinephrine concentration was significantly increased ( $P < 0.01$ ) in all of the treated groups including group 1 and group 2 on PND 21 *vs* the control group (204.87  $\pm$  1.83 ng/L and 155.42  $\pm$  0.086 ng/L *vs* 110.46  $\pm$  0.990 ng/L, respectively). Moreover, a significant change was detected in group 2 *vs* group 1 pups ( $P < 0.05$ ).

**Mean transglutaminase:** As shown in Table 4, the mean transglutaminase concentrations were significantly

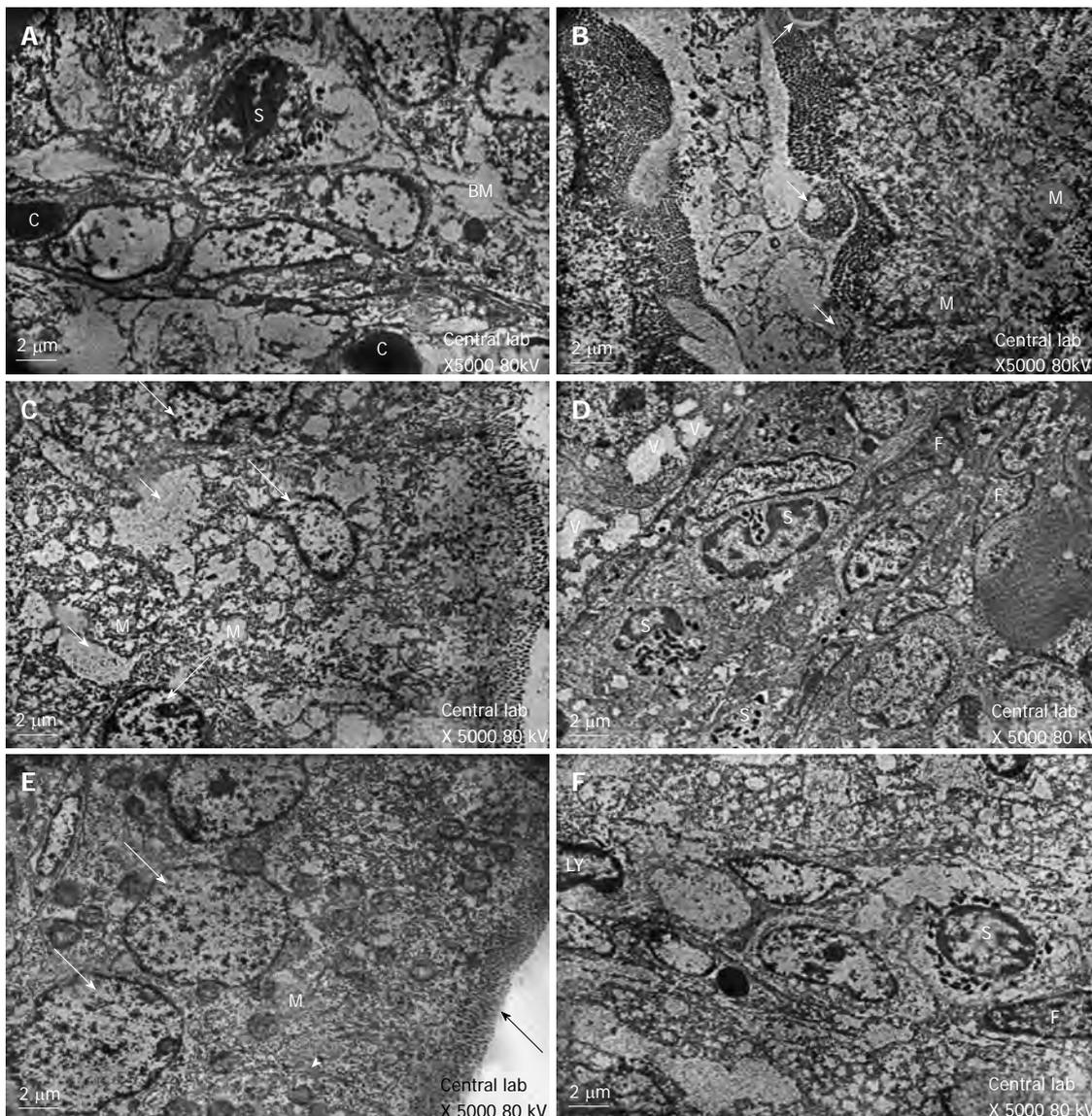
increased ( $P < 0.01$ ) in all of the treated groups including group 1 (suckling pups administered gliadin) and group 2 (human colostrum and gliadin) on PND 21 *vs* the normal controls (3.1  $\pm$  0.149 g/L and 1.6  $\pm$  1.021 g/L *vs* 0.9  $\pm$  0.066 g/L, respectively). Furthermore, the mean transglutaminase level was significantly ( $P < 0.01$ ) increased in the group 1 pups *vs* the group 2 pups. Moreover, a significant change was detected in the group 2 pups compared with the control pups. Treatment with human colostrum inhibited the increased level of transglutaminase in autistic pups with coeliac disease *vs* group 1 suckling pups.

**Mean calcium:** As shown in Table 4, the calcium level was decreased in group 1 *vs* the control group and group 2 (7.31  $\pm$  0.411 *vs* 10.91  $\pm$  0.241 and 10.12  $\pm$  0.819;  $P < 0.05$ ). No significant difference in the calcium level was observed between group 2 and the control group ( $P > 0.05$ ). Therefore, the human colostrum treatment was more effective than mother's milk in increasing the calcium level.

#### Transmission and scanning electron microscopy of the duodenal villi on PND 21

**Control group:** Transmission electron microscopy showed that the enterocytes exhibited a regular microvillus border, and cell junctions were typically observed between the enterocytes (Figure 2) along with scattered abundant mitochondria (Figure 2). The cores of the villi possessed normal appearances with normal capillaries and connective tissue cells (Figure 3A). The surface of the duodenal villi was examined using scanning electron microscopy and revealed some villi with intact tips and borders. The openings of goblet cells were observed on the surface of the villi (Figure 4A).

**Group 1:** The electron microscopic study showed localised areas of loss of the microvillus borders in the



**Figure 3** Transmission electron microscopy results showed. A: Transmission electron-micrograph of duodenum of rat of control group showing normal appearance of the core of the villi. Note eosinophil leucocytes (S) and close proximity of blood capillary (C) to the enterocyte basement membrane (BM); B: Transmission electron-micrograph of duodenum of breastfeeding rat group 1 showing local loss of microvillus border (long arrows) and swollen mitochondria with remnants cristae (M); C: Transmission electron-micrograph of duodenum of breastfeeding rat group 1 showing irregular nuclei (long arrow) with intercellular oedema (short arrow) and swollen mitochondria (M); D: Transmission electron-micrograph of duodenum of breastfeeding rat group 1 showing increased eosinophil leucocytes (S). Notice the nuclei of fibroblasts (F) and vacuolated enterocytes (V); E: Transmission electron-micrograph of duodenum of rat treated with human colostrum group 2 showing the enterocytes with vesicular nuclei (arrow) and regular microvillus border (black arrow). Notice the cell junction (head arrow) and abundant mitochondria (M); F: Transmission electron-micrograph of duodenum of rat treated with human colostrum showing normal appearance of the core of the villi with nuclei of lymphocyte (LY), nuclei of fibroblast (F) and eosinophil leucocytes (S).

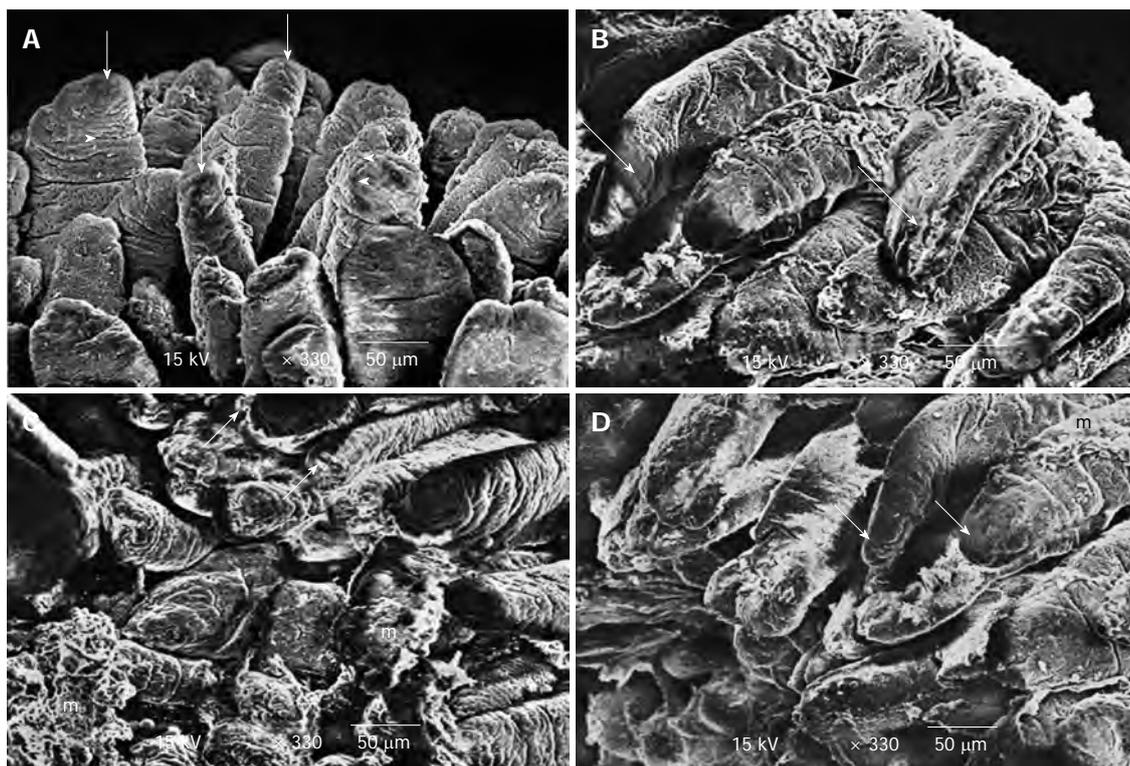
enterocytes with swollen mitochondria and vacuolation in the cytoplasm (Figure 3B). Irregular appearance of the nuclei of the enterocytes was also observed (Figure 3C), and the core of the villi showed cellular infiltration (Figure 3D). Scanning electron microscopy showed deformed villi (Figure 4B), villi with distorted appearances and the accumulation of mucous (Figure 4C).

**Group 2:** The ultrastructure of the enterocytes of the duodenal villi was similar to that of the control group. The microvillus border was regular and continuous. The enterocytes showed vesicular nuclei and abundant mito-

chondria (Figure 3E). The core of the villi appeared to be normal with normal capillaries (Figure 3F). Scanning electron microscopy showed that the villi of the duodenum of group 2 appeared to be normal and were similar to the control group (Figure 4D).

## DISCUSSION

In this investigation, the repeated administration of human colostrum until weaning in group 2 increased the mucosal restitution of duodenal tissue as shown in Figures 3E, 3F and 4D, which was observed by electron mi-



**Figure 4** Scanning electron microscopy results showed. A: Scanning electron-micrograph of duodenal villi of control group showing the villi with intact tips and border (short arrow). Notice the opening of goblet cells on the surface of the villi (arrow head); B: Scanning electron micrograph of duodenum of breastfeeding rat group 1 showing some of the villi are seen to be deformed (long arrow). Notice short villus (arrow head) is clearly seen; C: Scanning electron micrograph of duodenum of breastfeeding rat group 1 showing some villi with necrotic tips (short arrow). Notice distorted appearance of the other villi with accumulation of mucous (m); D: Scanning electron-micrograph of duodenum of rat treated with human colostrum group 2 showing duodenal villi with intact tips and borders (short arrow). Notice the presence of mucous on some villi (m).

scopy. However, the breast feeding in group 1 could not overcome the atrophy of the gut caused by coeliac disease in autistic rats as shown in Figures 3B-D, 4B and 4C. This suggested that epidermal growth factor (EGF) in colostrum (200  $\mu\text{g/L}$ ) and milk (30-50  $\mu\text{g/L}$ ) in humans and many other species acts as a luminal surveillance peptide that prevents gut damage<sup>[21]</sup> and stimulates the repair process at sites of injury<sup>[22]</sup>. Transforming growth factor- $\alpha$  (TGF- $\alpha$ ) is a 50-amino acid molecule present in human colostrum and milk at much lower concentrations (2.2-7.2  $\mu\text{g/L}$ )<sup>[23]</sup> than EGF and occurs in the gastrointestinal mucosa at sites of injury, which supports its role in mucosal growth and repair. TGF- $\beta$ -like molecules are present in high concentrations in both bovine milk (1-2 mg/L) and colostrum (20-40 mg/L)<sup>[24]</sup>. The concentrations are sufficient enough to mediate the ability to maintain gastrointestinal integrity in suckling neonates. The previous results are consistent with our previous report, which shows that treatment with bovine colostrum has a positive effect on duodenum injury in autistic rats with coeliac disease<sup>[25]</sup>. Insulin-like growth factor (somatomedins) (IGF-I) concentrations are much higher in bovine colostrum compared with human colostrum (500  $\mu\text{g/L}$  vs 18  $\mu\text{g/L}$ )<sup>[26,27]</sup>. And even lower concentrations in mature bovine milk (10  $\mu\text{g/L}$ ) are observed<sup>[28]</sup>. IGF-I is known to promote protein accretion (*i.e.*, it is an anabolic agent<sup>[29]</sup>) and is at least partially responsible for mediating

the growth-promoting activity of growth hormone. In rodents, two growth factors play an important role in the process of regeneration and proliferation of intestinal enterocytes: EGF and transforming TGF- $\alpha$ . However, TGF- $\alpha$  has not been found in rat maternal milk<sup>[30]</sup>. This may explain the decreased bone malformation in group 2, which was treated with human colostrum (results not shown), compared with group 1 rats. Growth factors are so named because they stimulate the growth of various cell lines *in vitro*; however, the functions of these peptide-based molecules are diverse. There are also marked species differences in the nature and concentration of growth factor constituents. For example, human colostrum contains much higher concentrations of EGF than bovine colostrum, while the reverse is true for insulin-like IGFs- I and II. In our investigation, the uptake of human colostrum in group 2 showed significant improvements compared with suckling pups in group 1 when assessed by body weight. The mean body weight measured in group 2 was highest at the end of weaning ( $29.6 \pm 1.75$ ) and significantly differed from the mean body weight observed on other days in group 1 and compared with the control. An additional consequence of different routes of immunoglobulin transmission relates to the changes in the relative contents of immunoglobulins, which occurs during the transition from colostrum to milk in certain species. For example, the profile of immunoglobulins in

human colostrum is similar to milk; the IgA level is high in both colostrum and milk (88%-90% of total immunoglobulin). IgA protects the gastrointestinal tract (GIT) from various diseases in new-borns, which is reflected in large changes in the profile of immunoglobulins during the transition from colostrum to mature milk<sup>[31]</sup>. IgA-deficient patients predominantly suffer from respiratory and gastrointestinal infections because secretory IgA has important functions in protecting mucosal surfaces<sup>[31]</sup>. Human colostrum has a low IgG content (2%), and the IgG required to provide systemic immunity is transferred across the placenta before birth<sup>[32]</sup>. In contrast, the colostrum IgG content in many other species is typically greater than 75% of the total immunoglobulin content.

Based on the aforementioned information, in our study, we used this unique model to induce gluten enteropathy. We found that the concurrent administration of IFN- $\gamma$  intraperitoneally and gliadin intragastrically in group 1 followed by a second intragastric dose of gliadin resulted in a failure to detoxify antigens from the gut, which may lead to cognitive impairments and coeliac crises on day 14. The impairments included loose stools with short and deformed duodenal villi in some pups, while other pups exhibited sloughing of the tips as observed by scanning electron microscopy. Local loss of the brush border, irregular nuclei and vacuolation of some enterocytes were observed using transmission electron microscopy on day 21. The previous result may be due to the presence of antigens in the diet that can cross into the mucosa more easily *via* a disrupted intestinal barrier. The antigens may cause local inflammatory reactions and generate proinflammatory cytokine signals that affect the GIT. The previously described damage was not observed in group 2, which was administered human colostrum until weaning, using scanning and transmission electron microscopy as shown in Figures 3E, 3F and 4D. This could be explained by the report from another group<sup>[33]</sup>, who revealed that the concentration of IgA anti-gliadin is the highest in colostrum and decreases after a month. This may diminish the effect of gluten peptides on the lamina propria and prevent the initiation of damage to intestinal villi that was observed in group 1.

Leptin levels, which supports duodenal protection in colostrum and milk, was correlated with the whole fat and choline phospholipid content of colostrum. The association between leptin and fat globule membrane content results from its protective properties during gut transit in the new-born animal<sup>[34]</sup>. Leptin receptor expression has been found in the small intestine, which suggests that the intestinal epithelium is a direct target of leptin action<sup>[35]</sup>. The concentration of leptin was 56% lower in mature milk (day 10) *vs* colostrum (13.90 g/L *vs* 6.14 g/L, respectively;  $P < 0.001$ ). The colostrum and milk leptin levels correlated with fat (0.90;  $P < 0.001$ ) and choline phospholipid (0.76;  $P < 0.05$ ). Thus, leptin is present in large quantities in colostrum, smaller and more variable quantities in untreated milk and is likely decreased in skim milk<sup>[3,4]</sup>. Our results revealed increased levels of

serotonin ( $33.3 \pm 0.244$ ), epinephrine ( $84.25 \pm 0.595$ ) and norepinephrine ( $184.29 \pm 8.44$ ) in autistic pups (study 1) due to maternal prenatal exposure to VPA. Furthermore, we observed a significant decrease in serum vitamin D and calcium levels in autistic neonatal rats in study 1 on PND 0 and group 1 on PND 21. Both epinephrine and norepinephrine were significantly increased in autistic pups on PND 0 and group 1 pups on PND 21 compared with the control group.

Colostrum is high in calcium and magnesium, which share bonding characteristics as functional groups. Both organic calcium and organic magnesium in colostrum enhance the absorption of other minerals and vitamins in our body. Colostrum also provides higher levels of vitamin D and vitamin A than normal milk<sup>[36]</sup>. In addition, vitamin D and IgD can help our body absorb a sufficient amount of calcium to enhance physiological functions<sup>[36]</sup>. Our investigation revealed that the uptake of human colostrum in group 2 inhibited the decrease in levels of vitamin D and calcium in autistic pups with coeliac disease as shown in Table 4.

An initial mucosal assault due to a gut infection or the toxic effects of gliadin may cause upregulation of tissue transglutaminase (tTG), which causes cross-linking of various proteins, including gliadin. This tTG-gliadin complex represents a "new antigen" (neoantigen), which could trigger the production of tTG antibodies<sup>[37]</sup>. Tissue TG plays a role in maintaining the integrity of both the intestinal crypt micro-architecture and the dermal-epidermal junction. Therefore, the development of villous atrophy in group 1 could be explained by the production of tTG antibodies ( $3.1 \pm 0.149$  g/L) due to repeated administration of gliadin, which may disturb the integrity of the duodenum in breastfeeding pups. However, human colostrum uptake inhibited the increase in the levels of transglutaminase antibody ( $1.6 \pm 1.021$  g/L) in autistic pups with coeliac disease.

The outcomes of the present investigation elucidated the effects of early-life nutrition from human colostrum on the functional maturation of the duodenal villi in autistic rats with coeliac disease, which might help to limit or prevent coeliac risk. There are numerous pathways that may lead to the diagnosis of ASD. In some, ASD may begin with genetic susceptibility, and in others, infection or immune abnormalities may play a key role. Further study of the reciprocal actions of the nervous, immune, and endocrine systems may help to unravel the mystery of ASD. Moreover, while many of the observations discussed herein are involved in the pathogenesis of autism, comprehensive studies of autism and coeliac disease with age-matched control individuals and their families are necessary to provide more conclusive results.

## COMMENTS

### Background

Coeliac disease (CD) exhibits a multifactorial aetiology, and both genetic and environmental factors contribute to disease development. CD is a human leuko-

cyte antigen (HLA)-associated disorder, and the majority of CD patients express HLA-DQ2 and HLA-DQ8 to a lesser extent. Disease development is a consequence of the ingestion of immunogenic fragments in gluten by genetically predisposed subjects. The treatment of CD involves a lifelong diet, which may cause difficulties, because avoiding gluten completely is almost impossible.

### Research frontiers

Many studies have been published regarding the preventive effect of breastfeeding in CD. An important systematic review and meta-analysis of observational studies on breastfeeding and CD by Akobeng *et al.* concludes that breastfeeding offers protection against the development of CD.

### Innovations and breakthroughs

Human colostrum may be an ideal antimicrobial for selective targeting in the gastrointestinal tract in autistic rats with coeliac disease.

### Applications

This investigation revealed that the uptake of human colostrum inhibited the decrease in levels of vitamin D and calcium in autistic pups with coeliac disease

### Peer review

This paper explores the effect of breastfeeding and human colostrums on the prevention of coeliac disease in autistic rats. The paper is well structured and the study is well designed. The article is sufficiently novel and interesting to warrant publication.

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**P- Reviewers** Cheung MC, Khajehei M **S- Editor** Zhai HH  
**L- Editor** A **E- Editor** Ma S



## Gastric antisecretory and antiulcer activity of bovine hemoglobin

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Received: December 30, 2012 Revised: March 9, 2013

Accepted: April 13, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate gastric antisecretory and gastro-protective activity of bovine hemoglobin (B-Hb) in rats.

**METHODS:** Adult Albino-Wistar rats were divided into groups of 6 animals each. B-Hb in doses of 100, 300 and 900 mg/kg body weight was tested for gastric acid secretion and antiulcer activity. Gastric secretions were measured 6 h after pylorus ligation in rats pretreated with B-Hb. The acidity was measured by titrating gastric contents against 0.01 mol/L NaOH to pH 7. Indomethacin ulcers were produced by oral administration of 30 mg/kg *bw* in the rats pretreated with B-Hb one hour before indomethacin. Six hours after indomethacin stomach removed and ulcer index was recorded. Ethanol ulcer were produced by 1 mL of ethanol in the rats pretreated with B-Hb 30 min before the ethanol.

One hour after ethanol stomach were cut open to score ulcers. Histological examination and analysis of gastric wall mucus, non-protein sulfhydryl groups (NP-SH), and myeloperoxidase (MPO) were carried in gastric tissue following ethanol administration.

**RESULTS:** In control rats pylorus ligation for 6 h resulted in the accumulation of  $8.1 \pm 0.61$  mL of gastric secretion. The treatment of the rats with 100, 300 and 900 mg/kg of B-Hb produced a significant decrease in the volume of gastric secretion  $5.6 \pm 0.63$ ,  $5.5 \pm 0.75$  and  $4.7 \pm 0.58$  mL respectively as compared to the control group [analysis of variance (ANOVA)  $F = 4.77$ ,  $P < 0.05$ ]. The lesion area in the control group was found to be  $22.4 \pm 3.2$  mm<sup>2</sup> six hours after the administration of indomethacin. Treatment of rats with B-Hb at doses of 100 mg/kg ( $24.3 \pm 3.29$  mm<sup>2</sup>), 300 mg/kg ( $16.2 \pm 1.45$  mm<sup>2</sup>) and 900 mg/kg ( $12.6 \pm 1.85$  mm<sup>2</sup>) produced a dose dependent decreased the lesion scores (ANOVA  $F = 4.50$ ,  $P < 0.05$ ). The ulcer index following one hour after 1 mL ethanol was  $7.1 \pm 0.31$ . Pretreatment of rats with B-Hb at the doses of 100 mg/kg ( $2.5 \pm 0.42$ ), 300 mg/kg ( $2.1 \pm 0.4$ ) and 900 mg/kg ( $0.7 \pm 0.21$ ) significantly inhibited the formation of gastric lesions (ANOVA  $F = 63.26$ ,  $P < 0.0001$ ). Histological examination of gastric mucosa following ethanol showed significant lesions in the form of gastric pits with detachment of the surface epithelium; vacuolation of epithelial cells and elongation of microvessels. The changes were dose-dependently attenuated by B-Hb. The treatment of rats with ethanol significantly decreased the Alcian blue binding capacity of gastric wall mucus ( $480 \pm 25.6$   $\mu$ g Alcian blue/g of tissue) as compared to control rats ( $667 \pm 25.8$   $\mu$ g). Pretreatment of rats with B-Hb at the doses of 100 mg/kg ( $516 \pm 31.6$   $\mu$ g/g), 300 mg/kg ( $558 \pm 28.8$   $\mu$ g/g) and 900 mg/kg ( $654 \pm 33.8$   $\mu$ g/g) significantly attenuated ethanol induced depletion of gastric wall mucus (ANOVA  $F = 8.05$ ,  $P < 0.005$ ). A significant and dose dependent increase of gastric mucosal NP-SH (ANOVA  $F = 19.62$ ,  $P < 0.001$ ) and decrease in MPO activity (ANOVA  $F =$

3.1,  $P < 0.05$ ) was observed in B-Hb treated rats.

**CONCLUSION:** B-Hb possesses significant gastric antisecretory and gastroprotective activity against experimentally induced gastric lesion. The gastroprotective effects of B-Hb are accompanied by inhibition of neutrophils activity, reduction of oxidative stress and maintenance of mucosal integrity.

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**Key words:** Bovine hemoglobin; Gastric ulcers; Indomethacin; Ethanol; Ischemic injury; Rats

**Core tip:** In the recent years the treatment strategies for gastric ulcer diseases have significantly changed, mirroring the revolution in the understanding of its pathogenesis. Improved oxygenation in critically ischemic gastric mucosa has emerged as a method of choice to accelerate epithelization of erosive and ulcerative defects. Bovine hemoglobin (B-Hb) has been used in a wide range of applications including restoration of tissue oxygenation in ischemic condition. Our data show that B-Hb has significant anti-gastric acid secretory and gastroprotective activity. Further studies are warranted to determine the role of B-Hb in prophylaxis/treatment of gastric ulcer disease.

Al Asmari AK, Al Omani S, Elfaki I, Tariq M, Al Malki A, Al Asmary S. Gastric antisecretory and antiulcer activity of bovine hemoglobin. *World J Gastroenterol* 2013; 19(21): 3291-3299 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3291.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3291>

## INTRODUCTION

A variety of noxious factors and substances like alcohol, drugs, psychological stress, smoking and bacterial infection by *Helicobacter pylori*, to which the man is exposed in the present day life are known to have deleterious effect on gastric mucosa<sup>[1]</sup>. Organized at several levels the mucosal defense system comprise the pre-epithelial mucosal layer, the epithelial cell barrier, the mucosal microvasculature, the supply of the mucosa by enteric, extrinsic sensory and extrinsic autonomic neurons and mucosal immune system<sup>[2]</sup>. Maintenance and repair of gastric mucosa is a dynamic process associated with proliferation and migration of epithelial cells and connective tissue to maintain/regain mucosal architecture<sup>[3]</sup>. It involves complex host of mechanisms which work in tandem to protect gastric mucosa from damage as well as trigger the mechanism to repair mucosal defects by proliferating and migrating epithelial cells and connective tissue resulting in reconstruction of mucosal architecture<sup>[4]</sup>. Numerous studies have demonstrated the importance of gastric mucosal haemo-dynamics as a defensive factor of the gastric mucosa against injury<sup>[5-7]</sup>. Treatment with some prostaglandin

derivatives such as prostaglandin E<sub>2</sub> have been shown to increase mucosal blood flow and protect gastric mucosa against indomethacin and ethanol induced gastropathy by improving mucosal haemodynamics<sup>[8,9]</sup>. Oxygen delivery to the gastric mucosa is not only a function of blood flow but also depends on oxygen content in the arterial inflow. A possible strategy would be to supplement oxygen using hyperbaric oxygen (HBO) to overcome ischemic injury<sup>[10]</sup>. The use of HBO in the treatment of peptic ulcer raised the efficacy of the multimodality therapy and accelerated the epithelization of erosive and ulcerative defects<sup>[11,12]</sup>. An alternative method to improve oxygenation in critically ischemic tissue has emerged, which consists of using oxygen carriers, such as hemoglobin (Hb) or Hb based products<sup>[13]</sup>. These biomaterials have been initially developed to avoid the drawbacks of blood transfusions including immunologic reactions, blood-borne transmitted infections, limited availability and restricted storage time<sup>[14]</sup>.

Hb is the iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates. In blood Hb carries oxygen from the respiratory organs to the rest of the body where it releases the oxygen to burn nutrients to provide energy to power the functions of the organism, and collects the resultant carbon dioxide to bring it back to the respiratory organs to be dispensed from the organism. In mammals, Hb makes up about 97% of the red blood cells dry content, and around 35% of the total content including water<sup>[15]</sup>. Hemoglobin has an oxygen binding capacity of 1.34 mL per gram of hemoglobin<sup>[16]</sup>, which increases the total blood oxygen capacity seventy-fold compared to dissolved oxygen in blood. A single mammalian hemoglobin molecule can bind (carry) up to four oxygen molecules<sup>[17]</sup>. Based on these properties cell free hemoglobin (CF-Hb) products have been successfully used in a variety of ischemic/hypoxic conditions<sup>[18,19]</sup>.

Bovine hemoglobin (B-Hb) has been used for a wide range of applications, including their use to enhance oxygen delivery to tissues during conditions of ischemia or hypoxia<sup>[20,21]</sup>. Strate *et al*<sup>[13]</sup> reported that B-Hb is much more potent than autologous red blood cells in restoring tissue oxygenation in ischemic conditions. In view of the superiority of CF-Hb compared to erythrocyte and whole blood we studied the efficiency of B-Hb against experimentally induced gastric mucosal injury.

## MATERIALS AND METHODS

Adult Albino Wistar rats of either sex, weighing 150-200 g and fed on standard chow diet were used. They were divided into groups of 6 animals each. The distribution of animals into groups and the treatment allotted to each group were randomized. All the experiments were started between 8:00 and 10:00 in the morning. The protocol of the study was approved by the Institutional Research and Ethical Committee.

The aqueous solution of ulcerogens and B-Hb were freshly prepared before administration. The concentra-

tions of drug were prepared in such a way that each rat received 0.5 mL of drug solution/100 g body weight.

### Gastric secretions

**Pylorus ligated (Shay) rats:** The animals were fasted for 36 h with access to water *ad libitum* before the pylorus was ligated under ether anesthesia, care being taken not to cause bleeding or to occlude blood vessels<sup>[22]</sup>. B-Hb in doses of 100, 300 and 900 mg/kg body weight was given by gavage (*ig*) half an hour before pylorus ligation by oral route. The animals were sacrificed at 6 h after the pylorus ligation. The stomachs were removed, contents collected, volume measured, centrifuged and subjected to analysis for titratable acidity against 0.01 mol/L NaOH to pH 7 using a pH meter and total acid output was calculated.

**Indomethacin-induced gastric lesion:** Indomethacin was suspended in 1% carboxymethylcellulose in water and administered by gavage at the dose of 30 mg/kg body weight<sup>[23]</sup>. B-Hb in the doses of 100, 300 and 900 mg/kg body weight was administered by gavage 1 h before indomethacin. The animals were sacrificed 6 h using ether after indomethacin administration. The stomach were removed and opened along the greater curvature. After washing with saline the gastric lesions were quantified by a person unaware of the treatment protocol. The ulcer were scored according to the method of Valcavi *et al.*<sup>[24]</sup>. The circular ulcer induced by indomethacin were assessed on the basis of their diameter: deep circular ulcers more than 8 mm diameter = 10; 7-8 mm = 8; 6-7 mm = 7; 5-6 mm = 6; 4-5 mm = 5; 3-4 mm = 4; 2-3 mm = 3; 1-2 mm = 2; and < 1 mm = 1. Deep linear ulcers 10 mm or more in length were scored 6, and linear ulcers less than 10 mm in length were scored 3. The scores of each single lesion were then summed up for determination of the ulcer index.

**Gastric lesions induced by ethanol (cytoprotection studies):** The animals were administered (*ig*) with 1 mL of absolute ethanol<sup>[25]</sup>. B-Hb in the doses of 100, 300 and 900 mg/kg body weight was given (*ig*) 30 min before the administration of ethanol. One hour after the administration of ethanol the animals were sacrificed and examined for the lesions in stomachs. The patchal lesions of stomach induced by ethanol were scored according to the method described by Schiantarelli *et al.*<sup>[26]</sup> using the following scale: 0 = normal mucosa; 1 = hyperemic mucosa or up to 3 small patches; 2 = from 4 to 10 small patches; 3 = more than 10 small or up to 3 medium-sized patches; 4 = from 4 to 6 medium-sized patches; 5 = more than 6 medium-sized or up to 3 large patches; 6 = from 4 to 6 large patches; 7 = from 7 to 10 large patches; 8 = more than 10 large patches or extensive necrotic zones. "small" was defined as up to 2 mm across (max diameter), "medium-sized" as between 2 and 4 mm across and "large" as more than 4 mm across.

Two separate batches of ethanol treated rats were used for biochemical and histological studies. The assay of gastric wall mucus, non-protein sulfhydryl group (NP-

SH), and myeloperoxidase (MPO) in the rats 1 h after ethanol exposure has been described below:

**Determination of gastric wall mucus:** Gastric wall mucus was determined according to the modified procedure of Corne *et al.*<sup>[27]</sup>. The glandular segment of the stomach was separated from the lumen of the stomach, weighed, and transferred immediately to 10 mL of 0.1% w/v Alcian blue solution (in 0.16 mol/L sucrose solution buffered with 0.05 mL sodium acetate at pH 5). Tissue was stained for two hours in Alcian blue, excess dye was removed by two successive rinses with 10 mL of 0.25 mol/L sucrose, first for 15 min and then for 45 min. Dye complexed with the gastric wall mucus was extracted with 10 mL of 0.5 mol/L magnesium chloride which was intermittently shaken for 1 min at 30 min intervals for 2 h. Four milliliters of blue extract was then vigorously shaken with an equal volume of diethyl ether. The resulting emulsion was centrifuged at 3600 *g* for 10 min and the absorbance of aqueous layer was recorded at 580 nm. The quantity of Alcian blue extracted per gram of wet glandular tissue was then calculated.

**Estimation of NP-SH:** Gastric mucosal NP-SH was measured according to the method of Sedlak and Lindsay<sup>[28]</sup>. The glandular part of stomach was homogenized in ice-cold 0.02 mmol/L ethylenediaminetetraacetic acid. Aliquots of 5 mL of the homogenates were mixed in 15 mL test tubes with 4 mL of distilled water and 1 mL of 50% trichloroacetic acid. The tubes were shaken intermittently for 10-15 min and centrifuged at 3000 *g*. Two milliliters of supernatant were mixed with 4 mL of 0.4 mol/L Tris buffer at pH 8.9; 0.1 mL of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) was added and the sample was shaken. The absorbance was read within 5 min of addition of DTNB at 412 nm against a reagent blank with no homogenates.

**Determination of MPO:** MPO activity in the gastric mucosa was measured according to the methods described earlier<sup>[29]</sup>. Preweighed tissue was homogenized (1:10 wt/vol) in 0.5% hexadecyltrimethyl ammonium bromide in 50 mmol potassium phosphate buffer (pH 6.0) before sonication in an ice bath for 20 s. Three freeze/thaw cycles were performed followed by sonication (20 s in ice bath). The samples were centrifuged at 17000 *g* (5 min, 4 °C) and MPO in the supernatant was assayed by mixing of 0.1 mL of supernatant with 2.9 mL of 50 mmol/potassium phosphate buffer (pH 6.0) containing 0.167 g/L *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The change in absorbance at 460 nm was measured for 4 min using ultraviolet (UV)-visible spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan).

**Histology of ethanol-induced gastric lesions:** The stomach was opened along the greater curvature, washed with saline and fixed in 10% neutral buffered formalin for 24 h. The specimens were then processed overnight

**Table 1** Effect of bovine hemoglobin on the volume of gastric secretion and acidity in 6 h pylorus ligated rats

| Treatment  | Dose of B-Hb (mg/kg) | Gastric secretion (mL)  | Total acid output (mEq)   |
|------------|----------------------|-------------------------|---------------------------|
| Control    | 0                    | 8.1 ± 0.61              | 498.7 ± 31.8              |
| Hemoglobin | 100                  | 5.6 ± 0.63 <sup>a</sup> | 471.1 ± 75.0              |
| Hemoglobin | 300                  | 5.5 ± 0.75 <sup>a</sup> | 438.4 ± 65.6              |
| Hemoglobin | 900                  | 4.7 ± 0.58 <sup>b</sup> | 287.6 ± 24.6 <sup>a</sup> |

Values are mean ± SE. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group using Dunnett's multiple test. B-Hb: Bovine hemoglobin.

for dehydration and clearing steps, using an automatic tissue processor (Shandon Processor MKII; Runcorn, Cheshire, United Kingdom). The specimens were embedded in paraffin blocks and sections of 5 µm thickness were stained with hematoxylin-eosin for light microscopy observations.

### Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Differences with *P* < 0.05 were considered as statistically significant.

## RESULTS

### Effect of B-Hb on gastric secretion in 6 h pylorus-ligated rats

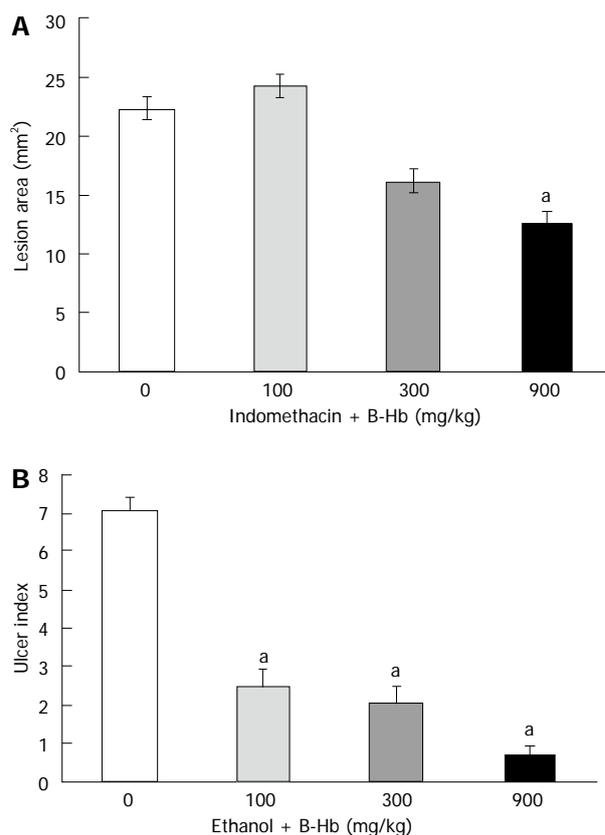
In control rats pylorus ligation for 6 h resulted in the accumulation of 8.1 ± 0.61 mL of gastric secretion and total acid output of 498.7 ± 31.8 mEq (Table 1). The treatment of the rats with 100, 300 and 900 mg/kg of B-Hb produced a significant decrease in the volume of gastric secretion 5.6 ± 0.63, 5.5 ± 0.75 and 4.7 ± 0.58 mL respectively as compared to the control group. Treatment with B-Hb also dose dependently reduced total acid output in the rats treated with 100 mg/kg (471.1 ± 75 mEq) and 300 mg/kg (438.4 ± 65.6 mEq). However a significant reduction in total acid output was observed in a dose of 900 mg/kg (287.6 ± 24.6 mEq) of B-Hb.

### Effect of B-Hb on indomethacin-induced gastric mucosal damage

The administration of indomethacin resulted in production of gastric lesions mainly in the glandular stomach of all the animals. The lesion area in the control group was found to be 22.4 ± 3.2 mm<sup>2</sup>. Pretreatment of rats with B-Hb at doses of 100 mg/kg (24.3 ± 3.29 mm<sup>2</sup>), 300 mg/kg (16.2 ± 1.45 mm<sup>2</sup>) and 900 mg/kg (12.6 ± 1.85 mm<sup>2</sup>) produced a dose dependent decrease of the lesion area (ANOVA *F* = 4.50, *P* < 0.05, Figure 1A).

### Effect of B-Hb on ethanol-induced gastric lesions

The treatment of rats with absolute ethanol produced extensive gastric lesion in the glandular mucosa of the stomach. These lesions were characterized by multiple hemorrhagic red bands (patches) of different sizes along



**Figure 1** Effect of bovine hemoglobin. A: On indomethacin-induced gastric mucosal damage; B: On ethanol-induced gastric mucosal damage. B-Hb: Bovine hemoglobin. <sup>a</sup>*P* < 0.05 vs B-Hb 0 mg/kg.

the axis of the glandular stomach. The ulcer index in the control group 1 h after ethanol administration was 7.1 ± 0.31. Pretreatment of rats with B-Hb at the doses of 100 mg/kg (ulcer index = 2.5 ± 0.42), 300 mg/kg (2.1 ± 0.4) and 900 mg/kg (0.7 ± 0.21) significantly inhibited the formation of gastric lesions (ANOVA *F* = 63.26, *P* < 0.0001, Figure 1B). Histological examination of gastric mucosa showed the appearance of these lesions in the form of gastric pits with detachment of the surface epithelium; vacuolation of epithelial cells and elongation of microvessels. Pretreatment with B-Hb dose-dependently prevented ethanol-induced mucosal damage (Figure 1).

### Effect B-Hb on ethanol-induced changes in gastric wall mucus

The treatment of rats with ethanol significantly decreased the Alcian blue binding capacity of gastric wall mucus (480 ± 25.6 µg Alcian blue/g of tissue) as compared to control rats (667 ± 25.8 µg). Pretreatment of rats with B-Hb at the doses of 100 mg/kg (516 ± 31.6 µg/g), 300 mg/kg (558 ± 28.8 µg/g) and 900 mg/kg (654 ± 33.8 µg/g) significantly enhanced the binding capacity of Alcian blue to gastric mucosa (ANOVA *F* = 8.05, *P* < 0.005, Table 2).

### Effect of B-Hb on ethanol-induced depletion of gastric mucosal NP-SH

The level of NP-SH in the gastric mucosa of control rats

**Table 2** Effect of bovine hemoglobin on ethanol induced changes in Alcian blue binding capacity, non-protein sulfhydryl groups levels and myeloperoxidase in gastric mucosa of rats

| Treatment   | Dose of B-Hb (mg/kg) | Alcian blue binding (mg/g tissue) | Non-protein sulfhydryl (mmol/g tissue) | Myeloperoxidase activity (DA/g tissue) |
|-------------|----------------------|-----------------------------------|--|--|
| Control     | 0                    | 667 ± 25.8                        | 4.59 ± 0.29                            | 15.94 ± 1.6                            |
| EtOH alone  | 1 mL/animal          | 480 ± 25.6 <sup>b</sup>           | 1.60 ± 0.12 <sup>b</sup>               | 24.54 ± 1.9 <sup>b</sup>               |
| EtOH ± B-Hb | 100                  | 516 ± 31.6                        | 3.11 ± 0.35 <sup>d</sup>               | 24.30 ± 4.2                            |
| EtOH ± B-Hb | 300                  | 558 ± 28.8 <sup>d</sup>           | 3.73 ± 0.42 <sup>d</sup>               | 14.25 ± 2.6 <sup>c</sup>               |
| EtOH ± B-Hb | 900                  | 654 ± 33.8 <sup>d</sup>           | 5.36 ± 0.34 <sup>d</sup>               | 17.21 ± 0.98 <sup>c</sup>              |

Values are mean ± SE. <sup>b</sup>*P* < 0.01 *vs* control; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 *vs* EtOH group using Dunnett's multiple comparison test. B-Hb: Bovine hemoglobin.

was  $4.59 \pm 0.29$   $\mu\text{mol/g}$  of tissue, which was significantly decreased to  $1.60 \pm 0.12$   $\mu\text{mol/g}$  of tissue following the administration of ethanol. A significant and dose dependent reversal of NP-SH was observed following administration of B-Hb in low ( $3.11 \pm 0.35$  mmol/g of tissue), medium ( $3.73 \pm 0.42$  mmol/g of tissue) and high dose ( $5.36 \pm 0.34$  mmol/g of tissue) (ANOVA *F* = 19.62, *P* < 0.001, Table 2).

#### Effect of B-Hb on ethanol-induced changes in gastric MPO activity

The MPO activity in the normal gastric mucosa was  $15.96 \pm 1.9$  mmol/g of wet tissue which increased significantly to  $24.54 \pm 1.9$  mmol/g following ethanol administration. The MPO activity was slightly reduced to  $24.30 \pm 4.2$  mmol/g in the rats treated with low dose of B-Hb prior to ethanol administration. However medium and high dose of B-Hb significantly reversed ethanol induced increase in MPO the value of MPO in these two groups being  $14.25 \pm 2.6$  mmol/g and  $17.21 \pm 0.98$  mmol/g respectively (ANOVA *F* = 3.21, *P* < 0.05, Table 2).

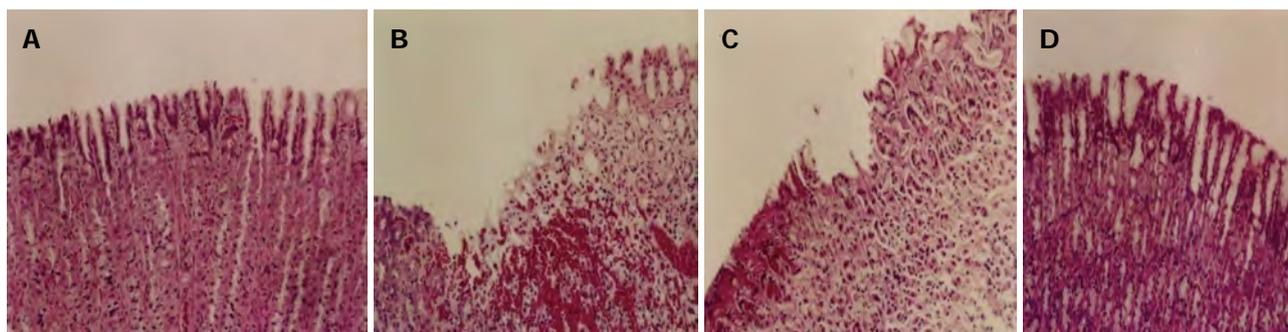
## DISCUSSION

The result of this study showed a dose dependent reduction in the volume and acidity of gastric secretions following intra-gastric administration of B-Hb (Table 1). Our findings are supported by several earlier investigators who showed that gastric hemorrhage or intra-gastric administration of blood or blood products exerts inhibitory effect on gastric acid secretions<sup>[30-32]</sup>. As early as 1953, Chandler *et al.*<sup>[33]</sup> reported that gastro-duodenal hemorrhage leads to a temporary absence of hydrochloric acid (achlorhydria) causing a significant decrease in gastric acid secretions. Fullarton *et al.*<sup>[34]</sup> also showed that gastro-duodenal blood infusion significantly inhibits pentagastrin stimulated gastric acid and pepsin secretion. Paradoxically, intra-gastric blood/blood products including hemoglobin which constitute a protein meal instead of stimulating gastric secretions result in gastric antisecretory activity<sup>[35,36]</sup>. Gastric acid secretion is an elaborate and dynamic process that is regulated by neural (efferent and afferent), hormonal (*e.g.*, gastrin), and paracrine (*e.g.*, histamine, ghrelin, somatostatin) pathways as well as mechanical (*e.g.*, distention) and chemical (*e.g.*, amino acids) stimuli<sup>[31]</sup>. It has been suggested that intra-gastric blood induced increase in plasma gastric inhibitory polypeptide, secretin or glucagon may

modulate gastric acid secretion in animals<sup>[32,34,37,38]</sup>. These mediators inhibit gastric acid secretions through somatostatin release<sup>[39-41]</sup>. However this hypothesis has been challenged by some other investigators<sup>[42]</sup>.

Pretreatment of rats with B-Hb dose dependently ameliorated indomethacin-induced gastric mucosal damage (Figure 1). Protective effect of cell free hemoglobin has been reported against a variety of experimentally induced disease models including cardiac ischemic reperfusion injury<sup>[18,43,44]</sup>, pancreatitis<sup>[45]</sup>, renal injury<sup>[46]</sup> and neuronal injury<sup>[18,19,47-49]</sup>. The mechanism by which B-Hb exerts its protective effect against indomethacin induced gastropathy is far from clear. Indomethacin causes gastric mucosal damage via several mechanisms, including the impairment of the barrier properties of the mucosa, suppression of gastric prostaglandin synthesis, reduction of gastric mucosal blood flow and interference with the tissue repair mechanism<sup>[50]</sup>. The ability of non-steroidal anti-inflammatory drugs (NSAIDs) to reduce gastric mucosal blood flow has been recognized for several decades<sup>[9]</sup>. Prostaglandins (PGs) of the E and I series are potent vasodilators that are continuously produced by the vascular endothelium, inhibition of PG synthesis by NSAIDs lead to a reduced vascular tone and hypoxic injury<sup>[51]</sup>. Cell free hemoglobin is able to readily diffuse in the microcirculation and transport O<sub>2</sub> to hypoxic tissue because of its high O<sub>2</sub> affinity, low viscosity and small mean diameter as compared to red blood cells<sup>[52]</sup>. Once in microcirculation Hb offloads oxygen to the ischemic tissue, which is unlike the vasoconstrictive effect of Hb on large vessels<sup>[53-56]</sup>. Hence the mitigation of pathogen induced ischemic injury by CF-Hb may be attributed to its ability to transient oxygen delivery<sup>[15,57]</sup> and preservation of energy metabolism<sup>[58]</sup>. Moreover, the presence of acid in the lumen of the stomach also contributes to the pathogenesis of indomethacin -induced ulcers and bleeding, by impairing the restitution process, interfering with hemostasis and inactivating several growth factors that are important in mucosal defense and repair<sup>[9]</sup>. Our experiments using Shay rats model clearly showed an inhibition of gastric acid secretion following B-Hb administration (Table 1). Thus the reduction of acid content in stomach by B-Hb may to some extent contribute to its gastroprotective effect against indomethacin induced ulcers.

The oral administration of absolute ethanol produced significant ulcers in glandular part of gastric mucosa of rats (Figure 2). Our histopathological studies of gastric



**Figure 2** Light micrographs showing the effect of bovine hemoglobin on ethanol-induced gastric lesions in rats ( $\times 200$ ). A: Normal mucosa; B: Ethanol produced lesion; C: Pretreatment of rats with bovine hemoglobin (B-Hb) 100 mg/kg; D: Pretreatment of rats with B-Hb 900 mg/kg.

mucosa showed a significant loss of glandular cells, disruption of epithelium, sub mucosal edema and infiltration of neutrophils following ethanol administration (Figure 2). A significant decrease in Alcian blue binding capacity of gastric mucosa following exposure to ethanol (Table 2) clearly suggests depletion of mucosal gel lining adhering to the gastric surface which is considered the first line of defense in stomach against endogenous and exogenous ulcerogens<sup>[59]</sup>. Numerous mechanisms have been proposed to explain necrotizing agent induced gastropathy<sup>[60]</sup>. Besides the direct deleterious effect of ethanol on gastric tissues, disruption of mucosal barrier also results from mucosal capillary necrosis, vascular congestion and thrombosis in the sub-epithelial microvasculature<sup>[61]</sup>. Oral administration of ethanol results in low or no blood flow to the stomach leading to transient hypoxic mucosal damage<sup>[62]</sup>. It is well accepted that gastric mucosal protective mechanism largely depend on appropriate microcirculation which help to orchestrate the defense mechanism at various levels of gastric mucosa<sup>[63]</sup>. Pre-treatment of animal with B-Hb dose dependently attenuated ethanol induced gastric ulcer with almost complete protection in higher dose of 900 mg/kg body weight (Figure 2). The gastroprotective effect of B-Hb against ethanol induced ulcers may to some extent be attributed to its ability to inhibit gastric acid secretions and enhanced oxygen delivery as discussed earlier. Moreover the cytoprotective effect of B-Hb was accompanied by attenuation of ethanol-induced increase in MPO (a marker of neutrophil activity) in glandular tissue of stomach (Table 2). Neutrophils are the major inflammatory cell type infiltrating the injured mucosa following exposure to ethanol<sup>[64]</sup>. Strategies to counteract the infiltration and/or activation of neutrophils have been shown to protect animals against gastric ulcers<sup>[65,66]</sup>. Activated neutrophils injure the microvasculature via the release of oxygen derived free radicals (ODFR) and proteases<sup>[67,68]</sup>. We observed a significant decrease in gastric NP-SH following ethanol administration clearly suggesting a massive generation of ODFR in stomach (Table 2). Our findings are in agreement with earlier reports showing depletion of sulfhydryls in ethanol-induced gastric lesions<sup>[69,70]</sup>. The treatment of rats with glutathione depletors has been shown to significantly potentiate ulcerogen-induced gastric mucosal injury<sup>[71]</sup>,

whereas increase in mucosal NP-SH exerts a gastroprotective effect<sup>[72,73]</sup>. These findings suggest that gastroprotective effects of B-Hb may to extent be attributed to its antioxidant activity. Dual effect of Hb as prooxidant and antioxidant has been reported earlier<sup>[74,75]</sup>. In fact, more than 100 years ago Hb was shown to readily react with  $H_2O_2$ <sup>[76]</sup> and peroxidase activity of Hb has been reported as early as by Wu<sup>[77]</sup>. Thus the active site of heme protein in Hb share peroxide cleavage properties of peroxidase to present antioxidant activity<sup>[78]</sup>. On the other hand the oxidation of Hb generates potentially cytotoxic products such as ferryl heme intermediate ( $Fe^{4+}$ ), methemoglobin ( $Fe^{3+}$ ), hemichromes and free heme or iron<sup>[79,80]</sup>. Comproportionation of cytotoxic ferryl hemoglobin with oxyhemoglobin has been shown to result in antioxidant function in which ferryl heme intermediate is quenched and resultant methemoglobin is re-reduced by methemoglobin reductase<sup>[81]</sup>. Moreover there is strong evidence to suggest that Hb exerts its cytoprotective effect through adaptive cytoprotection. The presence of Hb in the tissue has been shown to induce heme oxygenase-1 (HO-1) the enzyme responsible for heme degradation<sup>[82]</sup>. Studies with HO-1 knock out mice have demonstrated that HO-1 ameliorates NSAIDs induced gastric tissue damage<sup>[83,84]</sup>. It has been shown that HO-1 exerts its cytoprotective action against necrotizing agents by scavenging the prooxidant heme which is a major hemoglobin degradation product<sup>[84,85]</sup>. To sum up it may be suggested that cytoprotective effect of B-Hb is complex and involve multiple mechanisms.

In conclusion our data show that B-Hb has significant anti-gastric acid secretory and gastroprotective activity. Further studies are warranted to determine the role of B-Hb in prophylaxis/treatment of gastric ulcer disease.

## ACKNOWLEDGMENTS

The authors are thankful to the Prince Sultan Military Medical City and the Medical Services Department of Ministry of Defense for their encouragement and support.

## COMMENTS

### Background

In the recent years, the treatment strategies for gastric ulcer diseases have sig-

nificantly changed, mirroring the revolution in the understanding of its pathogenesis. Improved oxygenation in critically ischemic gastric mucosa has emerged as a method of choice to accelerate epithelization of erosive and ulcerative defects. Bovine hemoglobin (B-Hb) has been used in a wide range of applications including restoration of tissue oxygenation in ischemic condition.

### Research frontiers

Strategies to improve oxygen delivery to ischemic tissues could potentially protect gastric mucosa and other tissues from hypoxic injury. Cell free hemoglobin is able to readily diffuse in the microcirculation and transport O<sub>2</sub> to hypoxic tissue because of its high O<sub>2</sub> affinity, low viscosity and small diameter.

### Innovations and breakthroughs

Recent studies showed protective effect of cell free hemoglobin against disease models of ischemic reperfusion injury including neuronal injury, nephropathy, pancreatitis and myocardial infarction. This study first time demonstrated anti-secretory and gastric antiulcer activity of B-Hb. The gastroprotective effect could be attributed to the ability of B-Hb to improve oxygenation, reduction of oxidative stress and lowering of neutrophil activity.

### Applications

The result of this study suggests that bovine hemoglobin besides having significant anti-gastric acid secretory activity protects rats against ethanol and indomethamine induced gastric ulcers. B-Hb being animal protein can be safely explored clinically for the oral treatment of hyperacidity and gastric ulcer diseases.

### Terminology

Hb is the iron-containing oxygen-transport metalloprotein in red blood cells of all vertebrates. Hemoglobin is autonomous: it binds oxygen and release it without the need for any cofactor. Cell free Hb has been used for wide range of applications including enhanced oxygen delivery to ischemic tissue. Bovine hemoglobin is superior to autologous red blood cells in restoring tissue oxygen in ischemic conditions.

### Peer review

This is the first study showing antisecretory and gastric anti-ulcer activity of bovine hemoglobin. Reduction of oxidative stress and lowering of neutrophil activity might contribute to B-Hb induced gastroprotective effects. The results are valuable to explore pharmacological potential of B-Hb in a variety of ischemichypoxic conditions.

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P- Reviewer Vorobjova T S- Editor Gou SX L- Editor A  
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## Anticancer effects of sweet potato protein on human colorectal cancer cells

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Supported by The Earmarked Fund for China Agriculture Research System, No. CARS-11-B-19; "Technique of Processing and Utilization for Plant Proteins" from China-Argentina Science and Technology Cooperation Program, No. 2010DFA32690; Grant from the Capital Medical University, No. 2009ZR03 and No. 2012ZR17; the Importation and Development of High-Caliber Talents Project of Beijing Municipal Institutions

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Received: December 28, 2012 Revised: March 25, 2013

Accepted: April 27, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate the effects of proteins purified from sweet potato storage roots on human colorectal cancer cell lines.

**METHODS:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, Hoechst 33258 nuclear staining and Boyden transwell chamber methods were used to determine whether purified sweet potato protein (SPP) from fresh sweet potato roots affected proliferation, migration and invasion, respectively, of human colorectal cancer SW480 cells *in vitro*. The inhibitory effects of SPP on growth of human colorectal

cancer HCT-8 cells intraperitoneally xenografted in nude mice and spontaneous lung metastasis of murine Lewis lung carcinoma 3LL cells subcutaneously transplanted in C57 BL/6 mice were also investigated *in vivo*.

**RESULTS:** SPP inhibited the proliferation of SW480 cells in a dose-dependent manner, with an IC<sub>50</sub> value of 38.732 μmol/L ( $r^2 = 0.980$ ,  $P = 0.003$ ) in the MTT assay. Hoechst 33258 nuclear staining further revealed inhibition of cell viability and induction of apoptosis by SPP. The transwell assay disclosed significant reduction in migrated cells/field by 8 μmol/L SPP ( $8.4 \pm 2.6$  vs  $23.3 \pm 5.4$ ,  $P = 0.031$ ) and invaded cells/field through the ECMatrix by 0.8 μmol/L SPP, compared with the control ( $25.2 \pm 5.2$  vs  $34.8 \pm 6.1$ ,  $P = 0.038$ ). Both intraperitoneal (*ip*) and intragastric (*ig*) administration of SPP led to significant suppression of growth of intraperitoneally inoculated HCT-8 cells in nude mice to  $58.0\% \pm 5.9\%$  ( $P = 0.037$ ) and  $43.5\% \pm 7.1\%$  ( $P = 0.004$ ) of the controls, respectively, after 9 d treatment. Bloody ascites additionally disappeared after *ip* injection of trypsin inhibitor. Notably, *ig* and *ip* administration of SPP induced a significant decrease in spontaneous pulmonary metastatic nodule formation in C57 BL/6 mice ( $21.0 \pm 12.3$  and  $27.3 \pm 12.7$  nodules/lung vs  $42.5 \pm 4.5$  nodules/lung in controls, respectively,  $P < 0.05$ ) after 25 d treatment. Moreover, the average weight of primary tumor nodules in the hind leg of mice decreased from  $8.2 \pm 1.3$  g/mice in the control to  $6.1 \pm 1.4$  g/mice in the *ip* group ( $P = 0.035$ ).

**CONCLUSION:** SPP exerts significant antiproliferative and antimetastatic effects on human colorectal cancer cell lines, both *in vitro* and *in vivo*.

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**Key words:** Sweet potato protein; Colorectal cancer; Cell proliferation; Cell invasion; Metastasis

**Core tip:** Sweet potato protein (SPP) is a type of serine protease inhibitor that suppresses the activity of trypsin. The current study showed that SPP significantly inhibited proliferation, migration and invasion of human colorectal cancer SW480 cells *in vitro*. Moreover, the protein significantly suppressed the growth of intraperitoneally xenografted human colorectal cancer HCT-8 cells and volume of bloody ascites formed in nude mice. Spontaneous lung metastasis of a murine lung carcinoma cell line was significantly inhibited by SPP in mice. Notably, both intragastric infusion and intraperitoneal injection of SPP were effective in animal models.

Li PG, Mu TH, Deng L. Anticancer effects of sweet potato protein on human colorectal cancer cells. *World J Gastroenterol* 2013; 19(21): 3300-3308 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i21/3300.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3300>

## INTRODUCTION

Colorectal cancer (CRC) is one of the most commonly diagnosed cancer types in both men and women<sup>[1]</sup>. Annually, about 49380 Americans die of CRC, accounting for 9% of all cancer-related deaths<sup>[2]</sup>. Surgical resection is currently the primary curative therapy for CRC. However, surgery alone provides a high cure rate only for patients with early-stage disease. In the later stages of CRC, the presence of clinically occult micrometastases often leads to disease recurrence and death. Numerous natural or synthetic compounds have been tested for their ability to suppress degradation of the extracellular matrix (ECM) and subsequent invasion and metastasis of cancer cells, with the aim of preventing cancer metastasis<sup>[3,4]</sup>. Among these, protease inhibitors have been increasingly shown to have significant antimetastatic effects in various cancers<sup>[5-8]</sup>. The sweet potato [*Ipomoea batatas* (L.) Lam] is a dicotyledonous plant that belongs to the *Convolvulaceae* family. Its tuberous roots contain 0.49%-2.24% crude proteins on a fresh weight basis. Proteins isolated from sweet potato can be separated into sporamin A (31 kDa) and sporamin B (22 kDa) *via* non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which merge into a single band of about 25 kDa under reducing conditions<sup>[9,10]</sup>. Previous studies have identified the sweet potato protein (SPP) as a type of Kunitz-type trypsin inhibitor (KTI)<sup>[9]</sup> with potential therapeutic effects in a variety of cancer models. For instance, Huang *et al*<sup>[11]</sup> reported that KTI purified from sweet potato inhibited proliferation and induced apoptosis of NB4 promyelocytic leukemia cells. Additionally, Yao *et al*<sup>[12]</sup> showed that SPP inhibited proliferation and induced apoptosis of human tongue carcinoma Tca8113 cells *via* downregulation of the Akt/glycogen synthase kinase (GSK)-3 pathway. In addition, KTIs isolated from other sources, such as human urine and soybeans, have been shown to exert

antiproliferative, anti-invasion and antimetastatic activities in a variety of malignant cells, animal models and cancer patients<sup>[13-15]</sup>. The present study focused on the inhibitory effects of SPP on proliferation, migration and invasiveness of malignant cells in a number of CRC cell lines *in vitro* as well as *in vivo*.

## MATERIALS AND METHODS

### Cell culture

Human colorectal cancer SW480 cells were maintained in culture medium comprising RPMI 1640 supplemented with 10% fetal bovine serum (FBS),  $1 \times 10^5$  U/L penicillin and streptomycin. Cells were grown in T-75 culture flasks at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and 95% air.

### SPP preparation

SPP was purified from fresh sweet potato tuberous roots, as reported previously<sup>[16]</sup>. Purity of SPP exceeded 99% of the dry weight. A stock solution was freshly prepared before each experiment by dissolving SPP in culture medium, filtered through a sterile 0.22- $\mu$ m membrane, and further diluted with culture medium to achieve the final concentrations, as indicated.

### Protein and TI activity staining of SPP on 12% polyacrylamide gels

Purified SPP was detected using both protein and TI activity staining on 12% SDS-PAGE. For protein staining, samples were mixed with sample buffer (60 mmol/L Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, and 0.1% bromophenol blue) and  $\beta$ -mercaptoethanol. Coomassie brilliant blue R-250 stain was used. For TI activity, native gels were stained according to the method of Huang *et al*<sup>[11]</sup>. Briefly, upon completion of non-denaturing PAGE, the gel was immersed and shaken twice in 25% vol/vol isopropanol in 10 mmol/L Tris buffer (pH 7.9) for 10 min each. Next, the gel was dipped into 10 mmol/L hydrogen peroxide in the same buffer for at least 30 min with gentle shaking, and washed in 10 mmol/L Tris buffer (pH 7.9) for 10 min, followed by incubation in trypsin solution (50  $\mu$ g bovine trypsin/mL, 10 mmol/L Tris buffer pH 7.9) for 20 min at 37 °C. After rinsing with the same buffer to remove excess trypsin, the gel was incubated at 37 °C for at least 30 min in the dark with 160 mL substrate dye solution prepared immediately before use. The substrate dye solution consisted of 40 mg *N*-acetyl-D,L-phenylalanine  $\beta$ -naphthyl ester in 16 mL of *N,N*-dimethylformamide made up to 160 mL with 144 mL of 10 mmol/L Tris buffer (pH 7.9) in which 80 mg tetrazotized *O*-dianisidine was dissolved. The gel was destained with 10% acetic acid for at least 30 min.

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

SW480 cells were seeded into a 96-well plate (Corning, Cambridge, MA, United States) at a density of  $1.5 \times 10^4$

cells/well. After achieving confluence, cells were incubated in medium containing different concentrations of SPP. Following SPP treatment, 20  $\mu\text{L}$  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) was added to each well and incubated for a further 4 h. Next, 200  $\mu\text{L}$  dimethyl sulfoxide was added to cells in each well, mixed for 10 min, and optical density measured at 492 nm.

### Hoechst 33258 staining

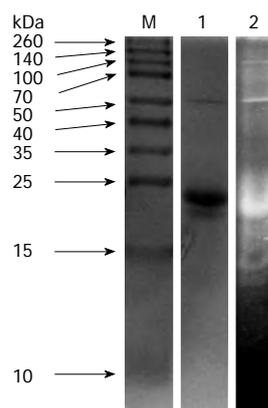
Cell viability was assayed morphologically using Hoechst 33258 (Sigma, St Louis, MO, United States) staining. SW480 cells were plated onto a 24-well plate ( $1.2 \times 10^5$  cells/well) and allowed to grow for 24 h. After confluence, cells were serum-starved for 16 h and treated with various concentrations of SPP for 48 h. Cells were fixed in 2% glutaraldehyde for 4 h and washed twice with 0.9% NaCl before staining with 1  $\mu\text{g}/\text{mL}$  Hoechst 33258 for 30 min under ice cooling in the dark. After washing twice with 0.9% NaCl, cells were observed under a fluorescence microscope (Olympus IX71) with excitation at ultraviolet (360 nm).

### Cell migration and invasion

SW480 cells were seeded into the upper chamber of a Transwell insert (Millipore, Billerica, MA, United States) in RPMI 1640 at a density of  $3 \times 10^5$  cells/well. Medium containing 10% FBS was placed in the lower chamber to act as a chemoattractant. After adherence, cells were treated with various concentrations of SPP for 10 h in serum-free medium. Cells migrating through 8.0  $\mu\text{m}$  polycarbonate membranes to the lower surface were stained with 0.1% crystal violet, whereas non-migratory cells remaining on the upper chambers were removed by scraping the upper surface of the membrane with a cotton swab. Migrating cells were quantified by counting stained cells under a microscope ( $\times 200$ ). Five random fields were selected for each well. The assay was performed in triplicate, and repeated three times. Invasion assays were performed in a similar manner to the migration assays, except that inserts were precoated with ECM substitute ECMatrix (Chemicon, Temecula, CA, United States) and cells were treated with SPP for 24 h.

### In vivo experiments

Two animal experiments were conducted. In the first experiment,  $2 \times 10^5$  human colorectal cancer HCT-8 cells were inoculated into the peritoneal cavity of 15 BALB/c nude mice (5 wk old,  $14 \pm 2$  g), which were randomly divided into three groups (5 animals per group). From the second day of inoculation, SPP was administered to two of the three groups either intraperitoneally (2  $\mu\text{mol}/\text{L}$  per kg/d) or intragastrically (80  $\mu\text{mol}/\text{L}$  per kg/d) for 9 d, while the remaining group served as a vehicle control receiving only intraperitoneally injected physiological saline during the experiment. After treatment with SPP for the indicated times, animals were sacrificed, and the number of tumor nodules in the peritoneal cavity was compared between groups. In the second animal experiment, we



**Figure 1** Protein (lane 1) and trypsin inhibitor activity (lane 2) staining of sweet potato protein on polyacrylamide gel electrophoresis gels (12%) with  $\beta$ -mercaptoethanol. A 10  $\mu\text{L}$  aliquot of staining of sweet potato was loaded in each lane. M: Molecular standards.

examined the effects of SPP on metastatic capacity of cancer cells. Lewis lung cancer cells were inoculated subcutaneously into the hind legs of 18 female C57BL/6 mice (5 wk old,  $14 \pm 2$  g) divided into three groups (6 animals per group). From the second day of inoculation, SPP was administered either intraperitoneally (2  $\mu\text{mol}/\text{L}$  per kg/d) or intragastrically (120  $\mu\text{mol}/\text{L}$  per kg/d) to animals for 25 d. After sacrifice, the number of the metastatic nodules formed in lungs of mice were counted under a stereomicroscope ( $\times 10$ ), and compared between the three treatment groups.

### Statistical analysis

Data were expressed as mean  $\pm$  SD, and analyzed using analysis of variance with DPS7.55 software (Refine Information Tech Company, Hangzhou, China). Two-tailed values of  $P < 0.05$  were considered significant.

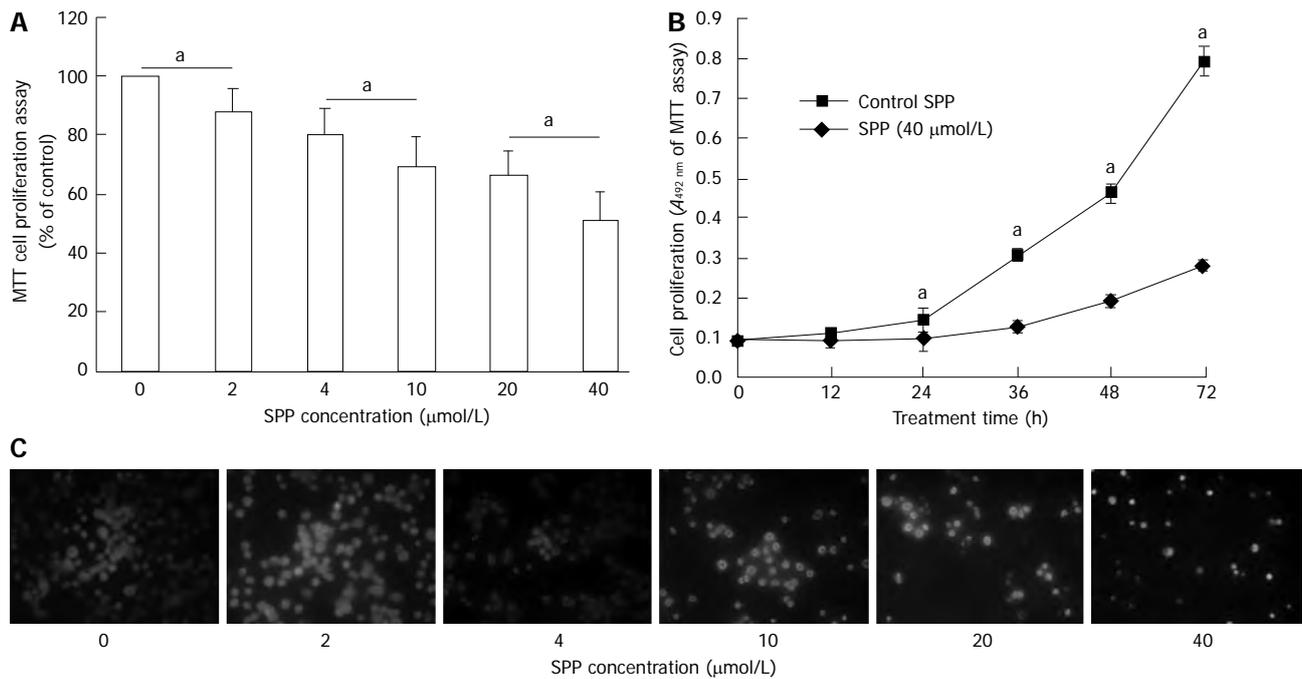
## RESULTS

### SPP purification and TI activity staining

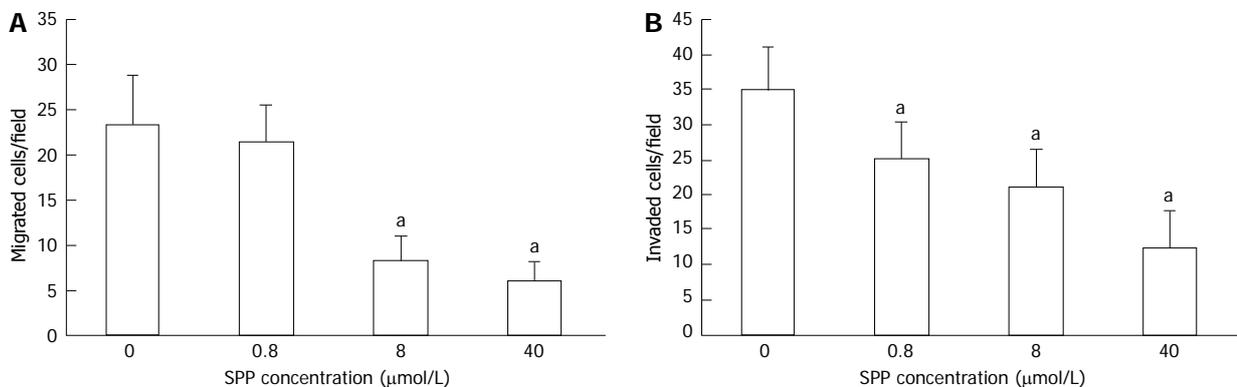
SPP was purified from fresh sweet potato tuberous roots, as reported previously<sup>[16]</sup>. Purity of SPP exceeded 99% of its dry weight. Protein (lane 1) and TI activity (lane 2) staining of purified SPP with  $\beta$ -mercaptoethanol treatment on 12% SDS-PAGE are depicted in Figure 1. Consistent with previous reports, extracted SPP had an average molecular weight of about 25 kDa. Both the monomeric protein and its 50-kDa dimer exhibited strong trypsin inhibitory activity *in vitro*.

### Effect of SPP on cell proliferation

Dose-dependent inhibition of SW480 proliferation was observed upon incubation of cells with increasing doses of SPP for 48 h. Doses of 2, 4, 10, 20 and 40  $\mu\text{mol}/\text{L}$  SPP reduced cell proliferation by 12% ( $P = 0.013$ ), 20% ( $P = 0.005$ ), 31% ( $P = 0.001$ ), 34% ( $P = 0.001$ ) and 49% ( $P = 0.001$ ), respectively (Figure 2A). Proliferation was inhibited in a time-dependent manner upon treatment of cells with 40  $\mu\text{mol}/\text{L}$  SPP (Figure 2B). After 48 h treat-



**Figure 2** Anti-proliferative effect of sweet potato protein on human colorectal cancer SW480 cells. **A:** Effects of various concentrations of sweet potato protein (SPP) on SW480 cell proliferation. We observed dose-dependent inhibition by SPP.  $^aP < 0.05$  between groups; **B:** Effect of various treatment times with 40 μmol/L SPP on SW480 cell proliferation. Time-dependent inhibition was observed.  $^aP < 0.05$  vs control; **C:** Hoechst 33258 nuclear staining. SW480 cells were incubated in the absence or presence of various concentrations of SPP for 48 h, stained with Hoechst 33258 dye, and observed under a fluorescent microscope (magnification,  $\times 400$ ). Images are representative of at least two independent experiments, with similar results.



**Figure 3** Effects of various concentrations of sweet potato protein on migration (**A**) and invasion (**B**) of SW480 cells in the Transwell assay. Inhibition of cell migration was observed with 0.8, 8 and 40 μmol/L sweet potato protein (SPP).  $^aP < 0.05$  vs control.

ment with various concentrations of SPP, fluorescent Hoechst 33258 nuclear staining indicated dose-dependent suppression of cell proliferation by SPP. Specifically, Hoechst 33258 staining revealed blue, round nuclei in viable cells and condensed or fragmented nuclei in apoptotic, compared to non-apoptotic cells. Apoptotic cells were detected when the SPP concentration exceeded 10 μmol/L (Figure 2C).

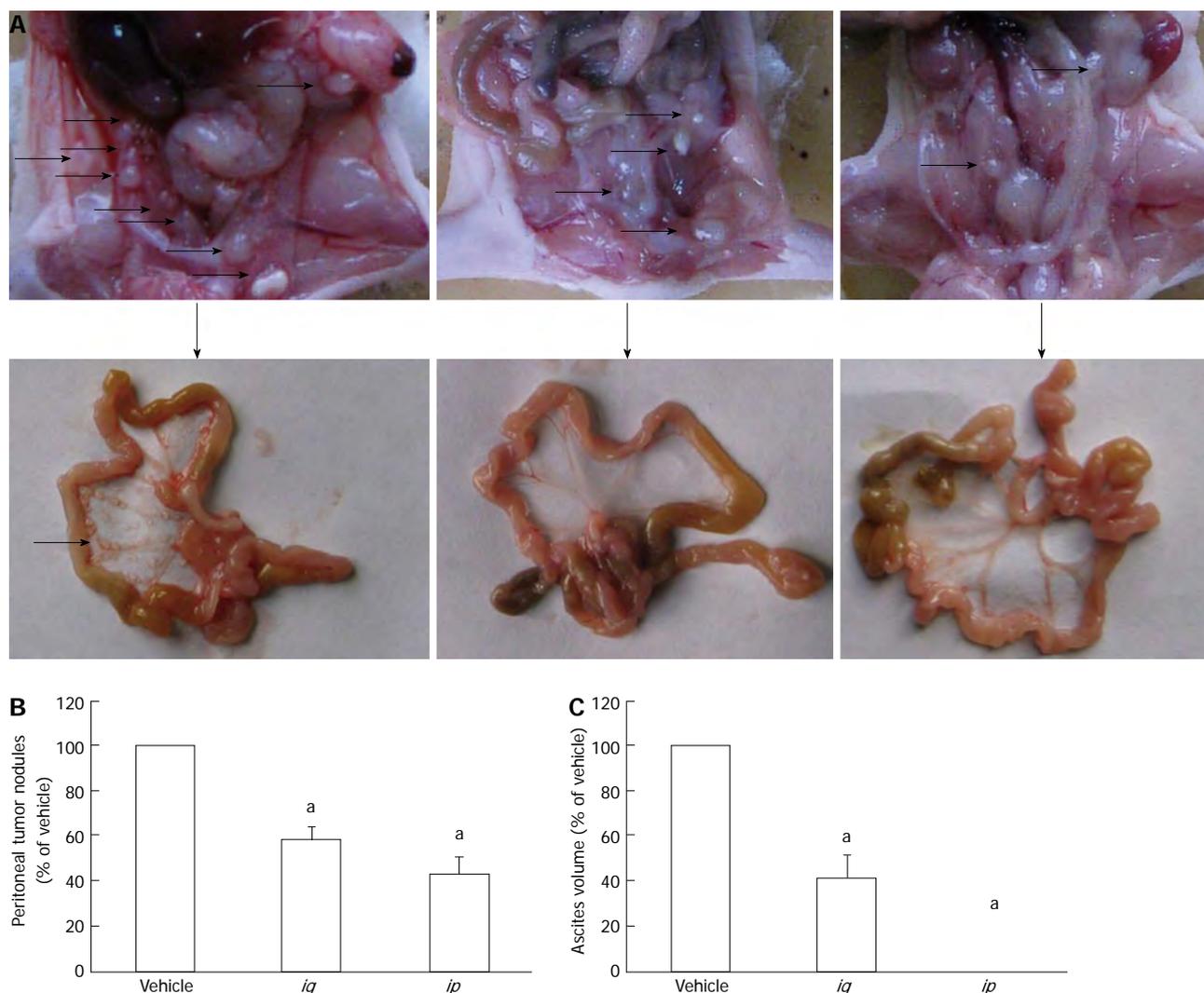
#### Effect of SPP on cell migration

Migration of SW480 cells through the 8-μm polycarbonate membrane of Transwell inserts was significantly decreased following treatment with SPP for 10 h. Doses of

0.8, 8 and 40 μmol/L SPP induced reduction of migrated cells/field from  $23.3 \pm 5.4$  in the control group to  $21.5 \pm 3.9$  ( $P = 0.068$ ),  $8.4 \pm 2.6$  ( $P = 0.031$ ) and  $6.1 \pm 2.1$  ( $P = 0.017$ ), respectively (Figure 3A).

#### Effect of SPP on cell invasion

Invasiveness of SW480 cells through the artificial basement membrane ECMatrix of Transwell inserts was significantly decreased upon incubation with SPP for 24 h. Doses of 0.8, 8 and 40 μmol/L SPP reduced the invaded cells/field from  $34.8 \pm 6.1$  in the control group to  $25.2 \pm 5.2$  ( $P = 0.038$ ),  $12.5 \pm 4.9$  ( $P = 0.024$ ) and  $6.1 \pm 2.1$  ( $P = 0.005$ ), respectively (Figure 3B).



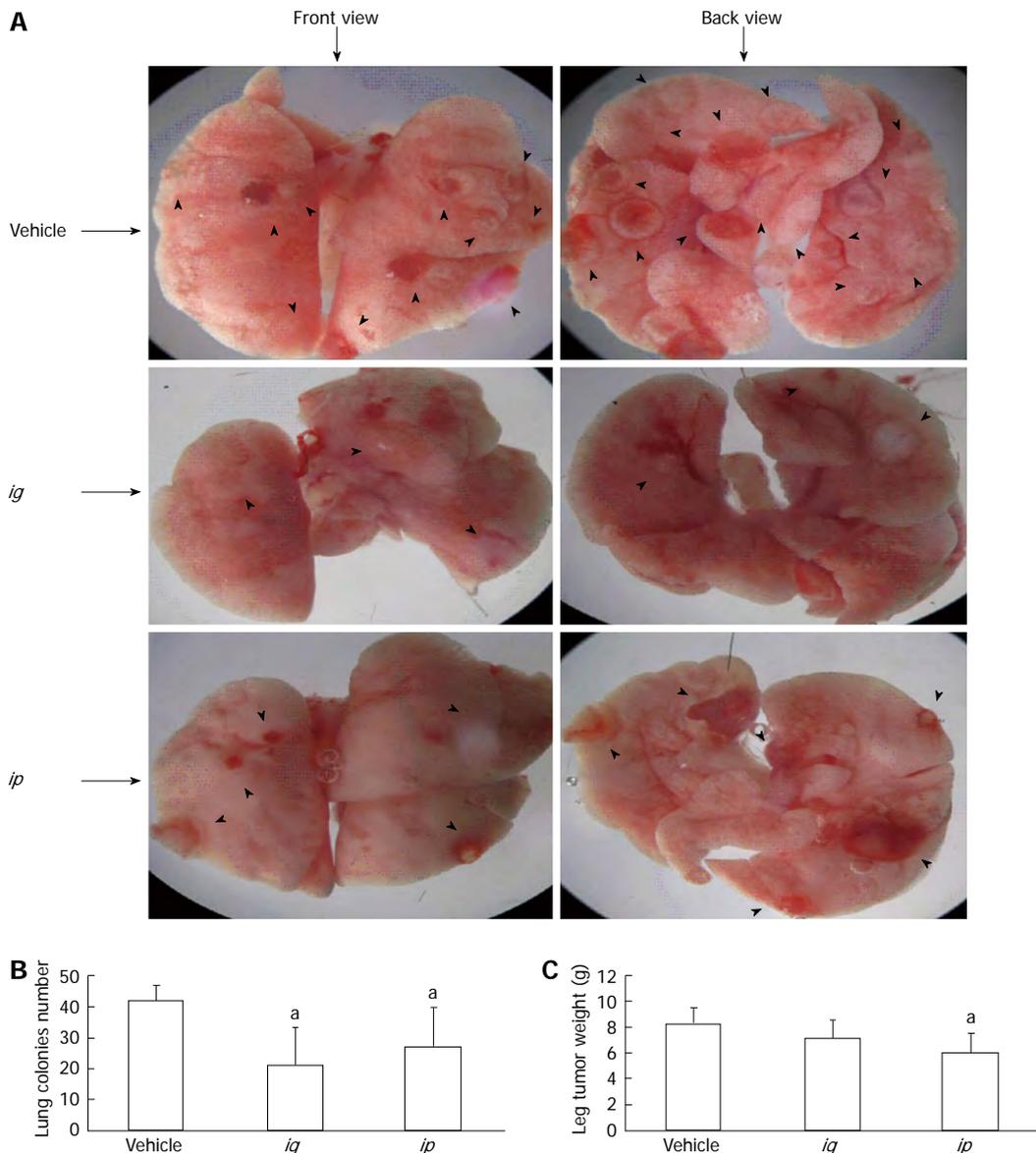
**Figure 4** Effect of sweet potato protein on growth of intraperitoneally inoculated human colorectal cancer HCT-8 cells in nude mice. A: Representative images of growth status of tumor nodules in the peritoneal cavity of nude mice after treatment with intraperitoneally injected or intragastrically infused sweet potato protein (SPP); B: Comparison of the number of tumor nodules formed in the peritoneal cavity of nude mice between different treatment groups; C: Volume of bloody ascites generated in the peritoneal cavity of nude mice. Arrows indicate the nodules formed. <sup>a</sup> $P < 0.05$  vs vehicle.

### Effects of SPP on malignant cell growth and metastasis in vivo

The growth of intraperitoneally inoculated human colorectal cancer HCT-8 cells was significantly suppressed by intraperitoneal (*ip*) or intragastric (*ig*) administration of SPP into nude mice (Figure 4). After 9 d inoculation, a significant number of tumor nodules formed in the vehicle group, which was markedly reduced to 58.0% ± 5.9% ( $P = 0.037$ ) and 43.5% ± 7.1% ( $P = 0.004$ ), respectively, compared to the vehicle, with *ig* infusion and *ip* injection of SPP (Figure 4B). Upon sacrifice, bloody ascites were found in the vehicle group, which were collected with a microsyringe, and the volumes compared between groups. The volume of ascities was significantly decreased following administration of SPP (Figure 4C). In the *ig* group, the volume decreased to 41.2 ± 10% of that of vehicle ( $P = 0.002$ ), and ascites was barely detectable in the *ip* group.

To investigate the inhibitory effect of SPP on the

metastatic capacity of malignant cells, murine Lewis lung carcinoma 3LL cells were inoculated subcutaneously into the hind legs of C57BL/6 mice, and primary tumor growth and formation of spontaneous lung metastatic colonies examined after 25 d of SPP treatment. As shown in Figure 5, after 25 d, apparent spontaneous pulmonary metastasis was evident in the vehicle-treated group (42.5 ± 4.5 nodules/lung), which was significantly inhibited following *ig* and *ip* administration of SPP into mice (21.0 ± 12.3 nodules/lung and 27.3 ± 12.7 nodules/lung, respectively,  $P < 0.05$ ). The antimetastatic effect was more significant in the *ig* than *ip* group (Figure 5B). A possible explanation for this phenomenon is that orally administered SPP is absorbed into the blood circulation and functions in the lung. However, this hypothesis requires further validation. The weights of primary tumor nodules in the hind leg of mice were additionally examined. The weight was 8.2 ± 1.3 g/mouse in the control group, which decreased to 6.1 ± 1.4 g in the *ip* group



**Figure 5** Effect of sweet potato protein on spontaneous metastasis of murine Lewis lung cancer 3LL cells in C57BL/6 mice. A: Representative images of lungs of mice after inoculation of cancer cells for 25 d; B: Number of spontaneous lung metastatic colonies formed after 25 d of inoculation; C: Weight of subcutaneously inoculated tumor nodules after 25 d. Arrowheads indicate the nodules formed. <sup>a</sup> $P < 0.05$  vs vehicle.

( $P = 0.035$ ; Figure 5C). The observed reduction in the *ig* group ( $7.1 \pm 1.5$  g/mouse,  $P > 0.05$ ) was not statistically significant, suggesting that *ip* administration had a more significant effect than *ig* administration.

In both animal experiments, changes in the body weight of mice were monitored. After tumor cell transplantation, body weight stopped increasing. However, SPP treatment did not significantly affect the growth of mice during the experimental period, compared with the vehicle group (Table 1). Moreover, no apparent adverse effects of SPP were observed.

## DISCUSSION

The present study showed that SPP promotes dose- and time-dependent inhibition of human colorectal cancer SW480 cell proliferation, migration and invasion. The an-

tiproliferative and antimetastatic effects of SPP were subsequently confirmed in nude and C57BL/6 mice *in vivo*.

SPP isolated from sweet potato had a strong trypsin inhibitory effect, as shown in Figure 1, consistent with previous findings<sup>[9]</sup>. In recent years, several families of proteases, including matrix metalloproteinases and serine and cysteine proteases, have been shown to play important roles in tumor invasion and metastasis, because formation of metastasis requires degradation of ECM<sup>[17]</sup>. The initial hypothesis that the inhibitors function in suppressing cancer invasion and metastasis was confirmed in subsequent studies. For instance, urokinase-type plasminogen activator (uPA), a serine protease, plays a critical role in cancer cell migration, ECM invasion, and metastasis in a variety of tumors<sup>[18-21]</sup>. In contrast, several serine protease inhibitors, including urinary KTI bikunin and soybean KTI, have been shown to suppress the expres-

**Table 1** Body weights of mice before and after sweet potato protein treatment

|           | BALB/c nude (g) |            | C57BL/6 (g) |            |
|-----------|-----------------|------------|-------------|------------|
|           | Before          | After      | Before      | After      |
| Vehicle   | 14.3 ± 0.9      | 14.6 ± 1.0 | 13.9 ± 0.6  | 14.1 ± 0.7 |
| <i>ig</i> | 14.6 ± 0.6      | 14.8 ± 0.6 | 13.4 ± 0.5  | 13.9 ± 0.7 |
| <i>ip</i> | 14.5 ± 0.5      | 14.7 ± 0.6 | 13.8 ± 0.5  | 13.9 ± 0.6 |

sion of uPA and its receptor, uPAR, at both the gene and protein levels<sup>[22,23]</sup>. Bikunin is reported to suppress invasiveness in a number of malignant cell lines by directly inhibiting tumor-cell-associated serine protease plasmin activity, as well as uPA and uPAR expression<sup>[24,25]</sup>. Notably, the therapeutic efficacy of orally administered bikunin against human ovarian cancer HRA cells growing in the peritoneum of nude mice and human cancer has been demonstrated<sup>[26]</sup>. Specifically, bikunin (30 mg/kg per day) induced a 40% decrease in tumor load in mice. In cancer patients, bikunin exhibited biological activity, as the post-treatment uPA levels in uterine cervical carcinoma tissue specimens were significantly decreased<sup>[26]</sup>. Analogous to SPP in the present study, soybean KTI is a natural trypsin inhibitor that has a similar effect as bikunin on the uPA signaling cascade and cancer cell invasion<sup>[27]</sup>. Dietary supplementation with soybean KTI (15 and 50 g/kg) also significantly reduced the tumor burden in an *in vivo* spontaneous metastasis assay in C57BL/6 mice<sup>[28]</sup>. KTIs isolated from sweet potato clearly have an anticancer effect. For example, Huang *et al.*<sup>[11]</sup> observed growth inhibition and apoptotic effects of TI from sweet potato in NB4 promyelocytic leukemia cells. More recently, Yao *et al.*<sup>[12]</sup> reported a significant antiproliferative effect of SPP in human tongue carcinoma Tca8113 cells. The group used the same method to isolate SPP from sweet potato roots as that used in the present study. The anticancer effects of SPP reported by our group are therefore consistent with earlier findings.

Several mechanisms have been proposed to explain the mechanism of action of SPP. First, as a serine protease inhibitor, SPP may directly suppress degradation of the ECM by tumor cells, similar to bikunin and soybean KTI. Second, it may exert its role indirectly by suppressing signaling pathways involved in the proliferation and invasion of cancer cells<sup>[12,15,23]</sup>. Yao *et al.*<sup>[12]</sup> have suggested that the effect of SPP results partly from induction of apoptosis of tongue cancer cells *via* downregulation of the Akt/GSK-3 pathway. Huang *et al.*<sup>[11]</sup> have demonstrated that sweet potato TI induces apoptosis in NB4 cells through a mitochondria-dependent pathway, associated with activation of the caspase-3 and -8 cascades. Moreover, an anti-inflammatory effect of KTIs has been reported, including suppression of proinflammatory cytokines, such as tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6<sup>[29]</sup>. This may additionally, in part, explain their effects on tumor development. Bikunin therapy leads to significant inhibition of angiogenesis-related molecules (vascular endothelial growth factor and fi-

broblast growth factor- $\beta$ ), and this antiangiogenic effect also partly explains the effect of KTIs in cancer cells<sup>[30]</sup>. Shakiba *et al.*<sup>[27]</sup> have reported that soybean KTI has a strong inhibitory effect on human umbilical vein endothelial cell migration and tubulogenesis in fibrin matrix. These findings collectively indicate that KTIs present multiple mechanisms of action that are cell type dependent. The results from the current study clearly show that SPP suppresses growth of cancer cells, both *in vitro* and *in vivo*. Moreover, the invasiveness of cancer cells is inhibited by SPP. However, the specific mechanisms of action involved in the anticancer activity of SPP remain to be established.

Another particularly interesting finding of the present study was that although *ip* injection of SPP had a more significant suppressive effect on primary tumors (Figure 5C), the antimetastatic effect against spontaneous lung metastasis of cancer cells was better in the *ig* than *ip* group (Figure 5B). One possible explanation for this phenomenon is that orally administered SPP is absorbed into the blood circulation and exerts its activity in the lung. This theory is supported by the finding that dietary supplementation of soybean and urinary KTIs is effective<sup>[26,28]</sup>. It is also possible that the SPP is digested in the gastrointestinal tract and its active peptides absorbed into the circulation. However, this hypothesis needs validation.

In summary, our results demonstrate that the SPP markedly inhibits proliferation, migration and invasion of human colorectal cancer SW480 cells *in vitro*. Moreover, SPP administered both orally and intraperitoneally exerts its effect on cancer cells *in vivo*. The antiproliferative activity of SPP may be triggered through induction of apoptosis of malignant cells, and anti-invasive activity through inhibition of the uPA signaling pathways. Antiangiogenic and anti-inflammatory activities may, at least in part, explain its effects on malignant cells in tumor-bearing animals. However, further studies are required to elucidate the mechanisms underlying the protective effects of SPP in CRC.

## COMMENTS

### Background

Colorectal cancer (CRC) is one of the major contributors to cancer-induced mortality worldwide. Sweet potato protein (SPP), a type of trypsin inhibitor (TI) that induces apoptosis and suppresses growth of various malignant cells, is a potentially effective anticancer agent for CRC.

### Research frontiers

Previous studies suggest that SPP inhibits growth and induces apoptosis in promyelocytic leukemia and human tongue carcinoma cells. A similar TI extracted from wampee (*Clausena lansium*) seeds has a growth inhibitory effect in human leukemia and hepatoma cells. However, the issue of whether SPP affects the growth and metastasis of CRC cells has remained unclear until now. In the current study, the antiproliferative and antimetastatic effects of SPP on human colorectal cancer cells were investigated *in vitro* and *in vivo*.

### Innovations and breakthroughs

Several recent studies have demonstrated that SPP affects the growth of malignant cancer cells, although the issue of whether SPP plays a role in CRC is yet to be established. The current study is believed to be the first thorough investigation focusing on the antiproliferative and antimetastatic effects of SPP on CRC cell lines, both *in vitro* and *in vivo*.

## Applications

Elucidation of the effects of SPP on CRC cell lines *in vitro* and *in vivo* may present an effective strategy for prevention or treatment of CRC in the clinic.

## Terminology

TIs are a group of peptides present in various sources, such as soybean, egg white and sweet potato, which mask or inhibit the active site of the trypsin molecule. Storage proteins are biological reserves of metal ions and amino acids used by organisms. Amino acids of storage proteins are used during the embryonic development of animals or plants.

## Peer review

This was a good experimental study in which authors investigated the effects of SPP on malignant cancer cells in different cell lines and animal models. The results are interesting, and support the utility of SPP as a potential therapeutic agent for preventing and treating CRC.

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P- Reviewer Zhang J S- Editor Zhai HH L- Editor Kerr C  
E- Editor Li JY



## Efficacy of adjuvant XELOX and FOLFOX6 chemotherapy after D2 dissection for gastric cancer

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Supported by National Natural Science Foundation of China, No. 30700805 and 81272643; Project 5010 from Sun Yat-Sen University, No. 20100816; Young Teacher Training Project of Sun Yat-Sen University, No. 09ykpy49

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Received: January 30, 2013 Revised: April 16, 2013

Accepted: May 7, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To compare the efficacy of capecitabine and oxaliplatin (XELOX) with 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX6) in gastric cancer patients after D2 dissection.

**METHODS:** Between May 2004 and June 2010, patients in our gastric cancer database who underwent D2 dissection for gastric cancer at the First Affiliated Hospital of Sun Yat-Sen University were retrospectively analyzed. A total of 896 patients were enrolled into this study according to the established inclusion and exclusion criteria. Of these patients, 214 received the XELOX

regimen, 48 received FOLFOX6 therapy and 634 patients underwent surgery only without chemotherapy. Overall survival was compared among the three groups using Cox regression and propensity score matched-pair analyses.

**RESULTS:** Patients in the XELOX and FOLFOX6 groups were younger at the time of treatment (median age 55.2 years; 51.2 years *vs* 58.9 years), had more undifferentiated tumors (70.1%; 70.8% *vs* 61.4%), and more lymph node metastases (80.8%; 83.3% *vs* 57.7%), respectively. Overall 5-year survival was 57.3% in the XELOX group which was higher than that (47.5%) in the surgery only group ( $P = 0.062$ ) and that (34.5%) in the FOLFOX6 group ( $P = 0.022$ ). Multivariate analysis showed that XELOX therapy was an independent prognostic factor (hazard ratio = 0.564,  $P < 0.001$ ). After propensity score adjustment, XELOX significantly increased overall 5-year survival compared to surgery only (58.2% *vs* 44.2%,  $P = 0.025$ ) but not compared to FOLFOX6 therapy (48.5% *vs* 42.7%,  $P = 0.685$ ). The incidence of grade 3/4 adverse reactions was similar between the XELOX and FOLFOX6 groups, and more patients suffered from hand-foot syndrome in the XELOX group ( $P = 0.018$ ).

**CONCLUSION:** Adjuvant XELOX therapy is associated with better survival in patients after D2 dissection, but does not result in a greater survival benefit compared with FOLFOX6 therapy.

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**Key words:** Gastric cancer; D2 dissection; Adjuvant; Capecitabine and oxaliplatin; 5-fluorouracil, folinic acid and oxaliplatin

**Core tip:** This original study retrospectively analyzed the efficacy of adjuvant chemotherapy and compared the effects of adjuvant capecitabine and oxaliplatin (XELOX) therapy with 5-fluorouracil, folinic acid and oxaliplatin

(FOLFOX6) therapy in gastric cancer patients undergoing D2 dissection. Propensity score matched-pair analysis was performed to account for biases associated with retrospective data. Adjuvant XELOX was significantly associated with improved survival after D2 dissection compared to surgery alone following multivariate Cox regression and propensity score matched analyses, however, the XELOX regimen did not result in a greater survival benefit compared with the FOLFOX6 regimen. Our findings suggest that adjuvant XELOX therapy should be considered in curable gastric cancer patients.

Wu Y, Wei ZW, He YL, Schwarz RE, Smith DD, Xia GK, Zhang CH. Efficacy of adjuvant XELOX and FOLFOX6 chemotherapy after D2 dissection for gastric cancer. *World J Gastroenterol* 2013; 19(21): 3309-3315 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i21/3309.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3309>

## INTRODUCTION

Gastric cancer (GC) is the fourth prevalent cancer and second most common cause of cancer-related deaths worldwide, with half of all patients in East Asia<sup>[1-4]</sup>. Radical resection remains the only curative treatment for GC<sup>[1,5,6]</sup>. Radical gastrectomy with D2 lymphadenectomy is the standard treatment for patients with resectable GC in Eastern countries<sup>[1,6,7]</sup> and is now recommended in Western countries<sup>[3,8-10]</sup>. However, even if radical resection is performed, about 50% patients have recurrence within 5 years of surgery<sup>[5,11,12]</sup> and 50%-90% of patients die due to disease relapse<sup>[13]</sup>. Therefore, novel approaches such as multimodality therapies are being explored to improve treatment outcome. Adjuvant chemotherapy has been intensively investigated for several decades, but definitive evidence is limited especially in patients after D2 dissection<sup>[14-16]</sup>.

The chemotherapeutic regimen after GC resection is heterogeneous throughout the world and the optimal regimen has not yet been determined<sup>[15,17]</sup>. S-1 plus cisplatin is considered the standard regimen for advanced GC in Japan<sup>[18,19]</sup>, while its application outside Japan remains uncertain. In the United States, the standard of care is adjuvant bolus 5-fluorouracil (5-FU)-based chemoradiotherapy for resected GC based primarily on the results of the Intergroup 116 trial<sup>[20]</sup>. In European countries, the epirubicin, cisplatin and 5-FU (ECF) regimen was associated with survival benefits in the MAGIC trial<sup>[21]</sup>, but not in the Intergroup CALGB 80101 trial<sup>[22]</sup>. Recently, the CLASSIC trial<sup>[23]</sup> carried out in 37 centers in South Korea, China and Taiwan showed that 6 mo of adjuvant capecitabine and oxaliplatin (XELOX) chemotherapy improved 3-year disease-free survival compared with surgery alone. However, this was a pre-specified interim efficacy analysis and not yet formally validated. Nevertheless, based on published data, adjuvant chemotherapy might improve survival, however, further definite evidence is needed, and a clearly superior strategy has not yet emerged. Adjuvant XELOX

and 5-FU, folinic acid and oxaliplatin (FOLFOX6) chemotherapy has been widely used in GC patients. However, few studies have directly compared their efficacy.

In the present study, we used our GC database prospectively established since 1994 and retrospectively analyzed the efficacy of adjuvant XELOX and FOLFOX6 therapy in GC patients undergoing D2 dissection. This study aimed to: (1) examine the benefit of adjuvant chemotherapy; (2) compare the prognosis of patients undergoing D2 dissection plus XELOX or FOLFOX6 with that of patients undergoing surgery only; and (3) account for biases associated with treatment selection of adjuvant chemotherapy in retrospective data through propensity score adjusted analysis.

## MATERIALS AND METHODS

### Patient population

From May 2004 to June 2010, 1083 patients in our database underwent gastrectomy with D2 nodal dissection for GC at the First Affiliated Hospital of Sun Yat-Sen University. This study was approved by the Ethical Review Committee of the First Affiliated Hospital of Sun Yat-Sen University. All patients signed an informed consent. The stage of gastric carcinoma was determined according to the TNM Classification of Malignant Tumors established by the International Union against Cancer 7<sup>th</sup> edition<sup>[24]</sup>.

### Inclusion and exclusion criteria

The inclusion criteria for this study were as follows: (1) histologically proven advanced GC, radical gastrectomy with D2 lymph-node dissection and R0 surgery; (2) patients aged between 20 and 75 years; (3) no preoperative chemotherapy, immunotherapy or radiotherapy; (4) patients receiving chemotherapy 4 wk after surgery; and (5) no synchronous or metachronous cancers.

Five exclusion criteria were employed: (1) age > 75 or < 20 years; (2) hepatic, renal, pulmonary or cardiac dysfunction; (3) severe postoperative complications, such as anastomotic fistula and pancreatic fistula; (4) less than 15 lymph nodes retrieved; and (5) loss to follow-up.

According to the inclusion and exclusion criteria, a total of 896 patients were included in the final analysis. Of these patients, 214 (23.9%) received XELOX chemotherapy (the XELOX group), 48 (5.4%) received FOLFOX6 chemotherapy (the FOLFOX6 group), and 634 (70.7%) underwent surgery only without chemotherapy (the surgery only group).

### Chemotherapy regimen

The XELOX regimen consisted of 3-wk cycles of oral capecitabine (1000 mg/m<sup>2</sup> twice daily on days 1-14 of each cycle plus intravenous oxaliplatin (130 mg/m<sup>2</sup> on day 1 of each cycle). The FOLFOX6 regimen consisted of 2-wk cycles of intravenous oxaliplatin 85-100 mg/m<sup>2</sup> and leucovorin 200 mg/m<sup>2</sup> over 2 h on day 1 of each cycle plus 5-FU 400 mg/m<sup>2</sup> bolus with infusional 5-FU 2400 mg/m<sup>2</sup> in 48 h of each cycle. The median chemo-

therapy duration was 8 cycles in the XELOX group and 7 in the FOLFOX6 group. All patients underwent weekly clinical evaluation and routine blood examinations during chemotherapeutic treatment. Chemotherapeutic adverse reactions were graded on a 0-4 scale for acute and sub-acute toxicity in accordance with the WHO guidelines for anti-cancer drugs.

### Follow-up

Follow-up assessments were performed every 3 mo for the first 2 years after surgery and then every 6 mo until the patient's death. The survival status of patients was ascertained in December 2011. Median follow-up was 39.7 mo (range 6-87.6 mo).

### Statistical analysis

Analyses were performed using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, United States). The overall survival was recorded from the date of surgery to the date of death from any cause or last follow-up. Survival curves and overall 5-year survival rates were established according to the Kaplan-Meier and Log-Rank methods. Hazard ratios for death were calculated by Cox regression analysis with backward model selection. The potential prognostic factors entered into the Cox regression model were as follows: chemotherapy (surgery only *vs* XELOX *vs* FOLFOX6); age (continuous variable); gender; tumor location (whole stomach *vs* upper *vs* middle *vs* lower *vs* remnant stomach); pathological T category (T1 and 2 *vs* T3 *vs* T4a *vs* T4b); pathological N category (N0 *vs* N1 *vs* N2 *vs* N3a *vs* N3b); macroscopic Borrmann type (I and II *vs* III *vs* IV and V); and tumor differentiation (undifferentiated *vs* differentiated). Two-sided *P* values were calculated for all tests. *P* values less than 0.05 were considered statistically significant.

To reduce selection biases associated with retrospective data, propensity score matched-pair analysis was performed with 1 to 1 matching (XELOX and surgery only; XELOX and FOLFOX6). The propensity score method was used to determine the probability of an individual patient having received a certain treatment as a function of several confounding covariates that were collapsed into a single predictor<sup>[25-27]</sup>. Individuals were matched for age, gender, tumor location, Borrmann type, number of retrieved nodes, tumor differentiation, pathological T category and pathological N category. In total, there were 370 patients in the XELOX *vs* surgery only analysis (*n* = 185 for adjuvant XELOX and *n* = 185 for surgery only) and 74 patients in the XELOX *vs* FOLFOX6 analysis (*n* = 37 for XELOX and *n* = 37 for FOLFOX6). Kaplan-Meier methods and Log-Rank analyses were then performed.

## RESULTS

The clinicopathological characteristics of the 896 patients are shown in Table 1. Compared to the surgery only group, patients in the XELOX and FOLFOX6 groups were younger at the time of treatment (median age 55.2

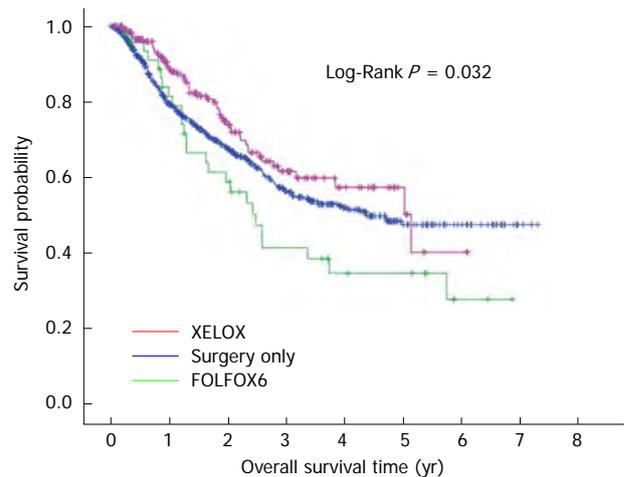


Figure 1 Overall survival analysis according to the Kaplan-Meier method for patients in the surgery only group (*n* = 634), the capecitabine and oxaliplatin group (*n* = 214) and the 5-fluorouracil, folinic acid and oxaliplatin group (*n* = 48). Overall survival was higher in the capecitabine and oxaliplatin (XELOX) group *vs* the surgery only group (*P* = 0.069) and 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX6) groups (*P* = 0.022).

years; 51.2 years *vs* 58.9 years), had more undifferentiated tumors (70.1%; 70.8% *vs* 61.4%), and more lymph node metastases (80.8%; 83.3% *vs* 57.7%), respectively. There were no significant differences in baseline features between the XELOX and FOLFOX6 groups.

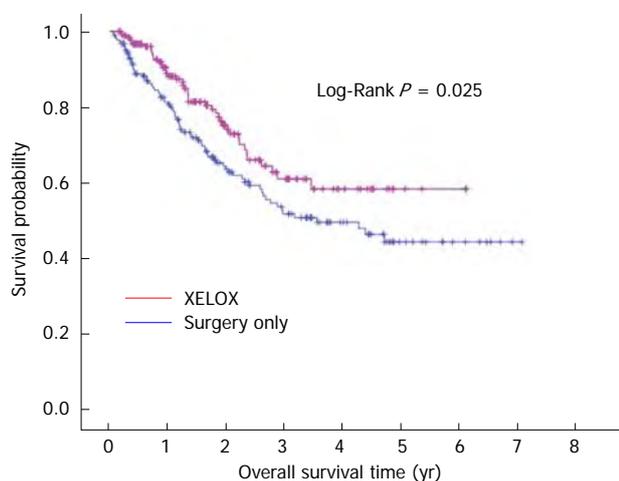
In the 896 patients, the overall mean and median survival were 57.8 and 52.7 mo, respectively. Overall 3-year and 5-year survival rates were 56.4% and 47.5% in the surgery only group, 61.5% and 57.3% in the XELOX group, and 41.3% and 34.5% in the FOLFOX6 group, respectively. Overall survival rate in the XELOX group was higher than that in the surgery only group (*P* = 0.069) and the FOLFOX6 group (*P* = 0.022) (Figure 1). Following multivariate analysis, adjuvant chemotherapy was found to have a prognostic influence on the hazard ratio (HR) for death (*P* = 0.001), and the HR for death was 0.564 (95%CI: 0.416-0.765; *P* < 0.001) for XELOX therapy (Table 2). In addition, pathological T category, pathological N category, tumor location, and macroscopic Borrmann type were also prognostic factors for overall survival. Recurrence rate was 24.3% (52/214) in the XELOX group, 31.3% (15/48) in the FOLFOX6 group and 32.8% (208/634) in the surgery only group. Recurrence rate in the XELOX group was lower than that in the surgery only (*P* = 0.021) and the FOLFOX6 (*P* = 0.36) groups.

In an attempt to eliminate treatment selection bias associated with retrospective data, a propensity score matched-pair analysis was performed using the following variables: age, gender, tumor location, Borrmann type, number of retrieved nodes, tumor differentiation, pathological T category and pathological N category. This resulted in a total of 370 patients for the XELOX *vs* surgery only analysis with 185 cases per treatment arm, and a total of 74 patients for the XELOX *vs* FOLFOX6 analysis with 37 cases per treatment arm. There were no significant differences (*P* > 0.05) among the matched

**Table 1 Patient characteristics *n* (%)**

|                               | Surgery only      | FOLFOX6          |                | XELOX             |                | <i>P</i> value <sup>1</sup> |
|-------------------------------|-------------------|------------------|----------------|-------------------|----------------|-----------------------------|
|                               | ( <i>n</i> = 634) | ( <i>n</i> = 48) | <i>P</i> value | ( <i>n</i> = 214) | <i>P</i> value |                             |
| Age (yr)                      |                   |                  |                |                   |                |                             |
| Mean ± SD                     | 58.9 ± 12.5       | 51.2 ± 13.2      | < 0.001        | 55.2 ± 11.4       | < 0.001        | 0.051                       |
| Median                        | 60                | 52               |                | 57                |                |                             |
| Tumor location, whole stomach | 23 (3.6)          | 5 (10.4)         |                | 7 (3.3)           |                | 0.124                       |
| Upper                         | 192 (30.3)        | 11 (22.9)        |                | 55 (25.7)         |                |                             |
| Middle                        | 148 (23.3)        | 16 (33.3)        |                | 61 (28.5)         |                |                             |
| Lower                         | 235 (37.1)        | 14 (29.2)        |                | 87 (40.7)         |                |                             |
| Remnant stomach               | 36 (5.7)          | 2 (4.2)          |                | 4 (1.9)           |                |                             |
| Macroscopic Borrmann type     |                   |                  | 0.035          |                   | 0.014          | 0.064                       |
| I                             | 39 (6.2)          | 0 (0.0)          |                | 13 (6.1)          |                |                             |
| II                            | 171 (27)          | 7 (14.6)         |                | 39 (18.2)         |                |                             |
| III                           | 333 (52.5)        | 30 (62.5)        |                | 138 (64.5)        |                |                             |
| IV and V                      | 91 (14.4)         | 11 (22.9)        |                | 24 (11.2)         |                |                             |
| Histological type             |                   |                  | 0.219          |                   | 0.013          | 0.919                       |
| Differentiated                | 245 (38.6)        | 14 (29.2)        |                | 64 (29.9)         |                |                             |
| Undifferentiated              | 389 (61.4)        | 34 (70.8)        |                | 150 (70.1)        |                |                             |
| Pathological T category       |                   |                  | < 0.001        |                   | < 0.001        | 0.087                       |
| T1 and 2                      | 117 (18.5)        | 0 (0.0)          |                | 9 (4.2)           |                |                             |
| T3                            | 73 (11.5)         | 1 (2.1)          |                | 23 (10.7)         |                |                             |
| T4a                           | 342 (53.9)        | 41 (85.4)        |                | 152 (71)          |                |                             |
| T4b                           | 102 (16.1)        | 6 (12.5)         |                | 30 (14)           |                |                             |
| Retrieved node                |                   |                  | 0.019          |                   | 0.004          | 0.339                       |
| Mean ± SD                     | 26.4 ± 15.9       | 32.1 ± 19.2      |                | 29.7 ± 14.5       |                |                             |
| Median (range)                | 24 (0-102)        | 31 (0-89)        |                | 27 (1-91)         |                |                             |
| Pathological N category       |                   |                  | 0.005          |                   | < 0.001        | 0.085                       |
| N0                            | 268 (42.3)        | 8 (16.7)         |                | 41 (19.2)         |                |                             |
| N1                            | 91 (14.4)         | 7 (14.6)         |                | 43 (20.1)         |                |                             |
| N2                            | 108 (17)          | 12 (25)          |                | 52 (24.3)         |                |                             |
| N3a                           | 89 (14)           | 9 (18.8)         |                | 56 (26.2)         |                |                             |
| N3b                           | 78 (12.3)         | 12 (25)          |                | 22 (10.3)         |                |                             |

<sup>1</sup>Capecitabine and oxaliplatin (XELOX) group *vs* 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX) group.



**Figure 2 Overall survival analysis according to the Kaplan-Meier method for patients in the D2 surgery only group (*n* = 185) and in the D2 surgery plus capecitabine and oxaliplatin group (*n* = 185) after propensity score matched-pair adjustment. XELOX: Capecitabine and oxaliplatin.**

variables by treatment group (results not shown). On matched analysis, adjuvant XELOX therapy significantly improved overall survival compared to surgery only with longer mean overall survival (51.2 mo *vs* 48.9 mo) and better 3-year (60.9% *vs* 51.7%) and 5-year survival (58.2% *vs* 44.2%, *P* = 0.025) (Figure 2). However, there was no

significant difference in overall survival between the XELOX and FOLFOX6 groups (*P* = 0.685). Overall 3-year survival was 42.7% in the FOLFOX6 group and 48.5% in the XELOX group with a median survival of 37.5 and 44.7 mo, respectively.

Grade 3/4 adverse reactions in the XELOX and FOLFOX6 groups are shown in Table 3. A total of 75 patients in the XELOX group (35%) experienced grade 3/4 adverse events similar to the rate in the FOLFOX6 group (19 patients, 39.6%, *P* = 0.618). More patients in the XELOX group experienced hand-foot syndrome than in the FOLFOX6 group (9.8% *vs* 0%, *P* = 0.018).

## DISCUSSION

This study showed that adjuvant XELOX was significantly associated with improved survival after D2 dissection for GC compared to D2 dissection alone, regardless of age, tumor differentiation, and nodal status. After adjustment for confounders in the propensity score analysis, adjuvant XELOX therapy improved 5-year overall survival by approximately 14% (*P* = 0.025) compared to surgery only, but was not associated with a greater survival benefit than FOLFOX6 therapy (48.5% *vs* 42.7%, *P* = 0.685). These results demonstrated that adjuvant XELOX therapy should be considered in curable GC patients.

D2 gastrectomy has been the standard surgical proce-

**Table 2** Multivariate survival analysis by Cox regressions

|                           | HR and 95%CI |           |       |         | Overall |
|---------------------------|--------------|-----------|-------|---------|---------|
|                           | HR           | Lower     | Upper | P value | P value |
| Adjuvant chemotherapy     |              |           |       |         | 0.001   |
| Surgery only              |              | Reference |       |         |         |
| FOLFOX6                   | 0.762        | 0.503     | 1.156 | 0.201   |         |
| XELOX                     | 0.564        | 0.416     | 0.765 | < 0.001 |         |
| Pathological N category   |              |           |       |         | < 0.001 |
| N0                        |              | Reference |       |         |         |
| N1                        | 1.613        | 1.049     | 2.483 | 0.030   |         |
| N2                        | 2.952        | 2.011     | 4.334 | < 0.001 |         |
| N3a                       | 3.938        | 2.702     | 5.739 | < 0.001 |         |
| N3b                       | 5.025        | 3.382     | 7.465 | < 0.001 |         |
| Tumor location            |              |           |       |         | 0.003   |
| Whole stomach             |              | Reference |       |         |         |
| Upper                     | 0.671        | 0.421     | 1.071 | 0.094   |         |
| Middle                    | 0.663        | 0.414     | 1.064 | 0.088   |         |
| Lower                     | 0.681        | 0.422     | 1.099 | 0.115   |         |
| Remnant stomach           | 1.454        | 0.791     | 2.675 | 0.228   |         |
| Macroscopic Borrmann type |              |           |       |         | < 0.001 |
| I and II                  |              | Reference |       |         |         |
| III                       | 1.199        | 0.875     | 1.642 | 0.258   |         |
| IV and V                  | 2.142        | 1.469     | 3.124 | < 0.001 |         |
| Pathological T category   |              |           |       |         | < 0.001 |
| T1 and 2                  |              | Reference |       |         |         |
| T3                        | 1.112        | 0.477     | 2.590 | 0.806   |         |
| T4a                       | 3.010        | 1.574     | 5.757 | 0.001   |         |
| T4b                       | 4.944        | 2.477     | 9.870 | < 0.001 |         |

HR: Hazard ratio; FOLFOX6: 5-fluorouracil, folinic acid and oxaliplatin; XELOX: Capecitabine and oxaliplatin.

ture for GC in Eastern countries for several decades. For accurate pathological N category, at least 15 nodes should be retrieved for pathological examination according to the International Union against Cancer 7<sup>th</sup> edition<sup>[24]</sup>. Accurate tumor stage supports correct prognostic evaluation. This is why patients with less than 15 nodes were excluded in the current study. However, few studies have compared D2 surgery only with D2 surgery plus adjuvant chemotherapy in patients with GC. The JCOG 9206-1 clinical trial<sup>[28]</sup> failed to demonstrate the survival benefits of adjuvant chemotherapy with intravenous mitomycin, fluorouracil, and cytarabine followed by oral fluorouracil after D2 or greater dissection. The JCOG 9206-2 phase III trial also showed no survival benefit with adjuvant intraperitoneal and intravenous cisplatin followed by oral fluorouracil in serosa-positive GC after D2 dissection compared to D2 dissection alone<sup>[29]</sup>. However, the phase III CLASSIC study<sup>[23]</sup> reported a significant improvement in 3-year disease-free survival with the XELOX regimen compared to surgery alone. Our study demonstrated that adjuvant XELOX significantly improved 5-year survival compared to D2 dissection only. As one center involved in the CLASSIC trial, results in the current study were consistent with the preliminary 3-year report of the CLASSIC trial and further confirmed the efficacy of adjuvant XELOX chemotherapy after D2 dissection in the Chinese population based on overall 5-year survival data. Compared to the CLASSIC trial, the 3-year overall survival rate was relatively low in our study (61.5% *vs* 83%

**Table 3** Grade 3/4 adverse reactions in 5-fluorouracil, folinic acid and oxaliplatin and capecitabine and oxaliplatin treatment groups *n* (%)

| Adverse reactions          | FOLFOX6<br>( <i>n</i> = 48) | XELOX<br>( <i>n</i> = 214) | P value |
|----------------------------|-----------------------------|----------------------------|---------|
| At least one adverse event | 19 (39.6)                   | 75 (35)                    | 0.618   |
| Vomiting                   | 5 (10.4)                    | 16 (7.5)                   | 0.555   |
| Anorexia                   | 5 (10.4)                    | 20 (9.3)                   | 0.788   |
| Oral mucositis             | 1 (2.0)                     | 3 (1.4)                    | 0.557   |
| Diarrhea                   | 4 (8.3)                     | 14 (6.5)                   | 0.751   |
| Hand-foot syndrome         | 0 (0.0)                     | 21 (9.8)                   | 0.018   |
| Peripheral neurotoxicity   | 9 (18.8)                    | 25 (11.7)                  | 0.232   |
| Leukocyte/neutropenia      | 7 (14.5)                    | 16 (7.5)                   | 0.153   |
| Thrombocytopenia           | 4 (8.3)                     | 14 (6.5)                   | 0.751   |
| ALT/AST increase           | 1 (2.0)                     | 5 (2.3)                    | 0.999   |

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; FOLFOX6: 5-fluorouracil, folinic acid and oxaliplatin; XELOX: Capecitabine and oxaliplatin.

in the XELOX group and 57.3% *vs* 78% in the control group). The main reasons for this may be related to the different population and advanced disease in our study. To eliminate selection biases associated with retrospective data, propensity score adjusted and matched-pair analyses were performed, and adjuvant XELOX therapy showed improved survival after D2 dissection in GC patients compared to surgery only. Our findings demonstrated that adjuvant XELOX was an effective therapy for patients with resectable GC.

In this study, adjuvant XELOX therapy was associated with a significantly higher overall survival than FOLFOX6 before propensity score adjusted analysis. However, this significant difference disappeared after propensity score matched analysis. A similar phenomenon occurred during a comparison between XELOX therapy and surgery only with or without propensity score analysis. These discrepant results showed that unbalanced features associated with retrospective data existed between these two groups, even if no significant unbalanced baseline factors were found before propensity score adjustment. The relatively small sample size in the FOLFOX6 group limited the efficacy of propensity score analysis and the reliability of our conclusion. Different chemotherapy regimens lead to different effects, and some chemotherapy regimens may not result in survival improvement<sup>[30]</sup>. The main differences between the XELOX and FOLFOX6 regimens were oral capecitabine and intravenous 5-FU. Although 5-FU is the most established single-agent drug in palliative chemotherapeutic GC care, the tumor response to 5-FU was reported to be inadequately predicted in SCID mouse models with sometimes no antitumor effects observed<sup>[31]</sup>. A retrospective study compared the effects of the XELOX regimen with the FOLFOX6 regimen in GC patients after D2 dissection and found no survival difference (5-year survival rate: 34% *vs* 29%) between these two regimens<sup>[32]</sup>. A meta-analysis showed that the HR for death for oral capecitabine-containing regimens was 0.94 (95%CI: 0.89-1.00; *P* = 0.0489) in patients with advanced

gastrointestinal cancers compared to 5-FU-containing regimens<sup>[33]</sup>. Our study showed no survival benefit for XELOX therapy compared to FOLFOX6 therapy and similar severe side effects were observed in these two groups. However, oral capecitabine was found to be more convenient and less dangerous than intravenous 5-FU. Few patients had nausea and vomiting during XELOX therapy, although more patients suffered from hand-foot syndrome. Most patients undergoing FOLFOX6 therapy did not complete all the required treatment courses due to frequent hospital visits for biweekly treatment and adverse effects related to eating, drinking and sleeping. This may be why more patients and doctors preferred the XELOX regimen.

In conclusion, this study demonstrated that adjuvant XELOX therapy was associated with survival benefits in GC patients after D2 dissection and provides a superior option for curable GC patients.

## ACKNOWLEDGMENTS

The authors would like to acknowledge the assistance of Dong-Lian Chen in maintaining our database.

## COMMENTS

### Background

The clinical treatment of gastric cancer (GC) is challenging and overall survival is poor. Adjuvant chemotherapy has been intensively investigated for several decades but definitive evidence is limited especially for patients after D2 dissection.

### Research frontiers

Adjuvant capecitabine and oxaliplatin (XELOX) and 5-fluorouracil, folinic acid and oxaliplatin (FOLFOX6) chemotherapy have been widely used in GC patients. However, few studies have directly compared their efficacy and the optimal regimen of adjuvant chemotherapy has not yet been determined.

### Innovations and breakthroughs

The authors found that adjuvant XELOX was significantly associated with improved survival after D2 dissection compared to surgery alone following multivariate Cox regression and propensity score matched analyses. However, the XELOX regimen did not result in a greater survival benefit than the FOLFOX6 regimen.

### Applications

The findings in this study demonstrated that adjuvant XELOX therapy should be considered in curable GC patients.

### Terminology

The propensity score method is used to determine the probability of an individual patient having received a certain treatment as a function of several confounding covariates that are collapsed into a single predictor.

### Peer review

The optimal chemotherapeutic regimen after resected advanced GC is still one of the clinical challenges. Based on previous studies, whether adjuvant chemotherapy can improve GC survival is still uncertain. Although XELOX (capecitabine and oxaliplatin) and FOLFOX6 (5-fluorouracil, folinic acid and oxaliplatin) regimens have been widely used in clinics, few studies have shown direct evidence to indicate their efficacy. This study compared the efficacy of XELOX and FOLFOX6 for GC patients after D2 dissection, and the findings have some scientific and clinical significance.

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## Differences in HER2 over-expression between proximal and distal gastric cancers in the Chinese population

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Supported by The National Natural Science Foundation of China, No. 81101815; the Science and Technology Development Project of Medicine in Nanjing, No. YKK08064; Jiangsu Health International Exchange Program and Young Talents Training Project of Health in Nanjing

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Received: January 4, 2013 Revised: February 22, 2013

Accepted: April 9, 2013

Published online: June 7, 2013

cases, according to the revised scoring criteria of HercepTest™ as used in the ToGA trial. Correlations between HER2 expression and clinicopathologic variables of proximal ( $n = 513$ ) and distal ( $n = 444$ ) GC were investigated.

**RESULTS:** Our results showed that HER2 expression was significantly higher in the proximal than in distal GC ( $P < 0.05$ ). Overall, HER2 expression was significantly higher in male patients ( $P < 0.01$ ), the Lauren intestinal type ( $P < 0.001$ ), low-grade ( $P < 0.001$ ) and pM1 ( $P < 0.01$ ) diseases, respectively. There was a significant difference in HER2 expression among some pTNM stages ( $P < 0.05$ ). In contrast, HER2 expression in the distal GC was significantly higher in male patients ( $P < 0.001$ ), low-grade histology ( $P < 0.001$ ), the Lauren intestinal type ( $P < 0.001$ ), and pM1 ( $P < 0.001$ ). In the proximal GC, however, higher HER2 expression scores were observed only in tumors with low-grade histology ( $P < 0.001$ ) and the Lauren intestinal type ( $P < 0.001$ ).

**CONCLUSION:** HER2 over-expression in GC of Chinese patients was significantly more common in proximal than in distal GC, and significantly correlated with the Lauren intestinal type and low-grade histology in both proximal and distal GC, and with pM1 disease and male gender in distal GC.

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**Key words:** HER2; Gastric cancer; Immunohistochemistry; Clinicopathology

### Abstract

**AIM:** To investigate HER2 expression and its correlation with clinicopathological variables between proximal and distal gastric cancers (GC) in the Chinese population.

**METHODS:** Immunostaining of HER2 was performed and scored on a scale of 0-3 in 957 consecutive GC

**Core tip:** In this study, immunostaining of HER2 was performed and scored according to the revised scoring criteria of HercepTest™ used in the ToGA trial in a very large cohort of gastric cancers (GC) patients (957 cases). Our results revealed that HER2 over-expression in GC of Chinese patients was significantly more common in proximal than in distal GC, and was significantly cor-

related with the Lauren intestinal type and low-grade histology in both proximal and distal GC, and with pM1 disease and male gender in distal GC.

Fan XS, Chen JY, Li CF, Zhang YF, Meng FQ, Wu HY, Feng AN, Huang Q. Differences in HER2 over-expression between proximal and distal gastric cancers in the Chinese population. *World J Gastroenterol* 2013; 19(21): 3316-3323 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3316.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3316>

## INTRODUCTION

HER2 gene amplification and over-expression, which possibly represents a negative prognostical factor<sup>[1,2]</sup> but a potential therapeutic target<sup>[3-7]</sup>, has been found in 6%-53.4% of gastric cancer (GC) in patients from Western countries<sup>[3,8]</sup>. As such, it is now recommended that all patients with GC should have their tumors tested for the HER2 status at the time of initial diagnosis<sup>[9]</sup>. At present, the tests for HER2 over-expression by immunohistochemistry (IHC) and HER2 gene amplification by fluorescence in situ hybridization (FISH) are the most common methods. As demonstrated in the phase III ToGA trial, patients with IHC 2+/FISH-positive or IHC 3+ tumors benefited from trastuzumab treatment, but cases with HER2 gene-amplified tumors, revealed by the FISH test, without HER2 over-expression (IHC 0 or 1+) did not show any survival gain<sup>[5]</sup>. The characteristics of HER2 expression in GC are different from those in breast cancer<sup>[5,10]</sup>. Therefore, in the phase III ToGA trial, the HER2 expression scoring system for breast cancer, which was proposed by the American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP), was modified for evaluation of HER2 expression in GC<sup>[11]</sup>, requiring IHC for HER2 testing before use of the FISH test<sup>[9,12,13]</sup>, because IHC seems to be more predictive of trastuzumab therapeutic responses than the FISH test alone in GC, compared with breast cancers<sup>[14]</sup>.

In China, GC remains one of the leading cancers. Despite recent advances in treatment, the outcome of patients with advanced GCs is poor. Because there exist considerable differences in GC histopathology, environmental factors, and the *Helicobacter pylori* status between Western and Chinese patients<sup>[15]</sup>, the need for a comprehensive investigation of the HER2 expression profile in GC of Chinese patients is urgent for better clinical management. Therefore, we carried out this study to fully investigate differences in HER2 expression and clinicopathologic features between proximal and distal GC with the same assessment criteria of IHC as used in the ToGA trial<sup>[11]</sup>.

## MATERIALS AND METHODS

### Case selection

We retrospectively searched a prospectively established

electronic pathology database for GC resection cases with HER2 immunostaining results over the period from January 2007 to August 2009 at the affiliated Nanjing Drum Tower Hospital, Nanjing University Medical School in Nanjing, China. A total of 957 consecutive cases were identified, including 513 proximal and 444 distal GC, according to the surgical resection methods of GC and gross specimen description. Because our recent research results in the same group of Chinese GC patients suggested that almost all proximal GC with esophageal involvement also included Siewert II-III GC, which are regarded as esophageal origin and stage-grouped as esophageal cancers<sup>[16,17]</sup>, they might be more accurately staged as gastric rather than esophageal cancers<sup>[18,19]</sup>. Therefore, the proximal GC in this study included both GC with epicenters entirely below the gastroesophageal junction (GEJ) and those invading through the GEJ into the distal esophagus as a minor component. Distal GC was defined as tumors with epicenters in the region from the incisura angularis to the antrum-pylorus. The Lauren classification of GC was followed to subgroup all cases in histopathology. All cases were staged, according to the staging rules for GC set out in the 7<sup>th</sup> edition of the American Joint Committee on Cancer Staging<sup>[20]</sup>. The patient demographics and pathologic information were retrieved from each pathology report. The patient private identification information was deleted and the study protocol was approved by the Medical Ethics Committee of the Hospital.

### Immunohistochemistry

A conventional immunostaining protocol was used for all cases. Briefly, paraffin-embedded tumor tissue blocks were cut at 4- $\mu$ m in thickness. Sections were deparaffinized, dehydrated, subjected to the antigen retrieval procedure, and then incubated with the primary rabbit antihuman HER2 polyclonal antibody (clone A0485, dilution: 1:2500, Dako, Denmark) for one hour at 37 °C. The HER2 immunoreactivity was visualized after a brief treatment with the EnVision Plus system kit (Dako). Both positive and negative controls were included in each run.

Three experienced pathologists independently evaluated HER2-stained slides blindly without the knowledge of patient clinicopathologic information. The HER2 immunoreactivity of neoplastic cells was scored according to the revised ToGA scoring criteria of HercepTest<sup>TM</sup> for GC<sup>[5,11]</sup>, which was based on the intensity of membrane staining and quantity of positive neoplastic cells on a scale of 0-3. In brief, no membranous reactivity in less than 10% of tumor cells was scored as 0; faint/barely visible complete or basolateral membranous reactivity in 10% or more of tumor cells was scored as 1+; weak-to-moderate complete or basolateral membranous reactivity in 10% or more of tumor cells was scored as 2+; strong complete or basolateral membranous reactivity in 10% or more of tumor cells was scored as 3+. A score of 0 or 1+ was considered negative while scores 2+ and 3+ were positive.

**Table 1 HER2 expression in gastric adenocarcinoma**

|          |               | n   | HER2 score |     |     |    | $\chi^2$ | P     | Univariate analysis |       |
|----------|---------------|-----|------------|-----|-----|----|----------|-------|---------------------|-------|
|          |               |     | 0          | 1   | 2   | 3  |          |       | r <sub>s</sub>      | P     |
| Gender   | F             | 244 | 149        | 58  | 19  | 18 | 10.106   | 0.001 | -                   | -     |
|          | M             | 713 | 366        | 176 | 98  | 73 |          |       |                     |       |
| Age (yr) | ≤ 70          | 682 | 370        | 164 | 82  | 66 | 1.347    | 0.418 | -                   | -     |
|          | > 70          | 275 | 145        | 70  | 35  | 25 |          |       |                     |       |
| pT       | T1            | 51  | 28         | 14  | 6   | 3  | 7.464    | 0.070 | -                   | -     |
|          | T2            | 145 | 79         | 38  | 19  | 9  |          |       |                     |       |
|          | T3            | 749 | 404        | 179 | 90  | 76 |          |       |                     |       |
|          | T4            | 12  | 4          | 3   | 2   | 3  |          |       |                     |       |
| pN       | N0            | 243 | 138        | 60  | 27  | 18 | 13.591   | 0.335 | -                   | -     |
|          | N1            | 140 | 63         | 34  | 23  | 20 |          |       |                     |       |
|          | N2            | 205 | 104        | 50  | 31  | 20 |          |       |                     |       |
|          | N3            | 369 | 210        | 90  | 36  | 33 |          |       |                     |       |
| pM       | M0            | 935 | 506        | 232 | 114 | 83 | 19.984   | 0.001 | 0.077               | 0.020 |
|          | M1            | 22  | 9          | 2   | 3   | 8  |          |       |                     |       |
| pTNM     | I             | 110 | 59         | 32  | 16  | 3  | 29.943   | 0.041 | 0.039               | 0.187 |
|          | II            | 267 | 151        | 57  | 30  | 29 |          |       |                     |       |
|          | III           | 558 | 296        | 143 | 68  | 51 |          |       |                     |       |
|          | IV            | 22  | 9          | 2   | 3   | 8  |          |       |                     |       |
| G        | Low           | 324 | 135        | 82  | 50  | 57 | 51.360   | 0.000 | -                   | -     |
|          | High          | 633 | 380        | 152 | 67  | 34 |          |       |                     |       |
| Lauren   | Intestinal    | 568 | 256        | 145 | 83  | 84 | 79.548   | 0.000 | -                   | -     |
|          | Diffuse/mixed | 389 | 253        | 88  | 34  | 7  |          |       |                     |       |

pTNM: Pathological tumor-node-metastasis; M: Male; F: Female.

**Table 2 Comparison of HER2 expression between proximal and distal gastric adenocarcinoma**

| Group    | n   | HER2 score |     |    |    | $\chi^2$ | P     |
|----------|-----|------------|-----|----|----|----------|-------|
|          |     | 0          | 1   | 2  | 3  |          |       |
| Proximal | 513 | 260        | 126 | 72 | 55 | 6.691    | 0.011 |
| Distal   | 444 | 255        | 108 | 45 | 36 |          |       |

**Statistical analysis**

The absolute and relative frequencies of qualitative variables were calculated in percentages.  $\chi^2$  tests for categorical variables were used and the differences were considered to be statistically significant if P values were less than 0.05. All analyses were performed using the SPSS version 20.0 software for Windows (SPSS Inc., Chicago, IL, United States).

**RESULTS**

**Clinicopathologic features**

The mean patient age was 63 years (range: 17-89 years). As shown in Table 1, most patients were male (75%) with a male-female ratio of 3.0; 29% of patients were older than 70 years. Most tumors were the Lauren intestine type (59%), high-grade histology (66%), and at advanced stages of pIII and pIV (61%). About 54% of GC was located in the proximal stomach (Table 2). None of the patients received neoadjuvant therapy before surgical resections.

**Overall HER2 immunoreactivity in GC**

In general, HER2 immunoreactivity of neoplastic cells

in GC was characterized by basolateral, lateral, and/or circumferential membranous, heterogeneous or diffuse, staining patterns (Figures 1-3). HER2 over-expression with score 3, and scores 2 and 3 were found in 9.5% (91/957) and 21.73% (208/957) of cases, respectively. Overall, HER2 expression with score 3 was significantly higher in male patients (10.24% *vs* 7.38% in females, *P* < 0.01), the proximal GC (8.11% *vs* 10.72% in distal GC, *P* < 0.05), the Lauren intestine type (14.79% *vs* 1.8% in the Lauren diffuse/mixed type, *P* < 0.001), histological low-grade histology (17.6% *vs* 5.37% in high-grade histology, *P* < 0.001), and pM1 (36.36% *vs* 8.88% in pM0, *P* < 0.01). Although there was a significant difference in HER2 expression between advanced and early GC, especially in stage pIV (p I : 2.7%; p II : 10.86%; p III : 9.12%; p IV : 38.1%; *P* < 0.05), the correlation between HER2 and pTNM stage was not statistically significant (*P* > 0.05) (Table 1).

**Differences in HER2 immunoreactivity between proximal and distal GCs**

HER2 expression was higher in proximal GC than in distal GC (10.7% *vs* 8.1% with score 3; 25% *vs* 18.2% with scores 2 and 3; *P* < 0.05) (Table 2). In the proximal GC, higher expression of HER2 with score 3 was found only in tumors with low-grade histology (17.24% *vs* 6.45%, *P* < 0.001), the Lauren intestine type (15.88% *vs* 0.58%, *P* < 0.001) than those with high-grade histology and the Lauren diffuse/mixed type (Table 3). In the distal GC, however, HER2 over-expression with score 3 was significantly higher in male patients (9.09% *vs* 5.88%, *P* < 0.001), distant metastasis (50% *vs* 6.94%, *P* < 0.001), histological low-grade (18.18% *vs* 4.33%, *P* < 0.001), the Lauren in-

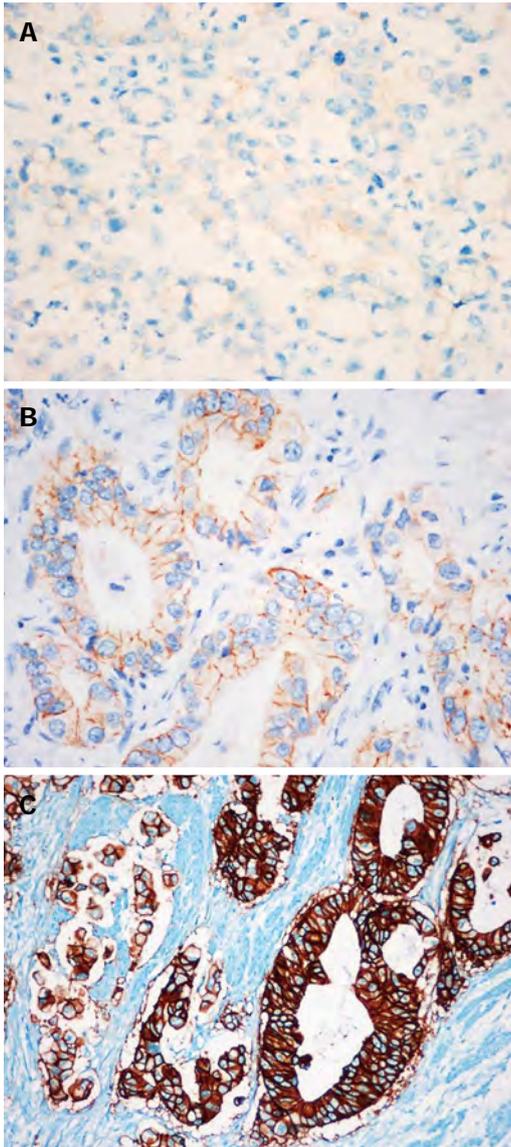


Figure 1 HER2 immunostaining in gastric cancers is scored as 1+, 2+ and 3+ in A, B and C, respectively. EnVision immunohistochemistry stain,  $\times 400$ .

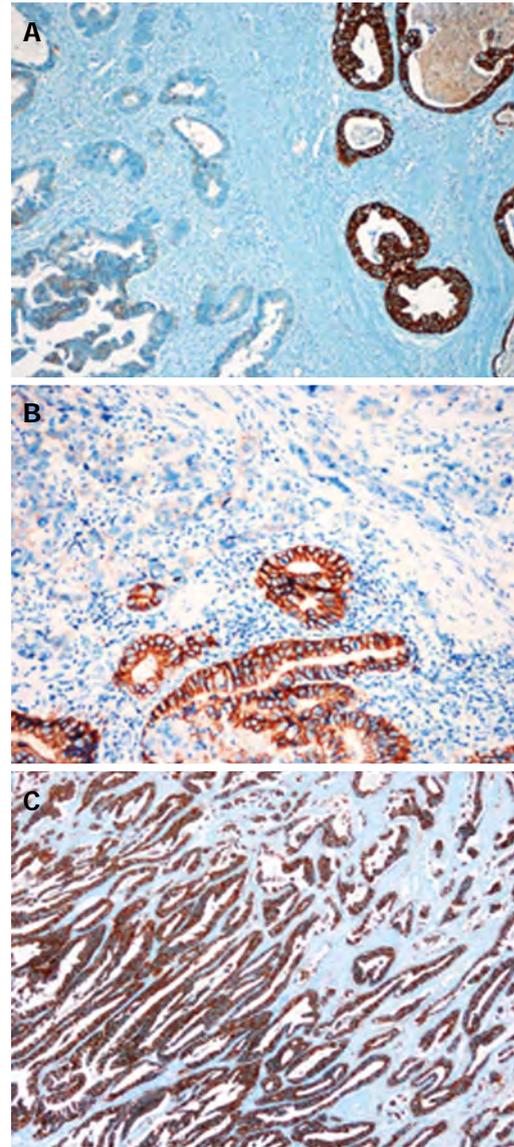


Figure 2 Heterogeneous (A and B,  $\times 200$ ) or diffuse (C,  $\times 100$ ) expression pattern of HER2 protein is discovered in gastric cancers. EnVision immunohistochemistry stain.

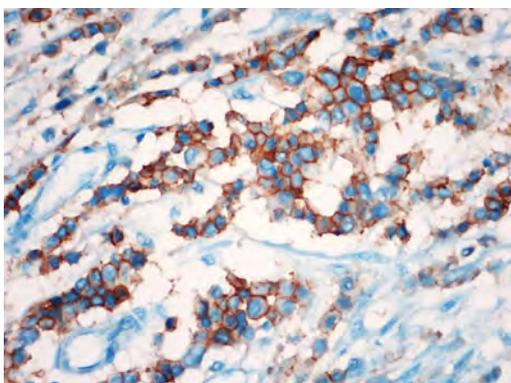


Figure 3 A rare diffuse and strong positive pattern of HER2 expression in the Lauren diffuse type gastric cancers. EnVision immunohistochemistry stain,  $\times 400$ .

testine type (13.16% *vs* 2.87%,  $P < 0.001$ ) GC than in female patients, pM0, high-grade histology, and the Lauren

diffuse/mixed type (Table 4).

## DISCUSSION

In this retrospective study using the revised IHC scoring criteria of HercepTest<sup>TM</sup> for HER2 expression in GC of Chinese patients, we compared HER2 protein expression profiles along with clinicopathologic features between 513 proximal and 444 distal GC in Chinese patients. The data showed that HER2 over-expression was significantly more common in proximal GC than in distal GC, and significantly correlated with the Lauren intestine type and low-grade histology in both proximal and distal GC, and with pM1 disease and male gender in distal GC.

In the recent literature, the frequency of HER2 over-expression in GC, determined by IHC, ranges widely from 6.8% to 26.8%<sup>[21]</sup>. In this study, HER2 over-expression

**Table 3 HER2 expression in proximal gastric adenocarcinoma**

|          |               | n   | HER2 score |     |    |    | $\chi^2$ | P     | Univariate analysis |       |
|----------|---------------|-----|------------|-----|----|----|----------|-------|---------------------|-------|
|          |               |     | 0          | 1   | 2  | 3  |          |       | r <sub>s</sub>      | P     |
| Gender   | F             | 108 | 57         | 28  | 13 | 10 | 0.884    | 0.232 | -                   | -     |
|          | M             | 405 | 203        | 98  | 59 | 45 |          |       |                     |       |
| Age (yr) | ≤ 70          | 371 | 189        | 92  | 48 | 42 | 1.647    | 0.476 | -                   | -     |
|          | > 70          | 142 | 71         | 34  | 24 | 13 |          |       |                     |       |
| pT       | T1            | 10  | 4          | 3   | 1  | 2  | 15.026   | 0.472 | -                   | -     |
|          | T2            | 61  | 30         | 16  | 11 | 4  |          |       |                     |       |
|          | T3            | 439 | 226        | 107 | 59 | 47 |          |       |                     |       |
|          | T4            | 3   | 0          | 0   | 1  | 2  |          |       |                     |       |
| pN       | N0            | 132 | 71         | 34  | 15 | 12 | 6.071    | 0.524 | -                   | -     |
|          | N1            | 70  | 31         | 16  | 13 | 10 |          |       |                     |       |
|          | N2            | 124 | 60         | 29  | 22 | 13 |          |       |                     |       |
|          | N3            | 187 | 98         | 47  | 22 | 20 |          |       |                     |       |
| pM       | M0            | 503 | 255        | 125 | 70 | 53 | 1.959    | 0.269 | -                   | -     |
|          | M1            | 10  | 5          | 1   | 2  | 2  |          |       |                     |       |
| pTNM     | I             | 43  | 20         | 14  | 6  | 3  | 6.790    | 0.133 | -                   | -     |
|          | II            | 129 | 74         | 26  | 16 | 13 |          |       |                     |       |
|          | III           | 331 | 161        | 85  | 48 | 37 |          |       |                     |       |
|          | IV            | 10  | 5          | 1   | 2  | 2  |          |       |                     |       |
| G        | Low           | 203 | 84         | 52  | 32 | 35 | 19.924   | 0.000 | -0.179              | 0.000 |
|          | High          | 310 | 176        | 74  | 40 | 20 |          |       |                     |       |
| Lauren   | Intestinal    | 340 | 150        | 85  | 51 | 54 | 39.351   | 0.000 | -0.210              | 0.000 |
|          | Diffuse/mixed | 173 | 110        | 41  | 21 | 1  |          |       |                     |       |

pTNM: Pathological tumor-node-metastasis; M: Male; F: Female.

**Table 4 HER2 expression in distal gastric adenocarcinoma**

|          |               | n   | HER2 score |     |    |    | $\chi^2$ | P     | Univariate analysis |       |
|----------|---------------|-----|------------|-----|----|----|----------|-------|---------------------|-------|
|          |               |     | 0          | 1   | 2  | 3  |          |       | r <sub>s</sub>      | P     |
| Gender   | F             | 136 | 92         | 30  | 6  | 8  | 11.510   | 0.000 | 0.148               | 0.000 |
|          | M             | 308 | 163        | 78  | 39 | 28 |          |       |                     |       |
| Age (yr) | ≤ 70          | 311 | 181        | 72  | 34 | 24 | 2.443    | 0.446 | -                   | -     |
|          | > 70          | 133 | 74         | 36  | 11 | 12 |          |       |                     |       |
| pT       | T1            | 41  | 24         | 11  | 5  | 1  | 3.983    | 0.131 | -                   | -     |
|          | T2            | 84  | 49         | 22  | 8  | 5  |          |       |                     |       |
|          | T3            | 310 | 178        | 72  | 31 | 29 |          |       |                     |       |
|          | T4            | 9   | 4          | 3   | 1  | 1  |          |       |                     |       |
| pN       | N0            | 111 | 67         | 26  | 12 | 6  | 9.645    | 0.255 | -                   | -     |
|          | N1            | 70  | 32         | 18  | 10 | 10 |          |       |                     |       |
|          | N2            | 81  | 44         | 21  | 9  | 7  |          |       |                     |       |
|          | N3            | 182 | 112        | 43  | 14 | 13 |          |       |                     |       |
| pM       | 0             | 432 | 251        | 107 | 44 | 30 | 29.728   | 0.000 | 0.133               | 0.000 |
|          | 1             | 12  | 4          | 1   | 1  | 6  |          |       |                     |       |
| pTNM     | I             | 67  | 39         | 18  | 10 | 0  | 39.702   | 0.164 | -                   | -     |
|          | II            | 138 | 77         | 31  | 14 | 16 |          |       |                     |       |
|          | III           | 227 | 135        | 58  | 20 | 14 |          |       |                     |       |
|          | IV            | 12  | 4          | 1   | 1  | 6  |          |       |                     |       |
| G        | Low           | 121 | 51         | 30  | 18 | 22 | 31.286   | 0.000 | -0.231              | 0.000 |
|          | High          | 323 | 204        | 78  | 27 | 14 |          |       |                     |       |
| Lauren   | Intestinal    | 228 | 106        | 60  | 32 | 30 | 40.352   | 0.000 | -0.209              | 0.000 |
|          | Diffuse/mixed | 209 | 143        | 47  | 13 | 6  |          |       |                     |       |

pTNM: Pathological tumor-node-metastasis; M: Male; F: Female.

with score 2 and 3 was found in 21.73% of cases, which was higher than that (15.9%) in another recent study using the same antibody and scoring criteria in Korean patients<sup>[8]</sup>. The discrepancy appears to result from the differences in tissue preparations because they used tissue microarray for HER2 immunostaining, which has a much lower sensitivity for HER2 immunoreactivity because of

the well-known heterogeneous expression of HER2 in GC<sup>[14,21-24]</sup>. In addition, different primary antibody clones, various immunostaining protocols, and diverse immunoreactivity scoring schemes may also contribute to variations described among recent studies<sup>[8,12,25]</sup>. This inconsistency indicates an urgent need for standardized HER2 immunostaining in GC for better reporting and comparison of

HER2 IHC results among different centers.

HER2 expression is known to differ among various clinicopathologic factors, such as patient gender, age, ethnicity, tumor location, type, and differentiation, *etc.*, as shown in this and previous studies<sup>[26-28]</sup>. Our data showed high HER2 expression in GC with low-grade histology and advanced pTNM stages. Similar to our results, a recent study in Japanese patients also described higher HER2 expression in male patients, tumors with the Lauren intestine type, and pM1 stage<sup>[26]</sup>. With a multivariate analysis, Janjigian *et al.*<sup>[29]</sup> reported a significantly higher frequency of HER2 immunopositivity in GC with liver metastasis and the Lauren intestine histology, but did not find significant differences in HER2 immunopositivity between resections and biopsies, or primaries and metastases. In that study, approximately 20% (78/381) of distant metastatic GC or GEJ cancers in Western patients were HER2-immunopositive (score 3+ or FISH-positive)<sup>[29]</sup>, a figure which is much lower than ours (36.4%, 8/22). However, the number of GC cases with distant metastasis was limited in this study and our results should be verified in studies with more qualified cases. Nonetheless, our data suggested that HER2 over-expression was more often seen in GC with the Lauren intestine type, low-grade histology, advanced pTNM stage, and in male patients.

In this study, we found a significantly higher frequency of HER2 over-expression in proximal GC than in distal GC. The result is similar to those reported recently in most other studies<sup>[30]</sup>. It must be pointed out that the vast majority of GEJ cancers in Chinese patients are not Barrett's esophagus-related and originate primarily in the proximal stomach, invading into the distal esophagus with clinicopathologic features of GC, as we reported previously<sup>[31]</sup>. Despite similar HER2 over-expression characteristics between Western GEJ cancers and Chinese proximal GC, there exist a number of differences in HER2 over-expression features between proximal and distal GC of Chinese patients in the present study. For example, in distal rather than proximal GC, HER2 over-expression in GC was more common in male patients and in tumors staged at pM1 than in female patients and in pM0 cases, respectively.

A major limitation in this observational study is the absence of the confirmatory FISH test for *HER2* gene amplification. However, unlike in breast cancer, HER2 expression with an IHC score of 0 or 1+ but with FISH positivity in GC tumors does not play a statistically significant role with regard to trastuzumab therapy<sup>[5]</sup>. GC with IHC 3+ HER2 status responds well to this treatment. Thus, the ToGA trial recommended testing *HER2* gene amplification by the FISH method only in GC cases with an IHC score of 2+<sup>[5]</sup>. Therefore, a confirmation FISH test might not be performed in GC with IHC 0, 1+ or 3+. However, our cohort is large with 957 GC resection cases and differences in HER2 expression are dramatically significant in many important clinicopathologic parameters. Therefore, the validity of our results should be

reasonably sound.

In summary, our data showed a significantly higher frequency of HER2 over-expression in proximal GC than in distal GC. HER2 over-expression was significantly correlated with low-grade histology and the Lauren intestine type in both proximal and distal GC, and with male gender and distant metastasis in distal GC.

## ACKNOWLEDGMENTS

This work was presented as a poster in an abstract form at the annual meeting of United States and Canadian Academy of Pathology in 2011 and Digestive Disease Week in 2011.

## COMMENTS

### Background

*HER2* gene amplification and over-expression is a potential therapeutic target and is found in 6%-53.4% of gastric cancer (GC) in patients from Western countries. As such, it is now recommended that all patients with GC should have their tumors tested for HER2 status at the time of initial diagnosis. In China, GC remains one of the leading cancers. Because there exist considerable differences in GC between Western and Chinese patients, the need for a comprehensive investigation of the HER2 expression profile in GC of Chinese patients is urgent for better clinical management.

### Research frontiers

The purpose of the present study was to investigate HER2 expression with the same assessment criteria of IHC as used in the ToGA trial and its correlation with clinicopathological variables between proximal and distal GC in the Chinese population.

### Innovations and breakthroughs

Our study represented a very large cohort of GC. In this study, HER2 expression in the overall GC was significantly higher in male patients, the Lauren intestinal type, low-grade and pM1 diseases. There was a significant difference in HER2 expression among some pTNM stages. Similar to some Western study results, our data showed that HER2 expression was significantly higher in proximal GC than in distal GC. Also, HER2 expression in the distal GC was significantly higher in male patients, low-grade histology, the Lauren intestinal type, and pM1. In the proximal GC, however, higher HER2 expression scores were observed only in tumors with low-grade histology and the Lauren intestinal type.

### Applications

Our data, derived from a comprehensive investigation of HER2 expression profile with the same ToGA criteria in GC resection specimens in a large cohort of homogeneous Chinese patients, provide pathologists and oncologists with more accurate study results than tissue microarray regarding HER2 over-expression characteristics in both proximal and distal GC, which is essential for better clinical management of patients with GC in the Chinese population.

### Terminology

ToGA trial: An international, open-label, randomized, controlled, phase III trial of Herceptin (Trastuzumab) in combination with chemotherapy compared with chemotherapy alone in patients with HER2-positive advanced gastric cancer, which was undertaken in 122 centers in 24 countries; Lauren classification: the Lauren classification is based on the histological features of gastric adenocarcinomas, and divides gastric adenocarcinomas into 3 types: intestinal type (the tumor consists of neoplastic glands arranged in tubules, acini, and papillae), diffuse type (the tumor cells are discohesive and many show the signet-ring morphology) and mixed type.

### Peer review

The authors reported statistically more frequent HER2 over-expression in proximal than distal GC. HER2 over-expression was also associated with some clinicopathological characteristics, such as gender, the Lauren intestinal type, low-grade dysplasia, and pM1 diseases in GC. This is a valuable paper which represents a very large cohort of cancers (957 cases). This provides

some real strength and confidence in the HER2 expression values. The data are consistent with but extend (for the Chinese population) the information already available.

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**P- Reviewers** Langdon S, Zhang HT **S- Editor** Wen LL  
**L- Editor** Logan S **E- Editor** Zhang DN



## Effect of endogenous insulin-like growth factor and stem cell factor on diabetic colonic dysmotility

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Supported by The National Natural Science Foundation of China, No. 30971354; The International Cooperation Project of Jiangsu Province Department of Health, No. SBZ201100103; The Graduate Innovation Foundation of Jiangsu Province, China, No. CXZZ11\_0704

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Received: January 30, 2013 Revised: March 23, 2013

Accepted: April 18, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To investigate whether the reduction of stem cell factor (SCF) is mediated by decreased endogenous insulin-like growth factor (IGF)-1 in diabetic rat colon smooth muscle.

**METHODS:** Sixteen Sprague-Dawley rats were randomly divided into two groups: control group and streptozotocin-induced diabetic group. After 8 wk of streptozotocin administration, colonic motility function and contractility of circular muscle strips were measured. The expression of endogenous IGF-1 and SCF was tested in colonic tissues. Colonic smooth muscle cells were cultured from normal adult rats. IGF-1 siRNA transfection was used to investigate whether SCF expression was affected by endogenous IGF-1 expression in smooth muscle cells, and IGF-1 induced SCF expres-

sion effects were studied. The effect of high glucose on the expression of endogenous IGF-1 and SCF was also investigated.

**RESULTS:** Diabetic rats showed prolonged colonic transit time ( $252 \pm 16$  min vs  $168 \pm 9$  min,  $P < 0.01$ ) and weakness of circular muscle contraction ( $0.81 \pm 0.09$  g vs  $2.48 \pm 0.23$  g,  $P < 0.01$ ) compared with the control group. Endogenous IGF-1 and SCF protein expression was significantly reduced in the diabetic colonic muscle tissues. IGF-1 and SCF mRNA expression also showed a paralleled reduction in diabetic rats. In the IGF-1 siRNA transfected smooth muscle cells, SCF mRNA and protein expression was significantly decreased. IGF-1 could induce SCF expression in a concentration and time-dependent manner, mainly through the extracellular-signal-regulated kinase 1/2 signal pathway. High glucose inhibited endogenous IGF-1 and SCF expression and the addition of IGF-1 to the medium reversed the SCF expression.

**CONCLUSION:** Myopathy may resolve in colonic motility dysfunction in diabetic rats. Deficiency of endogenous IGF-1 in colonic smooth muscle cells leads to reduction of SCF expression.

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**Key words:** Diabetes; Gastrointestinal motility function; Insulin-like growth factor-1; Stem cell factor; Smooth muscle cell

**Core tip:** Endogenous insulin-like growth factor (IGF)-1 levels in diabetic rat colonic tissues were decreased. Hyperglycemia may be involved in initiating this change. In colonic smooth muscle cells, IGF-1 had a direct effect on increasing stem cell factor mRNA and protein levels mediated by extracellular-signal-regulated kinase 1/2 signaling.

Wang Y, Xu XY, Tang YR, Yang WW, Yuan YF, Ning YJ, Yu YJ, Lin L. Effect of endogenous insulin-like growth factor and stem cell factor on diabetic colonic dysmotility. *World J Gastroenterol* 2013; 19(21): 3324-3331 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i21/3324.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3324>

## INTRODUCTION

Approximately 75% of diabetic patients with any type of diabetes mellitus (DM) present gastrointestinal symptoms<sup>[1,2]</sup>, particularly those who have poor glycemic control. Constipation and the use of laxatives are relatively common in patients with DM<sup>[3]</sup>. This disorder involves the depletion of interstitial cells of Cajal (ICCs), dystrophic changes of smooth muscle cells (SMCs), and impairment of enteric nervous systems<sup>[4-6]</sup>. ICCs are electrical active cells that generate and propagate electrical slow waves and serve as a bidirectional interface between nerves and smooth muscle<sup>[5-7]</sup>. ICCs damage has been shown in the stomach, jejunum, and colon of patients suffering from either type 1 or type 2 diabetes. ICCs depletion has been demonstrated in both patients with DM and laboratory animal models<sup>[8]</sup>, and ICCs depletion is a consequence of stem cell factor deficiency<sup>[9]</sup>.

It is well established that ICCs survival and function depend on the activation of c-kit, a receptor tyrosine kinase integral to ICCs. SCF, as a c-kit ligand, is produced locally within the tunica muscularis<sup>[10-12]</sup>.

*In vitro* experiments showed insulin-like growth factor-1 (IGF-1) prevented ICC deletion in long-term cultured smooth muscle<sup>[9]</sup>. In intact tissues, a significant proportion of tissue IGF-1 is actually produced by SMCs<sup>[13]</sup>. Therefore, local IGF-1 signaling may play a significant role in SCF expression. The aim of this research is to study the local colonic IGF-1 expression in DM rats and the endogenous IGF-1 effects on SCF production.

## MATERIALS AND METHODS

### Animals

Male SD rats (weighing 200-250 g) were used for the experiments. Smooth muscle cells were obtained from SD rat colons. Diabetic rats (streptozotocin, STZ-D,  $n = 8$ ) were induced by a single intraperitoneal injection of streptozotocin (STZ, 60 mg/kg body weight) dissolved in a citrate buffer, and age-matched control rats ( $n = 8$ ) received equal volumes of buffer by *ip* injection. Diabetes was confirmed 1 wk later by measurement of tail vein blood glucose levels with an AccuChek Compact Plus glucometer (Roche, IN, United States). Rats with final blood glucose levels > 16.7 mmol/L were included in the study. At 8 wk after STZ administration, all the experimental rats were sacrificed by cervical dislocation. Food and water were given *ad libitum*. All animals were maintained in a controlled environment with alternating 12 h light/dark cycles. All animal care, use, and experimental

protocols were approved by the Institutional Animal and Use Committee of Nanjing Medical University.

### Distal colonic transit

Eight weeks after STZ injection, colonic transit was measured using a bead expulsion test as described<sup>[14]</sup>. A glass bead (5 mm in diameter) was inserted through the anus, and pushed with a plastic rod into the distal colon for a distance of 3 cm, while each rat was under transient ethyl ether anesthesia. The time until bead expulsion was measured.

### *In vitro* studies of colonic smooth muscle contractility

Eight weeks after STZ injection, freshly obtained full-thickness distal colon tissues (about 5 cm from the anus) from control and diabetic rats were stored on ice for less than 1 h and then immersed in warm, oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs solution (in mmol/L: 118 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 11 D-glucose and 25 NaHCO<sub>3</sub>). The mucosal layers were removed by micro-dissection under a magnifying glass and discarded. Circular muscle strips (2 mm × 10 mm) were prepared. After connecting to an isometric force transducer (Alcott-Biotech), the strips were allowed to equilibrate for 30 min under an initial tension of 0.5 g. The bath temperature was maintained at 37 °C. Contractile responses to acetylcholine (Ach, 1 μmol/L, Sigma-Aldrich, St. Louis, MO, United States) were obtained. At least three muscle strips from the distal colon of each rat were used for muscle experiments. The mean of the three or more muscle strips exposed to Ach 1 μmol/L was determined for each rat.

### Cell culture and treatment

SMCs were isolated from colonic tissues and cultured as described previously<sup>[15]</sup>. Briefly, whole SD rat colons were dissected, and mucosa and serosa were quickly removed from muscle tissues. After digestion with type II collagenase, SMCs were cultured in DMEM medium (Gibco) with 10% fetal bovine serum. Cells of passage 2 or 3 were used for the studies. Before the experiments, cells were serum starved for 24 h and then stimulated with different concentrations of IGF-1 (R and D Systems, Minneapolis, MN, United States) for various time periods. Phosphorylated extracellular-signal-regulated kinase 1/2 (ERK1/2) and phosphorylated Akt were measured after IGF-1 (100 ng/mL) treatment for 15 min. ERK1/2 inhibitor PD98059 (50 μmol/L) and phosphoinositide 3-kinase inhibitor (PI3K) inhibitor LY294002 (50 μmol/L) (Cell Signaling, Danvers, MA, United States) were added to the medium 30 min before IGF-1 treatment. Culture medium containing 25 mmol/L D-glucose was used for high glucose stimulation, and mannitol (5 mmol/L D-glucose plus 19.5 mmol/L mannitol) was used for osmotic controls.

### siRNA transfection

IGF-1 siRNA was synthesized by GenePharma (Shanghai GenePharmaCo, China). The sequence for IGF-1 siRNA was as follows: anti-IGF-1-sense 5'-GCAG-

GAAACAAGACCUACATT-3', and anti-IGF-1-antisense 5'-UGUAGGUCUUGUUUCCUGCTT-3'. Negative control siRNA: sense 5'-UUCUCCGAACGUGUCAC-GUTT-3', and antisense 5'-ACGUGACACGUUCG-GAGAATT-3'. Primary colonic smooth muscle cells were grown in six-well plates to 30% confluence, washed with serum-free medium immediately before transfection, and 800  $\mu$ L serum-free medium was added to each well. siRNA (4  $\mu$ g) was mixed with 10  $\mu$ L of X-tremeGENE siRNA transfection reagent (Roche) in 200  $\mu$ L serum-free medium. The mixture was incubated for 20 min at room temperature and then added to cells, according to the manufacturer's instructions. Serum was added 4 h later to a final concentration of 10%. Cells were harvested at 72 h after transfection.

### Western blotting

Distal colonic tissues were dissected from the mucosa and serosa. The remaining tissues were mainly muscle layers and used for Western blotting<sup>[16]</sup>. Tissues and cultured cells were lysed and centrifuged at 12000 *g* for 20 min. Protein samples were run on a 12% polyacrylamide gel in Tris-HCl. Proteins were then transferred to nitrocellulose membranes for 1 h at 100 V. The membranes were blocked with 5% (w/v) skim milk for 1 h at room temperature, and probed with primary antibodies at 4 °C overnight, and then washed in Tris-buffered saline with 0.1% Tween 20 for 5 min three times. The membranes were probed with corresponding horseradish peroxidase-conjugated secondary anti-rabbit and anti-goat antibodies at 1:2000 dilutions. Protein bands were detected with enhanced chemiluminescence Western blotting reagents (Thermo Fisher Scientific, Rockford, IL, United States). The resulting bands were scanned with an Epson 2400 printer and analyzed using the ImageJ program. All the antibodies used in this study, except for IGF-1 (Abcam, Cambridge, MA, United States) and SCF (Santa Cruz, CA, United States), were from Cell Signaling Technology.

### Real-time polymerase chain reaction

Total RNA was isolated from colonic muscle layers and isolated SMCs, and quantified using Spectrophotometer Nanodrop 2000 (Thermo Scientific). RNA (500 ng) was submitted for cDNA synthesis using Prime Script RT Master Mix (Takara). The levels of isolated mRNA were measured by real-time polymerase chain reaction (PCR) using SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions. PCR was initiated at 95 °C for 30 s, followed by 40 cycles of denaturing at 95 °C for 5 s, annealing and extending at 60 °C for 30 s. After PCR, a dissociation curve was constructed at 60-95 °C. PCR was performed in triplicate. The oligonucleotide primers used were as follows: IGF-1, forward, GGCATTGTGGAT-GAGTGTG, reverse, GTCTTGGGCATGTCAGT-GTG; SCF, forward, TTCGCTTGTAAATGGCTTTGC; reverse, CAACTGCCCTTGTAAAGAC TTGCA; and 18S rRNA, forward, GCGAAAGCATTTGCCAAGAA, reverse, GGCATCGTTTATGGTCGGAAC. Results were

shown to be relative to 18S rRNA mRNA.

### Statistical analysis

The results are expressed as the mean  $\pm$  SE. The SPSS statistical package (version 14.0; SPSS Inc, Chicago, IL, United States) was used for statistical analysis. The differences between the two groups were analyzed using Student's *t* test, and differences between the three groups were analyzed using analysis of variance. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Effects of diabetes on colonic motility function

All STZ-induced rats showed hyperglycemia at the time of experiments, with their blood glucose concentrations being significantly higher than those of the age-matched rats which were not injected with STZ (*n* = 8) (Figure 1A). The body weight of STZ-treated (STZ-D) rats was significantly lower than the age-matched normal rats (*n* = 8) (Figure 1B).

The distal colon transit time was significantly increased in STZ-D group 8 wk after induction of diabetes (Figure 1C). Colonic circular smooth muscle strips from STZ-D rats showed weak contractility to Ach (Figure 1D). The maximum contractile tension of the STZ-D group was significantly reduced compared with control group (*n* = 8 each group) (Figure 1E).

### SCF and IGF-1 expression in STZ-D rat distal colonic tissue

Eight weeks after STZ administration, the rats were sacrificed and distal colonic tissues were harvested. Because the mucosa/submucosa and serosa had been removed, the protein and mRNA were mainly from the muscle layer. The protein levels of SCF and IGF-1 were significantly decreased in STZ-D rat distal colons (Figure 2A). SCF and IGF-1 mRNA levels were consistent with the protein results. SCF mRNA levels of diabetic rats were lower than those of control rats, and IGF-1 mRNA levels were also decreased in diabetic rat colonic tissues (Figure 2B).

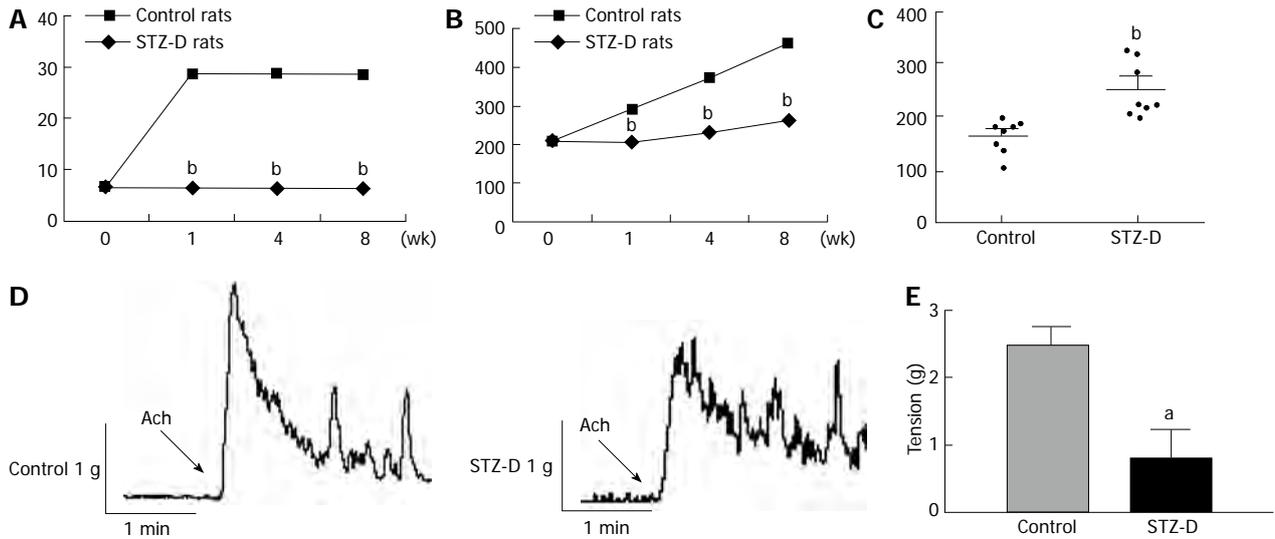
### Effects of IGF-1siRNA on SCF expression

siRNA knockdown of endogenous IGF-1 reduced SCF mRNA levels in SMCs, and SCF protein levels also showed a significant parallel reduction of IGF-1 levels compared with the controls (Figure 3).

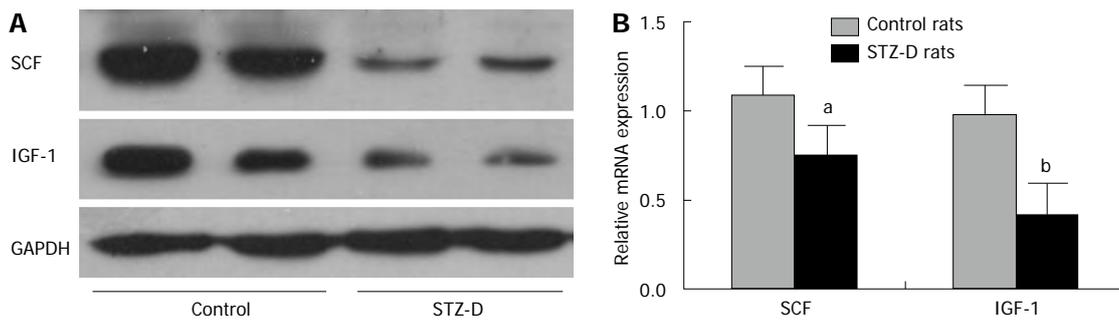
### IGF-1 increased expression of SCF in SMCs through ERK1/2 pathway

IGF-1 elicited a dose-dependent increase in the expression of SCF in rat colonic SMCs. The concentration of 50 ng/mL significantly increased the expression of SCF (*P* < 0.01). One hundred ng/mL and 150 ng/mL IGF-1 showed a maximal effect on the expression of SCF (*P* < 0.01); therefore, 100 ng/mL was used in subsequent experiments (Figure 4A).

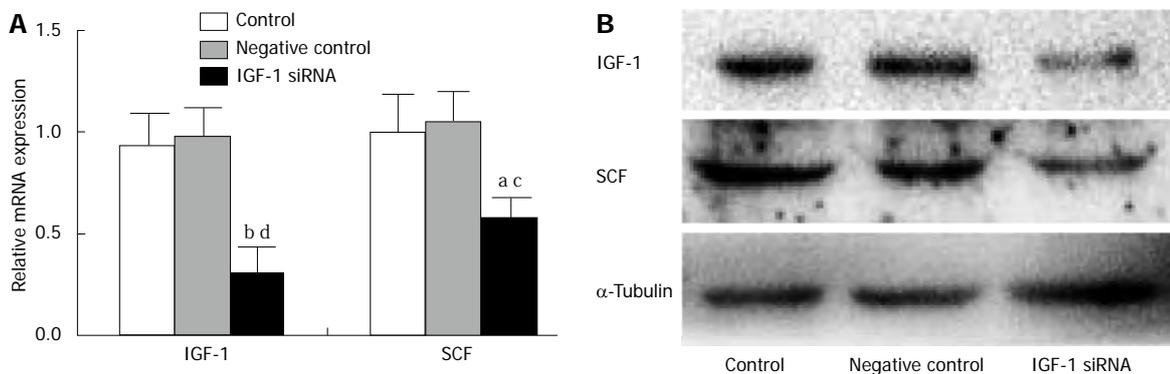
IGF-1 induced maximum SCF expression in SMCs at



**Figure 1** Colonic motility dysfunction in rats with streptozotocin-induced diabetes. A: Blood glucose levels of control group and rats with streptozotocin (STZ)-induced diabetes ( $n = 8$ ); B: The body weights of diabetic and age-matched control rats ( $n = 8$ ); C: Colonic transit time in STZ-D and control rats 8 wk after STZ injection ( $n = 8$ ); D: Circular muscle strip contractility from the representative distal colon of an STZ-D rats and control 8 wk after STZ injection and exposed to  $1 \mu\text{mol/L}$  ACh. Representative images are shown; E: Quantification of maximum contractile force of a circular muscle strip stimulated by  $1 \mu\text{mol/L}$  ACh ( $n = 8$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the control group.



**Figure 2** Stem cell factor and insulin-like growth factor-1 expression in streptozotocin-D rat distal colon. A: Stem cell factor (SCF) and insulin-like growth factor-1 (IGF-1) protein levels in streptozotocin (STZ)-D rat distal colon ( $n = 8$ ). Images are representative of at least 4 independent Western blotting, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control; B: SCF and IGF-1 mRNA levels in colonic tissue of control rats and STZ-D rats ( $n = 8$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the control group.

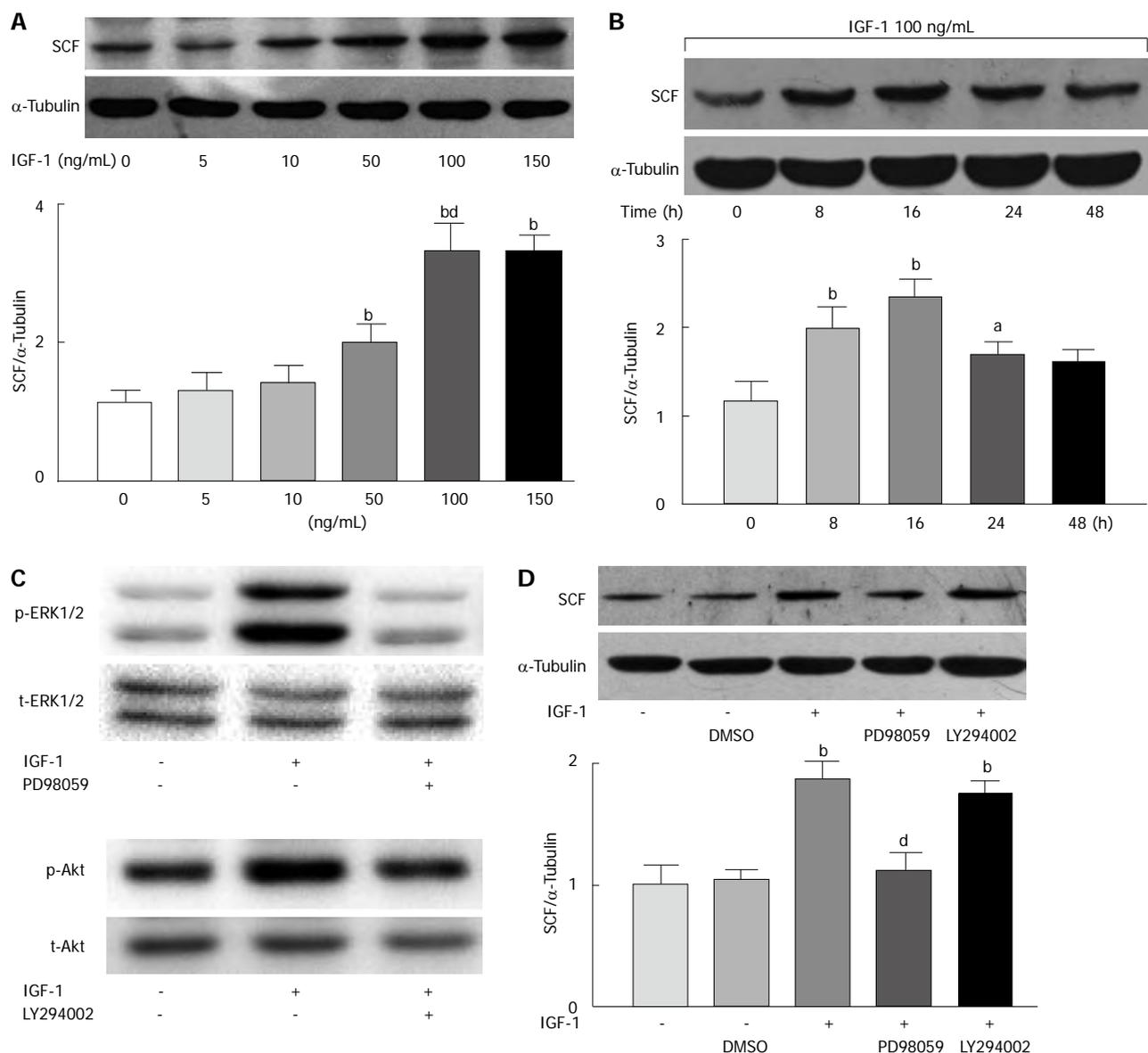


**Figure 3** Effects of insulin-like growth factor-1 siRNA on stem cell factor expression. A: Effects on stem cell factor (SCF) mRNA levels in colonic smooth muscle cells (SMCs) 72 h after of transfection with insulin-like growth factor-1 (IGF-1) siRNA (Each bar represents the mean  $\pm$  SE of 3 independent experiments). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs controls; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs negative controls; B: SCF protein expression in SMCs after IGF-1 knockdown for 72 h (figure is representative of 3 experiments).  $\alpha$ -Tubulin was used as a loading control.

16 h; while at 24 and 48 h, the stimulating effect of IGF-1 on SCF gradually diminished. By 48 h, the expression lev-

els had almost returned to baseline (0 h) (Figure 4B).

To determine whether IGF-1-induced SCF expression



**Figure 4** Insulin-like growth factor-1-induced stem cell factor expression through the extracellular-signal-regulated kinase 1/2 pathway in smooth muscle cells. **A:** Insulin-like growth factor-1 (IGF-1) effects on the expression of stem cell factor (SCF) as a function of concentration after treatment with IGF-1 for 24 h. <sup>b</sup>*P* < 0.01 vs 0 ng/mL IGF-1; <sup>d</sup>*P* < 0.01 vs 50 ng/mL IGF-1; **B:** IGF-1 effects on SCF expression as a function of time. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs 0 h; **C:** Phosphorylation of extracellular-signal-regulated kinase 1/2 (ERK1/2) and Akt after 100 ng/mL IGF-1 treatment for 15 min; **D:** Effects of PD98059 and LY294002 on SCF expression by IGF-1 treatment. <sup>b</sup>*P* < 0.01 vs the no treatment group; <sup>d</sup>*P* < 0.01 vs IGF-1 treatment group. Western blotting represent 3 independent experiments. α-Tubulin was used as a loading control (each bar represents the mean ± SE of 3 independent experiments).

was mediated by Akt-dependent or ERK1/2-dependent mechanisms, we measured the phosphorylation of Akt and ERK1/2. Both of them could be phosphorylated by IGF-1 (100 ng/mL). We then used a PI3K/Akt inhibitor (LY-294002, 50 μmol/L) and an ERK1/2 inhibitor (PD-98059, 50 μmol/L) before IGF-1 (100 ng/mL) was added to the media. Sixteen hours after IGF-1 stimulation, the ERK1/2 inhibitor significantly inhibited IGF-1-induced SCF expression (*P* < 0.01) while the PI3K/Akt inhibitor showed no effect (Figure 4C).

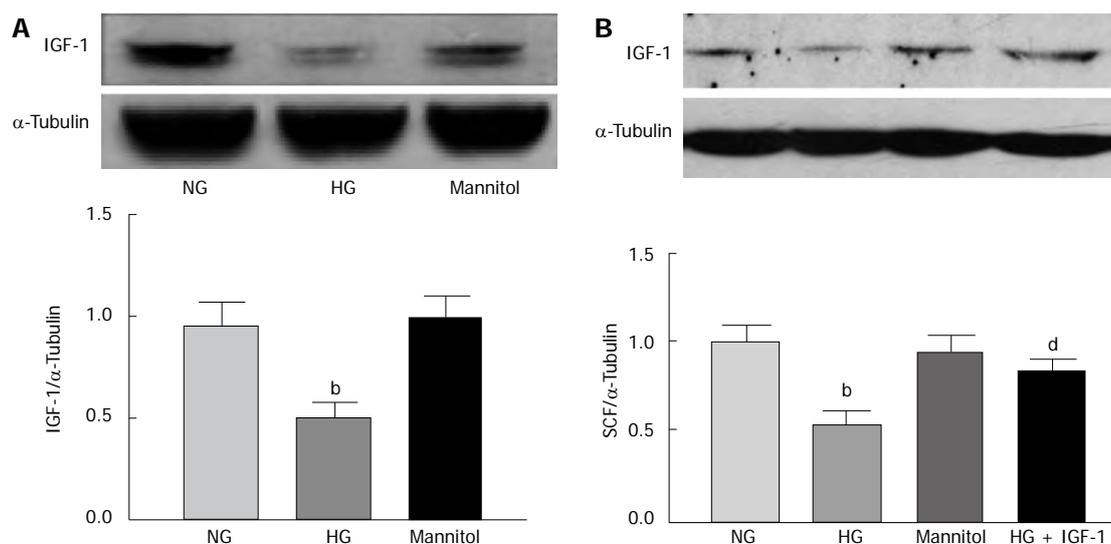
### High glucose suppresses endogenous IGF-1 and SCF expression

Compared with cultures for 24 h in normal glucose (5

mmol/L) medium, cultures in DMEM containing 25 mmol/L glucose significantly inhibited IGF-1 expression in cultured colonic SMCs. Mannitol was used as an osmotic control which did not have similar inhibitive effect (Figure 5A). High glucose concentrations also suppressed SCF expression, which was reversed by the addition of exogenous IGF-1 (100 ng/mL) (Figure 5B).

## DISCUSSION

Intestinal transit is often disturbed in diabetes (rapid or slow)<sup>[17]</sup>, and symptoms such as diarrhea and constipation occur more frequently than in the general population<sup>[18]</sup>. The prevalence of GI symptoms was found to be more



**Figure 5 High glucose inhibited endogenous insulin-like growth factor-1 and stem cell factor expression.** A: Expression of endogenous insulin-like growth factor-1 (IGF-1) in SMCs cultured in a high glucose (HG) medium for 24 h. <sup>b</sup> $P < 0.01$  vs the normal glucose (NG) group; B: Stem cell factor (SCF) expression in smooth muscle cells (SMCs) exposed to HG, and effects of the addition of exogenous IGF-1 (100 ng/mL). <sup>b</sup> $P < 0.01$  vs the NG group; and <sup>d</sup> $P < 0.01$  vs HG group. The Western blotting are representatives of 4 independent experiments.  $\alpha$ -Tubulin was used as a loading control (each bar represents the mean  $\pm$  SE of 3 independent experiments).

common in patients who had long histories of diabetes<sup>[19]</sup>. In diabetic animal models, it has been proven that 2 mo of DM is adequate to impair gastrointestinal motility. The spontaneously diabetic NOD/LtJ mouse, as a type 1 DM model, developed gastroparesis 2 mo after the onset of diabetes<sup>[20]</sup>, and a type 2 DM model *db/db* mouse developed disturbed motility of the stomach, small intestine, and colon 7 wk after the development of DM<sup>[21]</sup>.

Intestinal contractility is dependent on not only intact smooth muscle but also trophic factors, such as SCF and IGF-1, which protect the gastrointestinal slow wave generating ICCs<sup>[9]</sup>, and promote gastrointestinal growth<sup>[22]</sup>, respectively. In the present study, we found that SCF levels in colonic tissue of diabetic rats were significantly reduced, which is consistent with previous reports; in which NOD/LtJ mice had significantly reduced gastric mRNA levels of SCF<sup>[9]</sup> and diabetic *db/db* mice had lower mRNA levels of SCF in the small intestine and colon compared with age-matched control mice 7 wk after the development of type 2 DM<sup>[21]</sup>. Reduction of SCF production in the gastrointestinal tract of diabetic rats appears to play a significant role in decreasing ICCs network densities that have been described in human diabetes<sup>[9]</sup>. SCF has been shown to be produced locally by SMC and neurons within the tunica muscularis<sup>[9,23]</sup>. Decreases in IGF-1 signaling have been considered to be responsible for the decrease in SCF levels. Horváth *et al.*<sup>[9,24]</sup> have shown that IGF-1 treatment of murine gastric tunica muscularis tissues could reverse SCF reductions in long-term organ culture, and decreased levels of SCF were correlated with smooth-muscle atrophy. The observed pro-survival effects of insulin and IGF-1 on mature ICCs were probably indirect because ICCs do not express insulin or IGF-1 receptors<sup>[9]</sup>, while SMCs express both insulin and IGF-1 receptors. Therefore, both insulin and IGF-1 may induce SMCs to produce SCF.

IGF-1 is an endogenous growth factor that plays a central role in the growth and development of visceral and vascular smooth muscle<sup>[25,26]</sup>. Existing evidence suggests that mesenchymal cells, including  $\alpha$ -smooth muscle actin-positive myofibroblasts and smooth muscle cells, are primary sources of locally expressed IGF-1 in the intestine<sup>[27,28]</sup>. Local intestinal IGF-1 has autocrine effects on the growth of human intestinal muscle cells<sup>[29]</sup>. In this study, we found that endogenous IGF-1 was decreased in diabetic colonic tissues. Meanwhile, SCF expression in colonic tissue was paralleled by endogenous IGF-1, which suggested that a lack of endogenous IGF-1 might lead to low levels of SCF expression.

IGF-1 binds to the cellular membrane IGF-1 receptor leading to stimulation of proliferation and inhibition of apoptosis through PI3K/Akt and mitogen-activated protein kinase pathways<sup>[15,30]</sup>. In the present study, IGF-1 could cause both ERK1/2 and Akt phosphorylation, but IGF-1 promoted SCF expression mainly through ERK1/2-dependent signaling, not the PI3K/Akt pathway.

Both type 1 and type 2 diabetes patients have hyperglycemia as a basic feature, and chronic hyperglycemia is associated with dysfunction, damage, and failure of several organs<sup>[31]</sup>. This study showed that high glucose impaired the production of trophic factors in colonic SMCs. Whether the mechanism underlying the effects is oxidative stress caused by high glucose remains to be determined.

The current study is an extension of those described by Horváth *et al.*<sup>[9]</sup>. The significant differences between that study and the current one are: (1) we showed evidence supporting the presence of dysfunction of smooth muscle contractility in animals, which is more compelling evidence of myopathy than just a reduction of myh11 mRNA levels; (2) in cell culture studies, we demonstrated that IGF-1 had a direct effect on increasing SCF mRNA and protein levels, and this effect was mediated

by ERK1/2 signaling; additionally; and (3) we found that endogenous IGF-1 levels in diabetic rat colonic tissues were decreased, and that hyperglycemia may be involved in initiating this change.

In conclusion, we have demonstrated that the myopathy may resolve in colonic motility dysfunction in diabetic rats. Deficiency of endogenous IGF-1 in SMCs caused reduction in SCF expression, which is a critical developmental, growth, and survival factor for ICCs.

## COMMENTS

### Background

Most diabetic patients experience gastrointestinal symptoms, particularly those who are under poor glycemic control. Constipation is relatively common in patients with diabetes mellitus, and involves the depletion of interstitial cells of Cajal (ICCs), dystrophic changes of smooth muscle cell, and impairment of enteric nervous system.

### Research frontiers

ICCs are the primary electrical pacemakers for rhythmic contractile activity. ICC depletion has been demonstrated in both patients with diabetes mellitus and laboratory animal models, and is a consequence of stem cell factor (SCF) deficiency. Cell culture experiments have shown that addition of insulin-like growth factor-1 (IGF-1) prevented ICC deletion in long-term cultured smooth muscle cells.

### Innovations and breakthroughs

This study showed evidence supporting the presence of dysfunction of smooth muscle contractility in animals, which is compelling evidence of myopathy. In cell culture studies, the authors demonstrated that IGF-1 had a direct effect on increasing SCF mRNA and protein levels, and this effect was mediated by extracellular-signal-regulated kinase 1/2 signaling; and that endogenous IGF-1 levels in diabetic rat colonic tissues were decreased, and that hyperglycemia may be involved in initiating this change.

### Applications

The results of this study suggest that compensation of endogenous IGF-1 is a potential therapeutic option that could prevent the development of diabetic colonic dysmotility.

### Terminology

SCF is a cytokine that binds to the c-Kit receptor. SCF can exist both as a transmembrane protein and a soluble protein. This cytokine plays an important role in hematopoiesis, spermatogenesis, and melanogenesis. In gastrointestinal tract, SCF is mainly produced by smooth muscle cells and neurons.

### Peer review

This is a good descriptive study in which authors showed that myopathy may resolve in colonic motility dysfunction in diabetic rats, and deficiency of endogenous IGF-1 in colonic smooth muscle cells caused reduction of SCF expression. The results are interesting and suggest that endogenous IGF-1 is a potential therapeutic target for diabetic colonic dysmotility.

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**P- Reviewers** Kawakami K, Torres-Aleman I **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Ma S



## PRSS1\_p.Leu81Met mutation results in autoimmune pancreatitis

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Supported by National Natural Science Foundation of China, No. 81201362, No. 81201590, No. 21275028; Putian Municipal Science and Technology Bureau Project, No. 2009S3-3; Fujian Medical Innovations, No. 2012-CXB-21; Education Department of Fujian Province, No. JA12133, No. JA12143; National High Technology Investigation Project Foundation of China, No. 2012AA022604

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Received: December 13, 2012 Revised: February 18, 2013

Accepted: March 6, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To describe protease serine 1 (*PRSS1*) gene mutations in patients with autoimmune pancreatitis (AIP) and the clinical features of AIP.

**METHODS:** Fourteen patients with AIP, 56 with other chronic pancreatitis, 254 with pancreatic cancer and 120 normal controls were studied. The mutations and polymorphisms of four genes involved with pancreatitis or pancreatic cancer, *PRSS1*, *SPINK1*, *CFTR* and *MEN1*, were sequenced. The pathogenic mechanism of AIP was investigated by comparing the wild-type expression system with the p.81Leu→Met mutant expression system.

**RESULTS:** Two novel mutations (p.81Leu→Met and p.91Ala→Ala) were found in *PRSS1* gene from four patients with AIP. *PRSS1*\_p.81Leu→Met mutation led to a trypsin display reduction (76.2%) combined with phenyl agarose (Ca<sup>2+</sup> induced failure). Moreover, the ratio of trypsin/amyase in patients with AIP was higher than in the patients with pancreatic cancer and other pancreatitis. A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts.

**CONCLUSION:** Autoimmune pancreatitis may be related to *PRSS1* gene mutations.

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**Key words:** Autoimmune pancreatitis; Molecular mechanism; p.81Leu→Met; *PRSS1*

**Core tip:** Novel mutations (p.81Leu→Met and p.91Ala→Ala) were found in protease serine 1 gene from the patients with autoimmune pancreatitis. Trypsinogen abnormal activation resulted in multiple organ injuries. And this offers direct evidence in support of the trypsinogen gene mutation and abnormal immune system.

Gao F, Li YM, Hong GL, Xu ZF, Liu QC, He QL, Lin LQ, Weng SH. *PRSS1* p.Leu81Met mutation results in autoimmune pancreatitis. *World J Gastroenterol* 2013; 19(21): 3332-3338 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3332.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3332>

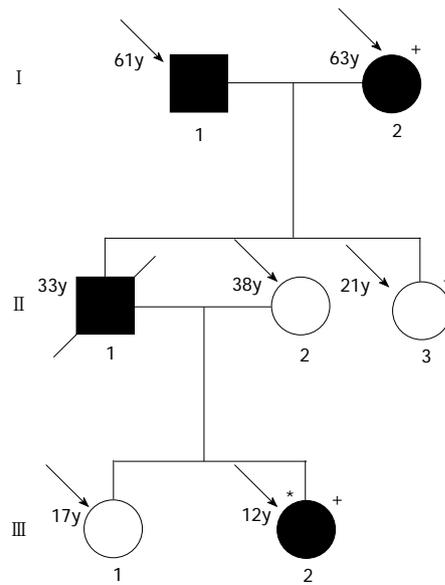
## INTRODUCTION

Most of the earlier literature about autoimmune pancreatitis (AIP) came from Japan<sup>[1-3]</sup>. AIP has been referred to a variety of names including sclerosing pancreatitis, tumefactive pancreatitis, and nonalcoholic destructive pancreatitis, depending in part on the specific pathologic findings and the presence of extrapancreatic manifestations. However, it is generally believed that the pathologic heterogeneity may reflect different stages or manifestations of the same disease. Immunoglobulin G4 (IgG4) positive plasma cells infiltration is considered a marker for the disease and can be detected in the pancreas and a variety of other tissues<sup>[3-7]</sup>. Unfortunately, serum IgG4 increase was not found in all patients with AIP and more than half of the patients with AIP had normal serum IgG4<sup>[8-10]</sup>. It is urgent to find some more specific diagnosis technology (including the molecular markers). Genetic mutation is often involved in immune system disorders, and protease serine 1 (*PRSS1*), cystic fibrosis conductance regulator (*CFTR*), serine protease inhibitor Kazal type 1 (*SPINK1*) and multiple endocrine neoplasia 1 (*MEN1*) mutation is followed by pancreatitis or pancreatic cancer<sup>[11-15]</sup>. We are keen on identifying these genes targeted by the inflammatory process in AIP. Although trypsin was historically believed to be immunologically active, it is now continued to be verified that abnormal activation of trypsin can be recognized by the immune system. This study aimed to determine whether *PRSS1* gene p.T81M mutation contributes to the functions of calcium-induced trypsinogen activation and to explore its role in autoimmune pancreatitis.

## MATERIALS AND METHODS

### Patients

This study was approved by the Fujian Medical University Ethics Committee and all study participants gave informed consent for DNA analyses. Clinical information for the survey was obtained by personal interviews using a structured questionnaire and/or clinical trials. AIP diagnostic criteria were as follows: (I) pancreatic imaging studies show diffuse narrowing of the main pancreatic duct with irregular wall (more than 1/3 of length of the entire pancreas); (II) laboratory data demonstrate abnormally elevated levels of serum gamma globulin and/or IgG, or the presence of autoantibodies; and (III) histopathologic examination of the pancreas shows fibrotic changes with lymphocyte and plasma cell infiltration. For diagnosis, criterion I (pancreatic imaging) must be presented with criterion II (laboratory data) and/or III (histopathologic



**Figure 1** p.L81M mutation of the protease serine 1 gene in autoimmune pancreatitis family. Hatched symbols: Patients with chronic pancreatitis; Striped symbols: Individuals with suspected chronic pancreatitis; Arrows: Subjects who were available for genetic analysis; Plus: Presence of heterozygous mutation; Asterisk: Index patient.

findings)<sup>[16-19]</sup>. Total one pedigree (Figure 1) and 12 unrelated patients with AIP, 56 with chronic pancreatitis, 254 with pancreatic cancer and 120 normal controls seen in the past six years were studied.

### DNA extraction and molecular genetic analysis

Genomic DNA was extracted from peripheral blood and other tissue specimens using a QIAamp DNA mini kit (Qiagen, Hilden, Germany). Primer pairs and experimental condition were used to generate specific fragments according to the references<sup>[12,15]</sup>. The polymerase chain reaction (PCR) products were purified for sequencing after electrophoresis on an agarose gel. For sequencing, a Perkin Elmer Big Dye Sequencing kit (Perkin-Elmer, Shelton, CT, United States) and an ABI PRISM7700 sequencer (Perkin-Elmer ABI, Foster, CA, United States) were used.

### Pancreatitis/pancreatic cancer-associated gene detection

Four genes involved in pancreatitis/pancreatic cancer, *PRSS1*, *SPINK1*, *CFTR* and *MEN1*, were sequenced according to references<sup>[12-15]</sup>. A 20  $\mu$ L mixture was prepared for each reaction and contained 1  $\times$  HotStarTaq buffer, 2.0 mmol/L  $Mg^{2+}$ , 0.2 mmol/L dNTP, 0.2  $\mu$ mol/L of each primer, 1 U HotStarTaq polymerase (Qiagen Inc., Valencia, CA, United States) and 1  $\mu$ L template DNA. The cycling program was 95  $^{\circ}C$  for 15 min; 11 cycles of 94  $^{\circ}C$  for 15 s, 62  $\pm$  0.5  $^{\circ}C$  per cycle for 40 s, 72  $^{\circ}C$  for 1 min; 24 cycles of 94  $^{\circ}C$  for 15 s, 57  $^{\circ}C$  for 30 s, 72  $^{\circ}C$  for 1 min, and 72  $^{\circ}C$  for 2 min. PCR purification was completed using SAP and *Exo* I 1 U SAP, and 6 U *Exo* I was added into 8  $\mu$ L PCR products. The mixture was incubat-

**Table 1** Clinical data of the patients with autoimmune pancreatitis

| Number                 | 1 (I 1) | 2 (II 2) | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    |
|------------------------|---------|----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Type                   | 1       | 1        | 2     | 2     | 1     | 1     | 2     | 2     | 2     | 1     | 1     | 2     | 2     | 1     |
| Sex                    | M       | F        | M     | M     | F     | F     | M     | M     | F     | M     | F     | F     | F     | M     |
| Age/onset              | 61/59   | 63/52    | 70/58 | 59/52 | 68/62 | 70/62 | 53/46 | 46/46 | 60/48 | 59/52 | 62/60 | 62/55 | 48/42 | 33/32 |
| Weight loss (kg/12 mo) | 5       | 3        | 3     | 4     | 6     | 2     | 8     | 3     | 5     | 8     | 6     | 3     | 6     | 9     |
| Nausea/vomit           | +/-     | -/-      | +/+   | -/-   | +/+   | -/-   | +/+   | -/-   | +/-   | -/-   | +/-   | -/-   | +/-   | -/-   |
| IgG (0-16)             | 12.5    | 6.9      | 11    | 9.6   | 8.5   | 6.3   | 23.5  | 26.9  | 21    | 15.2  | 11.3  | 26.3  | 18.5  | 6.9   |
| IgG4 (0.08-1.4 g/L)    | 2.53    | 0.82     | 0.89  | 1.12  | 2.69  | 0.77  | 1.75  | 0.25  | 0.96  | 4.25  | 2.01  | 0.75  | 0.63  | 0.56  |
| Glucose (mmol/L)       | 14.32   | 4.56     | 5.14  | 18.69 | 4.33  | 6.55  | 4.25  | 6.35  | 7.15  | 8.66  | 4.12  | 5.6   | 4.23  | 5.02  |
| Trypsin (ng/mL)        | 28.65   | 63.55    | 52.45 | 33.65 | 56.55 | 32.12 | 23.15 | 56.99 | 87.02 | 74.52 | 63.05 | 56.23 | 78.06 | 12.66 |

F: Female; M: Male; IgG: Immunoglobulin G.

ed at 37 °C for 60 min, followed by incubation at 70 °C for 10 min.

### Pancreatic tissue pathology

Pancreatic tissues were stained with haematoxylin and eosin, modified gomori trichrome, periodic acid-Schiff stain and IgG4 special staining.

### Detection of serum trypsin and amylase

The serum trypsin was tested with ELISA kits (R and D Systems, Minneapolis, MN, United States) and amylase with latex-enhanced nephelometric immunoassay (Dade Behring Marburg GmbH, Germany).

### Functional experiments on mutants

The complete mutated (p.T81M) and wild-type *PRSS1* cDNA were introduced into plasmid pMD18-T (TaKaRa, China) and transformed into *Escherichia coli* DH5 competent cells. Primers were designed for PCR amplification. The forward primer was 5'-TGCAATTGTATGGCAC-CATTCGACGATGATGACAAGAT-3' and the reverse primer was 5'-GAGTCGACTCAGCTAATTAAGCT-TAGTG-3'. In addition, *Mun* I and *Sal* I digestion sites were designed in the forward and reverse primers, respectively. The expression products underwent isolation, purification and renaturation. Benzoyl L-arginine ethyl ester served as a substrate, and absorbance ( $A_{253}$ ) was measured at 253 nm within 30 min. The specific enzyme activity was calculated as follows: specific activity = enzyme activity/mg of protein =  $\Delta A_{253}/t \times 1000/(\epsilon \times t \times 0.001)$ , where t refers to time (min) and  $\epsilon$  refers to the amount of proteinase ( $\mu$ g) during the detection.

### $^{45}\text{Ca}^{2+}$ binding assay

Binding of  $^{45}\text{Ca}^{2+}$  to wild-type recoverin and the L81M mutant was investigated as described previously<sup>[20]</sup>. In brief, 100 mol/L protein was dissolved in 20 mmol/L HEPES-KOH, pH 7.5, 100 mmol/L NaCl and 1 mmol/L DTT, and then it was transferred to centricon 10 devices.  $^{45}\text{Ca}^{2+}$  was added, the samples were centrifuged for 1 min, and radioactivity of the filtrate was counted. Next, non-radioactive  $\text{Ca}^{2+}$  was added and the centrifugation procedure was repeated. Protein-bound  $\text{Ca}^{2+}$  versus free  $\text{Ca}^{2+}$  was determined from the excess  $\text{Ca}^{2+}$  in the protein

sample in the ultrafiltration.

### Treatment and follow-up

Informed consent was obtained before treatment. Glucocorticoids were administered empirically [oral prednisone (40 mg) once daily with a 5-mg taper every 2 wk]. At the same time, oral acid-suppressing agents and calcium were also given.

## RESULTS

### Clinical data of patients with AIP

The auxiliary test results of the patients with AIP (male/female = 7:7) are shown in Table 1. There was significant weight loss (2-9 kg/12 mo) and abnormally increased serum IgG4 level: 5/14 (+).

### Molecular genetic analysis

In the affected patients, novel mutations were found in the genes coding for *PRSS1* (Figure 2). They were *PRSS1\_c.247 C > A* (p.81Leu→Met) (No. 1, 2, 6 and 7) and *PRSS1\_c.279 C > A* (p.91Ala→Ala) (No. 7). None of these mutations were found in the normal controls and other patients with chronic pancreatitis and pancreatic cancer.

### Pathological analysis

Histopathologic examination of the pancreas revealed a characteristic lymphoplasmacytic infiltrate of lymphocytes and IgG4 positive plasma cells, and interstitial fibrosis and acinar cell atrophy in later stages. However, localization and the degree of duct wall infiltration were variable. It has been proposed that a cytologic smear is rich in inflammatory cells. The sensitivity and the specificity of these criteria for differentiating AIP from neoplasia are unknown. A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts (Figure 3A). The number of pancreatic acini was markedly reduced (Figure 3B) (immunohistochemistry by CK, CD3, CD20, CD38, CD68 and vimentin).

### Monitoring of serum enzyme

Patients were treated with glucocorticoids for 3-6 mo,

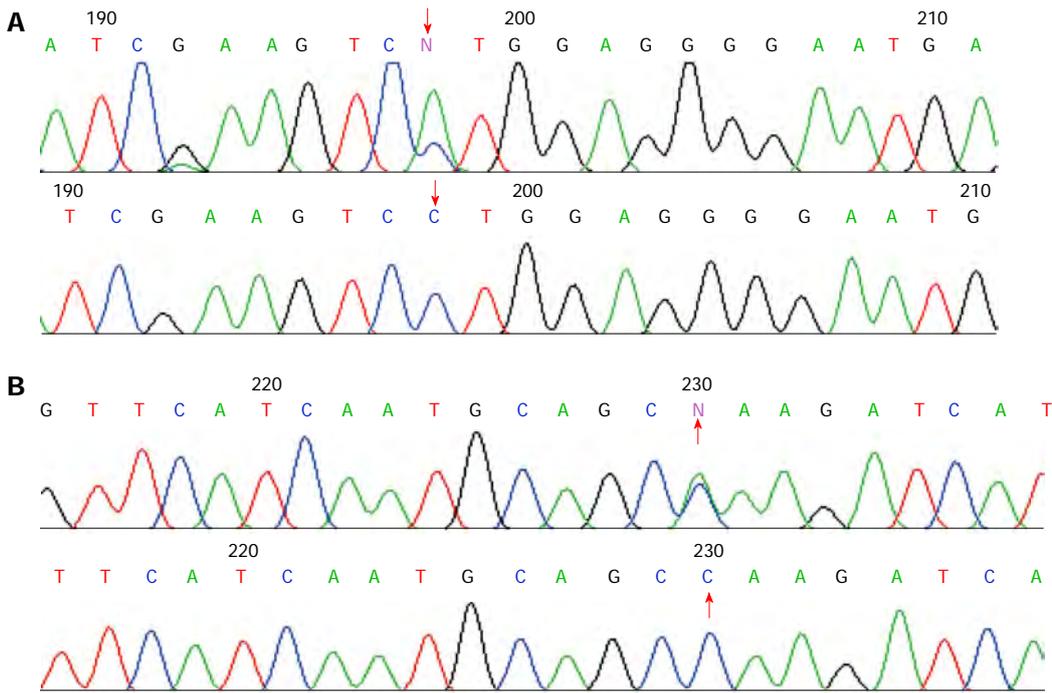


Figure 2 Sequencing of gene mutations from the patients with autoimmune pancreatitis. A: The sequencing c.247 C > A of *PRSS1* gene mutation (p.81Leu→Met); B: Sequencing c.279 C > A of *PRSS1* gene silent mutation (p.91Ala→Ala). The red arrow indicates the base mutation.

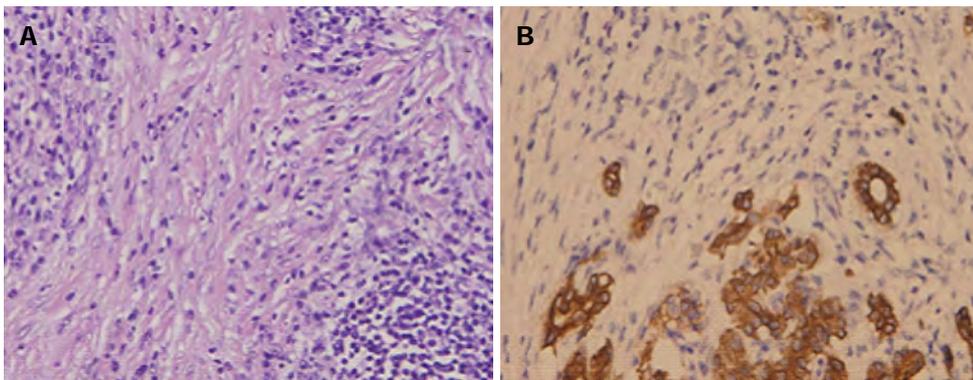


Figure 3 Histopathologic examination of the pancreas. A: A large number of lymphocytes and plasma cells were found in the bile ducts accompanied by hyperplasia of myofibroblasts (hematoxylin-eosin, × 20); B: The number of pancreatic acinar cells was markedly decreased (immunohistochemistry staining of cytokeratin, × 20).

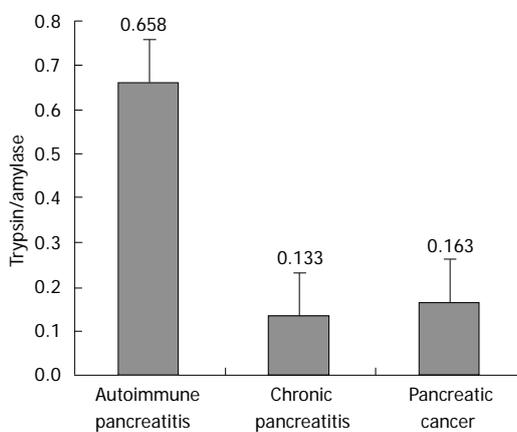


Figure 4 Ratio of trypsin/amyase among the three groups.

and the jaundice improved. Throughout the course of the disease, the trypsin (ng/mL)/amylase (U/L) ratio was higher in patients with AIP ( $0.658 \pm 0.309$ ) than in patients with pancreatic cancer ( $0.163 \pm 0.087$ ) or other types of chronic pancreatitis ( $0.133 \pm 0.095$ ) (Figure 4).

#### Radiologic features

Computed tomography found a diffusely enlarged hypodense pancreas or a focal mass and retroperitoneal lymph node enlargement that may be mistaken for a pancreatic cancer. Magnetic resonance imaging revealed diffusely decreased signal intensity and delayed enhancement on dynamic scanning. The characteristic endoscopic retrograde cholangiopancreatographic finding was segmental or diffuse irregular narrowing of the main pan-

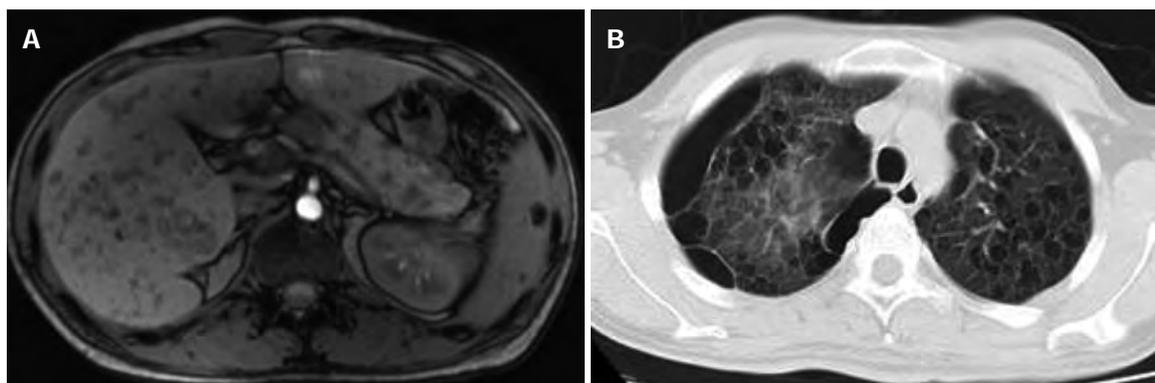


Figure 5 Polycystic lesions in the liver, gallbladder, pancreas, and spleen, and retroperitoneal lymphadenopathy and bullae. A: Abdominal magnetic resonance imaging figure showed diffuse swelling; B: Computed tomography findings image of lung.

creatic duct. The characteristic magnetic resonance cholangiopancreatographic finding was partial intrahepatic bile duct dilatation or narrowness and multiple focal high signal intensity in the liver. Multiple cysts occurred in the liver, pancreas, spleen and other organs (Figure 5).

#### Activity of the products of the mutated gene

A UV spectrophotometer was used to measure the activity of trypsinogen before and after enterokinase activation at 253 nm ( $\Delta OD_{253}$ ). The activity of products of the mutated gene remained unchanged after enterokinase activation. Using the aforementioned formula, the calculated specific activity of renatured recombinant trypsin was 126-183 BAEE U/mg, and the calculated specific activity of wild-type trypsin was 123-165 BAEE U/mg after enterokinase activation, showing that p.T81M mutation did not affect the activity of trypsin.

#### Interaction with phenyl-agarose

According to the literature<sup>[20]</sup>, the binding of recoverin to phenyl-agarose is thought to depend on the  $Ca^{2+}$ -induced exposure of hydrophobic residues and not on the presence of the myristoyl group. Could the non-myristoylated L81M variant bind to phenyl-agarose in a fashion similar to the wild-type? In fact, the binding capacity of the mutant protein was lower (76.2% of that of the wild-type protein).

#### Patient follow-up

Six patients (No. 2, 3, 5, 6, 7 and 10) were treated with glucocorticoids for 3-6 mo, and the jaundice improved. The serum levels of total and direct bilirubin were reduced significantly. Wheezing was markedly improved and the body weight increased (2-5 kg/mo). The symptoms of the other 8 patients were improved to different degrees.

## DISCUSSION

AIP shares many presenting symptoms with pancreatic carcinoma. Fortunately, AIP can be effectively treated and cured. Serum IgG4 levels are usually abnormally increased<sup>[6,9,21-24]</sup>, but increased serum IgG4 levels are

also found in patients with pancreatic cancer and some patients without AIP. Indeed, genetic analyses identified a specific gene, *PRSS1*, for hereditary pancreatitis and other types of chronic pancreatitis in 1996. Some *PRSS1* mutations enhance trypsinogen autoactivation, explaining the young age of patients at onset of AIP. Other mutations may render some patients more susceptible to pancreatitis in the presence of other insults to the pancreas<sup>[10-12]</sup>. In Japan, a strong association with the HLA-DRB1\*0405/DQB1\*0401 haplotype has been identified<sup>[25]</sup>. However, the relationship has not been reported in other ethnic groups, which prompted us to search for AIP-related genes<sup>[25-30]</sup>.

Although p.T81A mutant protein is not associated with functional activity, it binds to the sites that are quite dissimilar from 56Q-57W-58V-59V (*i.e.*, classical  $Ca^{2+}$ -binding sites)<sup>[20]</sup>. On the basis of this observation, a refined model of the role of the myristoyl group as an intrinsic allosteric modulator is proposed.  $Ca^{2+}$  stabilizes the hydrophobic pore structure of the trypsin molecule and increases folding to permit formation of two ionic bonds with  $\beta$  trypsin. Asp102, His57, Ser195 catalytic triad and Asp189, Gly216, Gly226 displayed by the substrate binding pocket of the trypsin are more stable, thereby improving the efficiency of the enzyme catalysis<sup>[20,31-33]</sup>.

There is no controversy that trypsinogen activation plays a very important role in early pancreatitis. However, what activates trypsinogen is not entirely clear. Recent researches have focused on the relationship between the original concentration of intracellular calcium and trypsin activation within acinar cells. Trypsinogen activation starts in the apical part of the acinar cells after supramaximal cholecystokinin stimulation. It is highly dependent on the release of calcium ions within subcellular structures<sup>[32-35]</sup> and on repetitive calcium transients. Normally, trypsin cannot be activated by calcium. Mutation at the trypsin calcium binding point blocks such a conventional activation pathway<sup>[20]</sup>. The gene mutation significantly increases the catalytic activity of trypsin but has no effect on the expression level of the mutant. Increased trypsin synthesis and secretion results in ectopic activation, leading to the occurrence of pancreatitis.

The abnormal increase in serum IgG4 is considered to be an indication of AIP. Unfortunately, serum IgG4 level is normal in more than half of the patients with AIP. In this study, most of our patients with AIP had normal serum IgG4 levels. Our findings suggest that patients with AIP can present with a variety of clinical phenotypes, and that genetic heterogeneity and clinical heterogeneity are features of AIP. In addition, studies of mutations in the *PRSS1* gene have helped elucidate the molecular mechanism underlying the pathogenesis of AIP. Furthermore, high trypsin/amyase ratio contributes to the diagnosis of AIP.

## COMMENTS

### Background

Autoimmune pancreatitis (AIP) is an autoimmune chronic pancreatitis which is characterized by infiltration of lymphocytes and plasma cells, and pancreatic fibrosis and dysfunction. AIP and pancreatic cancer have similar clinical manifestations and findings on imaging, thus AIP is often misdiagnosed as pancreatic cancer, resulting in unnecessary surgical intervention. AIP not only poses a diagnostic challenge for clinicians, but can lead to severe and irreversible injury to patients.

### Research frontiers

The etiology of AIP is poorly understood. It has been reported that genetic factors play an important role in the pathogenesis of AIP. Parkdo *et al* reported that substitution of aspartic acid at the 57<sup>th</sup> position of haploid DQβ1 of the histocompatibility leukocyte antigen is closely related to the recurrence of AIP. The trypsinogen genes are inserted in the TCR vβ site; thus, the trypsinogen gene and TCR vβ gene share the same loci, and this arrangement is conserved in humans, mice, and chickens.

### Innovations and breakthroughs

*PRSS1*<sub>p.81Leu→Met</sub> mutation leads to a trypsin display reduction (76.2% combined with phenyl agarose (Ca<sup>2+</sup> induced failure) which induced premature activation of trypsinogen and caused AIP-related polycystic lesions in the lungs, liver, gallbladder, pancreas, and spleen. Although trypsin was historically believed to be immunologically active, it is now continue to be verified that abnormal activation of trypsin can be recognized by the immune system.

### Applications

The authors' findings suggest that AIP can present with polycystic lesions in multiple organs, which can be found in patients with hereditary AIP, as well as those with sporadic AIP. In addition, functional studies have elucidated the molecular mechanisms underlying the pathogenesis of AIP due to the presence of mutations of the *PRSS1* gene. Additional clinical studies are required to investigate the clinical heterogeneity and molecular pathogenesis of AIP.

### Peer review

This paper reports the novel mutations (p.81Leu→Met and p.91Ala→Ala) found in *PRSS1* gene from patients with AIP. This finding is intriguing, but some of the cases described in the paper may not be AIP for the reasons written in major comments.

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P-Reviewer Goto N S-Editor Zhai HH L-Editor Ma JY  
E-Editor Ma S



## Gene expression profiles in peripheral blood mononuclear cells of ulcerative colitis patients

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Supported by Grants from National Natural Science Foundation of China, No. 81260074/H0310 and 81160055/H0310; Confederate Special Foundation of Science and Technology, Department of Yunnan Province and Kunming Medical College, No. 2011FB183 and 2007C0010R; and Medical Academic Leader of Yunnan Provincial Bureau of Health, No. D-201215

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Received: July 10, 2012 Revised: September 27, 2012

Accepted: March 15, 2013

Published online: June 7, 2013

### Abstract

**AIM:** To identify peripheral blood mononuclear cell (PBMC) gene expression profiles of ulcerative colitis (UC) patients, using oligonucleotide microarrays, to gain insights into UC molecular mechanisms.

**METHODS:** The Human OneArray microarrays were used for a complete genome-wide transcript profiling of PBMCs from 12 UC patients and 6 controls. Differential analysis per gene was performed with a random variance model; *t* test and *P* values were adjusted to control the false discovery rate (5%). Gene ontology (GO) was deployed to analyze differentially expressed

genes at significant levels between patients and controls to identify the biological processes involved in UC.

**RESULTS:** Comparative analysis revealed that 4438 probes (4188 genes) were differentially expressed between the two groups, of which 3689 probes (3590 genes) were down-regulated whereas 749 probes (598 genes) were up-regulated. Many dysregulated genes in our data have been reported by previous microarray studies carried out on intestinal mucosa samples, such as *S100A8*, *CEACAM1* and *S100A9*. GO enrichment analysis revealed 67 high enrichment up-regulated categories and one significant down-regulated category. The up-regulated genes were mainly involved in immune and inflammatory response, cell cycle and proliferation, DNA metabolism and repair.

**CONCLUSION:** Gene expression profiling of PBMCs from patients with UC has highlighted several novel gene categories that could contribute to the pathogenesis of UC.

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**Key words:** Ulcerative colitis; Microarray; Gene ontology; Peripheral blood mononuclear cells

**Core tip:** Evidence indicates that peripheral blood immune cells play a vital role in the pathogenesis of ulcerative colitis (UC). This study identified genome-wide gene expression profiles of peripheral blood mononuclear cells in UC, and gene ontology analysis highlighted the significance of several categories related to immune and inflammatory responses, cell cycle and proliferation, and DNA repair. The gene enrichment analysis provides greater understanding of the processes that may be involved in UC.

Miao YL, Xiao YL, Du Y, Duan LP. Gene expression profiles in peripheral blood mononuclear cells of ulcerative colitis patients. *World J Gastroenterol* 2013; 19(21): 3339-3346 Available from:

## INTRODUCTION

Ulcerative colitis (UC) is a form of chronic inflammatory bowel disease (IBD) characterized by chronic and recurrent inflammation of the colonic mucosa, often resulting in intermittent bloody diarrhea and abdominal pain<sup>[1]</sup>. UC used to be considered western population-specific. However, recent investigation has revealed that the incidence and prevalence of the disease in Asia have increased significantly<sup>[2]</sup>. Taking China as an example, the number of 140120 cases of UC have been observed in the last 15 years, with an 8.5-fold increase during the last 5 years compared with the first 5 years<sup>[3]</sup>; conservative speculation from the reported cases indicate the incidence has reached 11.6/100000<sup>[4]</sup>.

Microarray analysis of global gene expression at the RNA level has generated new insights into the biological phenomena under investigation. Previous studies have successfully applied microarray technology to investigate the gene expression profile of resected specimens<sup>[5-7]</sup> and endoscopic pinch mucosal biopsies<sup>[8-15]</sup> from IBD, with the goal of identifying diagnostic or prognostic markers and therapeutic target genes of the diseases. The principal dysregulated genes revealed some similar biological processes, but there is little overlap between the individual genes among these data. In contrast to most studies utilizing intestinal tissue, taking peripheral blood mononuclear cells (PBMCs) is more accessible as this source of gene expression material offers the possibility of sampling any individual for future diagnosis. What is more important, PBMCs may be considered as a part of the intestinal immune system and contribute to the exacerbation and perpetuation of inflammation in UC. Firstly, PBMCs are found migrating between blood and intestine<sup>[14]</sup>, and labeled peripheral granulocytes and monocytes can be accumulated rapidly in the diseased gut of IBD patients<sup>[15]</sup>; this movement has a positive correlation to the disease activity<sup>[14]</sup>. Secondly, peripheral mononuclear cells in active IBD are found to release increased amounts of many proinflammatory mediators<sup>[16]</sup>. *In vitro* study found a cytotoxic effect in human fetal colon cells that was released by PBMCs in UC patients<sup>[17]</sup>. Leukocytapheresis therapy, through removing a portion of activated immunocytes from the peripheral blood<sup>[18]</sup>, indeed achieves a super effect and a lower incidence of adverse effects than high-dose steroids in a proportion of IBD patients<sup>[19]</sup>.

Given that PBMCs may play a vital role in the pathogenesis of UC and that the mechanism is still obscure, we hope to clarify the molecular processes involved in the etiopathogenesis of UC that are represented by differentially expressed genes. We recruited twelve UC patient and six healthy controls to compare gene expression profile differences in PBMCs and then, through system biology

tools, some useful information was found hidden behind the lists of significantly up- and down-regulated genes that may be further illustrate the pathogenesis of UC.

## MATERIALS AND METHODS

### *Patient selection and blood sample processing*

The diagnoses of patients were based on a combination of clinical features, endoscopy and pathology findings using standard diagnostic criteria<sup>[4,20]</sup>. Two groups of patient samples were recruited at the First Affiliated Hospital of Kunming Medical University. Group one was composed of 12 UC patients (half males and half females, mean age  $36.25 \pm 10.98$  years; range 19-60 years, Table 1) and 6 healthy controls (3 females, mean age  $35.17 \pm 14.88$  years; range 19-60 years) for microarray analysis. Median duration of disease was 4.33 years (range 1-12 years). At the time of recruitment, four patients were not receiving therapy, eight patients were receiving 5-aminosalicylic acid treatment, and none of them had taken corticosteroids or immunodepressants during the three months preceding their enrolment. The Sutherland Disease Activity Index was used to assess disease activity; all the patients were in active stage (scores ranging from 5 to 12). Group two was extended to 21 patients (mean age  $30 \pm 9.58$  years; range 20-46 years) and 21 age- and sex-matched healthy individuals (mean age  $32 \pm 11.3$  years; range 24-50 years) for the subsequent reverse transcription polymerase chain reaction (RT-PCR) analysis intending to verify and investigate the epidemiology of interesting markers.

To avoid the potential effect of diseases or conditions other than UC, which may lead to gene expression changes in PBMCs, the following strict exclusion criteria were implemented to screen combined diseases of (1) immune deficiency disease; (2) autoimmune-related diseases; (3) hematological diseases; and (4) any treatment or disease that would be an apparent factor affecting gene expression in these cells (*e.g.*, chronic obstructive pulmonary disease, allergy, smoking, chronic alcohol consumption, hormonal or other medications, *etc.*). Controls were persons who receiving a regular medical checkup at the First Affiliated Hospital of Kunming Medical University with the excluded diseases or factors mentioned above.

After obtaining approval of the Institutional Review Board and Ethics Committee, and informed consent of all 60 individuals, blood samples (10 mL) were collected at the Clinical Laboratory of First Affiliated Hospital of Kunming Medical University, and then PBMCs were isolated immediately. Each PBMC biopsy was added to 1 mL TRIzol Reagent (Invitrogen, CA, United States) and then kept at  $-80^{\circ}\text{C}$  until isolation of the RNA.

### *RNA isolation and microarray experiments*

The RNA amplification, labeling, hybridization, and data extraction were performed at the National Engineering Research Center for Biochip at Shanghai. Briefly, total RNA was extracted from PBMCs with Trizol Reagent (Invitrogen, United States), then purified to remove ge-

Table 1 Subject characteristics

| No. | Age (yr) | Sex | Duration (yr) | Extent             | SDAI | Therapy    |
|-----|----------|-----|---------------|--------------------|------|------------|
| 1   | 19       | F   | 1             | Proctitis          | 5    | No therapy |
| 2   | 27       | F   | 5             | Proctosigmoiditis  | 7    | 5-ASA      |
| 3   | 30       | F   | 2             | Proctosigmoiditis  | 6    | No therapy |
| 4   | 34       | F   | 2             | Proctosigmoiditis  | 7    | No therapy |
| 5   | 40       | F   | 4             | Left sided colitis | 9    | 5-ASA      |
| 6   | 45       | F   | 3             | Left sided colitis | 8    | 5-ASA      |
| 7   | 22       | M   | 3             | Proctosigmoiditis  | 7    | 5-ASA      |
| 8   | 33       | M   | 7             | Pancolitis         | 10   | 5-ASA      |
| 9   | 37       | M   | 2             | Proctosigmoiditis  | 8    | No therapy |
| 10  | 40       | M   | 5             | Pancolitis         | 9    | 5-ASA      |
| 11  | 48       | M   | 6             | Left sided colitis | 8    | 5-ASA      |
| 12  | 60       | M   | 12            | Pancolitis         | 12   | 5-ASA      |

M: Male; F: Female; 5-ASA: 5-Aminosalicylic acid; SDAI: Sutherland disease activity index.

nomic DNA contamination and concentrated using an Rneasy Plus Mini kit (Qiagen, Hilden, Germany). RNA quality was assessed by determining the 26S/18S ratio using a Bioanalyzer 2100 (Agilent, CA, United States). Two micrograms of RNA from each subject of group one was converted into cyanine-5 labeled target cRNA, then hybridized to the Human OneArray microarray (Phalanx Biotech Group, Taiwan, China) containing 32050 oligonucleotides: 30968 human genome probes and 1082 experimental control probes. A complete description of the array is available at [www.phalanxbiotech.com](http://www.phalanxbiotech.com). The followed procedure was washing, scanning and extracting gene expression results (DNA Microarray Scanner G2565B, Agilent, United States) according to the manufacturer's protocol. Raw intensity values were normalized and log-transformed by GeneSpring GX 10 software (Agilent Technologies, United States).

### Statistical analysis

We applied the rostral ventromedial medulla (RVM) *t* test to filter the differentially expressed genes for the control and experimental groups because the RVM *t* test can raise degrees of freedom effectively in the cases of small samples. After the significant analysis and false discovery rate (FDR) analysis, we selected the differentially expressed genes according to the  $P < 0.01$  as a threshold. Gene ontology (GO) analysis was applied in order to organize dysregulated genes into hierarchical categories according to the key functional classification of the National Center for Biotechnology Information, and to uncover the gene regulatory network on the basis of biological process and molecular function<sup>[21]</sup>. Generally, Fisher's exact test and  $\chi^2$  test were used to classify the GO category, and the FDR<sup>[22]</sup> was calculated to correct the *P* value, since the smaller the FDR, the smaller the error in judging *P* value. We chose only GO categories that had a *P* of  $< 0.01$ . Within the significant category, the enrichment Re was given by  $Re = (m/n)/(N_r/N)$  where *m* is the number of differential genes within the particular category, *n* is the total number of genes within the same category, *N<sub>r</sub>* is the number of differential genes in the entire microarray, and *N* is the total number of genes in the microarray<sup>[23]</sup>.

### Validation of microarray results by semi-quantitative RT-PCR

For the second part of our study, mRNA from 21 UC patients and 21 age- and sex-matched controls was used for RT-PCR. Primers for PCR validations were designed using Primer-3 software<sup>[24]</sup>. Primer sequences are presented in Table 2. Total RNA was extracted from PBMCs and purified using the Rneasy Kit according to the manufacturer's instruction (Qiagen, United States) and converted to cDNA by moloney murine leukemia virus reverse transcriptase with oligo(dT) primer in the presence of RNasin (Stratagen, United States). After reverse transcription, 1/10<sup>th</sup> of the cDNA was used for each PCR reaction with 0.2  $\mu$ mol/L of each primer, 100  $\mu$ mol/L dNTPs, 2 mmol/L MgCl<sub>2</sub>, and 1 U Taq polymerase (Promega, Madison, WI, United States). Cycling conditions were the same for all primer pairs: 94 °C for 2 min, and then 30 cycles at 94 °C for 30 s followed by 55 °C for 45 s and 72 °C for 45 s.  $\beta$ -Actin served as a quantitative control and its sense primer is 5'-TTC-CAGCCTTCCTTCCTGG-3' and antisense primer, 5'-TTGCGCTCAGGAGGAGCAAT-3'. PCR products were fractionated by agarose electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light. Densitometric analysis was done using Bio-Rad's Quantity One software. Statistical significance was tested using Student's *t* test.

## RESULTS

### Analysis of differentially regulated genes in UC

Identifying differential expression genes was achieved in 12 UC cases and 6 age- and sex-matched healthy controls by using RVM *t* test with a FDR of 0.5% and  $P < 0.01$ . Comparative analysis revealed that 4438 probes (4188 genes) were differentially expressed between the two groups, of which 3689 probes (3590 genes) were down-regulated whereas 749 probes (598 genes) were up-regulated. The data presented here confirm 10 (*SERPINB2*, *IGHG3*, *FUT4*, *Meis1*, *ZNF145*, *NFKB1A*, *APBB2*, *IGHM*, *COPG2*, *PTGDS*) of 71 reported genes identified by Burczynski *et al.*<sup>[25]</sup> with similar design of our study. Excepting *SERPINB2*, we also observed the up-regulation of another fundamental inhibitor of the fibrinolytic process: *SERPINE1* fold change (FC) 3.15,  $P = 3.8 \times 10^{-6}$ .

Some dysregulated genes found in mucosal biopsies of UC also appeared in the present PBMC study, such as genes for up-regulating of S100 family proteins (*S100A8*, FC 2.24,  $P = 0.0016$ ) and *S100A9* (FC 3.24,  $P = 0.00041$ ), as reported by Lawrance *et al.*<sup>[7]</sup> and Noble *et al.*<sup>[12]</sup>, and carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*, FC 4.59,  $P = 0.00027$ ) observed by Costello *et al.*<sup>[8]</sup> and Dooley *et al.*<sup>[26]</sup>. In general, compared to four recently published papers that applied microarrays to explore gene expression profiles of endoscopic pinch mucosal biopsies with UC, our data confirmed 7 over-expressed (27%) and 14 down-regulated (22%) genes reported by Wu *et al.*<sup>[11]</sup>. Another microarray study found

**Table 2** Primers used for semiquantitative reverse transcription-polymerase chain reaction

| Genes   | Unique ID     | Forward primer sequence       | Reverse primer sequence       |
|---------|---------------|-------------------------------|-------------------------------|
| CEACAM6 | PH_hs_0020590 | 5'-GGGGACTTCTGTATTATGCTAAC-3' | 5'-GTGGTGCTAAATGCTACATACTC-3' |
| ELF4    | PH_hs_0009109 | 5'-CAGATGGGGTCTGATACTGG-3'    | 5'-ACGTGTGTATTGCATCATCG-3'    |
| MEFV    | PH_hs_0010414 | 5'-GGACATTGGAGACATCTGC-3'     | 5'-CGCAGGGTTCTGAGAAGTA-3'     |
| APAF1   | PH_hs_0024223 | 5'-AGAGACTGAGGCTGATGGTG-3'    | 5'-TTAAAGCCAGGCAGTGAATC-3'    |
| SLC1A6  | PH_hs_0032730 | 5'-CACCATGGTCATTGTGCTTA-3'    | 5'-GTGCCATGAGGGACTGTAG-3'     |
| KCNJ11  | PH_hs_0032311 | 5'-AGGAGGACGGACGTTACTCT-3'    | 5'-GGAATCTGGAGAGATGCTGA-3'    |

differences in 143 genes, of which our data confirmed 27 (18.9%)<sup>[12]</sup>. Twenty-six up- and 9 down-regulated genes out of 272 differentially regulated genes reported by Costello *et al.*<sup>[8]</sup> were seen in our data. Among the eight up-regulated genes reported by the only microarray study based on an Asian UC population carried out by Okahara *et al.*<sup>[10]</sup>, up-regulation of the *TFF1* gene was the only consistent finding. Although there is little overlap between the individual genes identified compared to those microarray studies due to different microarray platforms, specimens, and race, all the studies point to the involvement of one major biological process: the immune and inflammatory response.

To analyze functional differences represented by differentially expressed genes, we conducted a GO analysis. A  $P < 0.01$  in the Fisher's exact tests was selected as the significant criteria. Using this threshold, a total of 67 high-enrichment GOs targeted by over-expressed genes and one term corresponding to under-expressed genes (nucleosome assembly) were identified. Over-expressed genes were mainly segregated to three critical biological processes: (1) immune and inflammatory response (immune response, inflammatory response, innate immune response, positive regulation of natural killer cell-mediated cytotoxicity); (2) cell cycle and proliferation [cell cycle, cell proliferation, natural killer (NK) cell proliferation, natural killer T (NKT) cell proliferation, cell differentiation, cell division, cell cycle arrest]; and (3) DNA metabolism and repair (response to DNA damage stimulus, DNA repair, DNA recombination). The GO terms and enrichment are shown in Figure 1.

### Validation of selected microarray results

To validate the microarray data results, we chose six genes to quantify by RT-PCR in 21 UC cases and 21 controls. In agreement with the microarray data, of the four up-regulated genes, three were found to be expressed to a significantly greater extent in UC by RT-PCR (*CEACAM6*:  $0.0491 \pm 0.0353$  vs  $0.955 \pm 0.141$ ,  $P < 0.01$ ; *ELF4*:  $0.458 \pm 0.143$  vs  $0.185 \pm 0.0703$ ,  $P < 0.01$ ; *MEFV*:  $0.451 \pm 0.245$  vs  $0.161 \pm 0.0629$ ,  $P < 0.01$ ). Another up-regulated gene, *APAF1*, also showed the same tendency with microarray ( $0.253 \pm 0.168$  vs  $0.104 \pm 0.0308$ ,  $P < 0.05$ ). Of two down-regulated genes, *SLC1A6* was found to be significantly lower in UC than in controls ( $0.144 \pm 0.0749$  vs  $0.356 \pm 0.127$ ,  $P < 0.01$ ), and *KCNJ11* was not found to be significantly different between the two groups ( $0.227 \pm 0.0549$  vs  $0.294 \pm 0.0965$ ,

$P > 0.05$ ) (Figure 2).

## DISCUSSION

Clarifying the molecular mechanisms leading to UC remains a major challenge.

Definite diagnosis of UC mainly depends on colonoscopy and a following invasive biopsy due to deficiency of molecular markers for UC, and current first-line therapies generally target downstream of the inflammatory cascade. For these reasons, it is exigent to generate new biologic agents aimed at specific inflammatory processes and to explore disease specific molecular markers to facilitate diagnosis. To identify the molecules involved in activation behavior of PBMCs in UC, we compared the gene expression profiles of PBMCs between UC patients and healthy individuals using microarray technology. In addition, GO enrichment analysis, which was based on the significant dysregulated genes, has been developed to reveal whether particular functions were enriched.

To the best of our knowledge, there is only one published study on PBMC gene expression profiles in UC. Comparison of that study with the present Phalanx array screening on 30968 genes, which represents the first gene expression analysis in an Asian population, shows consistency with data from Burczynski *et al.*<sup>[25]</sup> who found that the most highly expressed gene commonly elevated in both IBDs was that for the protease inhibitor SERPINB2: we also observed the upregulation of *SERPINE1* in UC patients. Another gene for fundamental inhibitor of the fibrinolytic process, *SERPINE1* (also called *PAI-1*) showed upregulation in our data. Increased plasma levels of PAI-1 have been found in IBD patients<sup>[26]</sup>, this suggests that these molecules might contribute to the imbalance of the coagulation system and thromboembolic events in UC. Another consistent finding was the up-regulated expression of two immunoglobulin encoding sequences, *IgHG3* and *IGHM*. This correlates with the finding that elevated serum immunoglobulin G (IgG) levels are found in patients with UC, and is reminiscent of the finding that IgG antibodies to organ specific autoantigens such as colonic epithelial cells and antineutrophil cytoplasmic are identified in the colon of UC patients, and that these antibodies originate in the periphery.

A response to the peripheral antigens which have switched isotype to cross react with antigens of the flora or/and autoantigens which are normally segregated by the endothelial barrier<sup>[27]</sup> may result in mucosal inflamma-

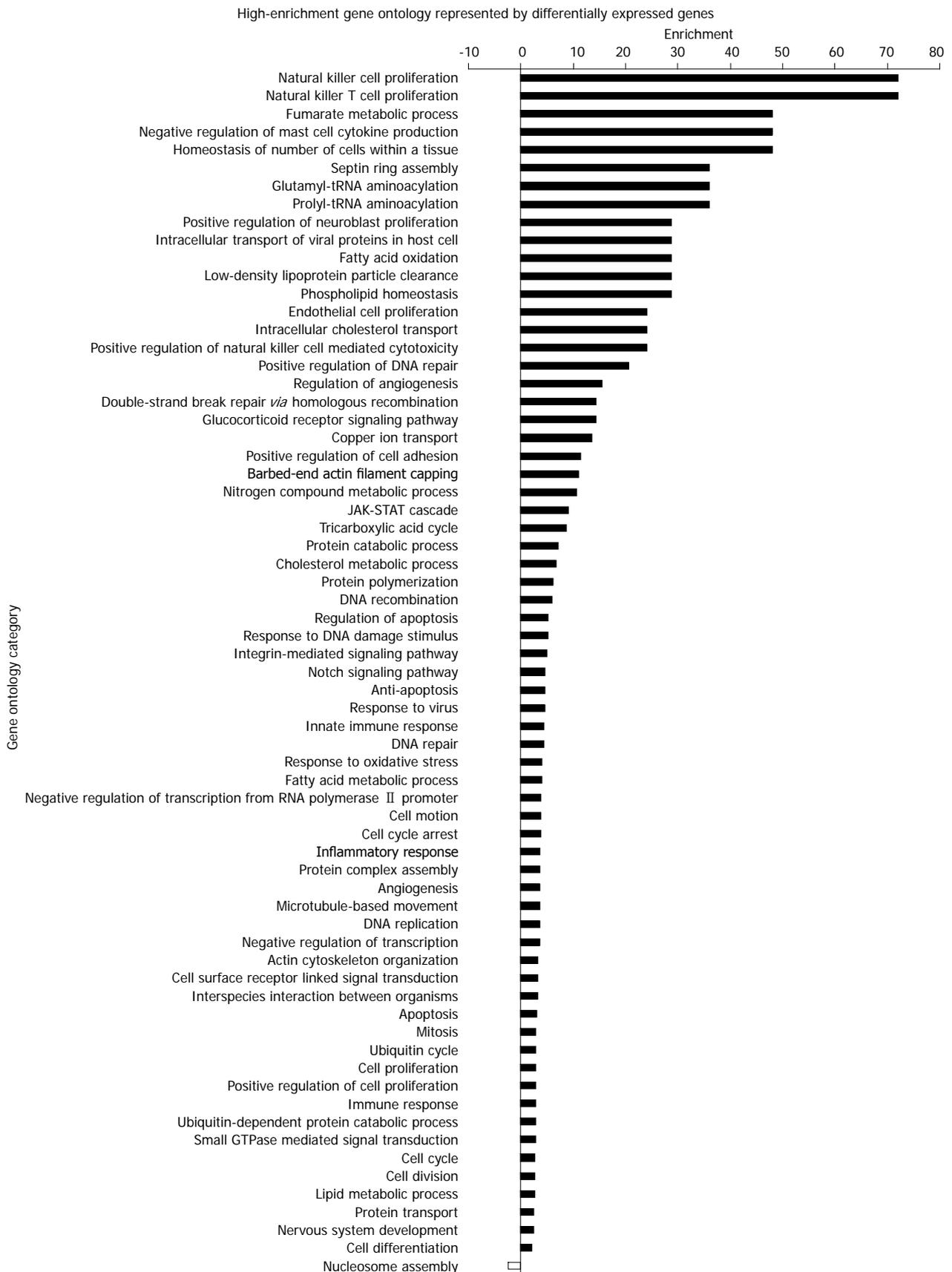
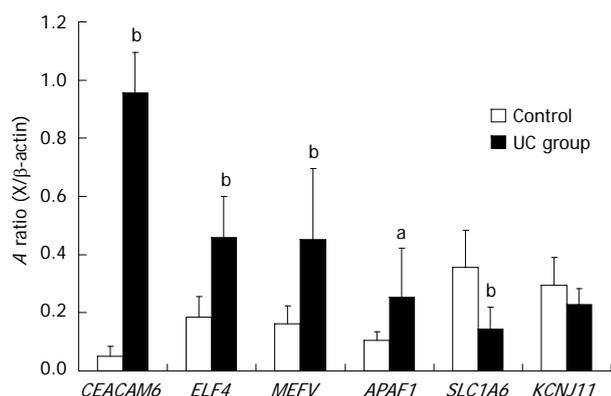


Figure 1 High-enrichment gene ontology represented by differentially expressed genes. A  $P < 0.01$  in the Fisher's exact test was selected as the significance criterion. Over-expressed genes were mainly segregated to three critical biological processes: immune and inflammatory response, cell cycle and proliferation, DNA metabolism and repair. JAK-STAT: Janus kinase-signal transducer and activator of transcription.



**Figure 2** Semiquantitative relative reverse transcription-polymerase chain reaction validation of 6 selected genes. The values were normalized against  $\beta$ -actin. Graph bars indicate the averaged absorbance (A) ratios between each gene and  $\beta$ -actin. Values represent the mean  $\pm$  SE. Significance levels were: <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control subjects. UC: Ulcerative colitis.

tion. If this is the case, IgG-producing cells will expand through the antigen stimulation. This can explain the many up-regulated genes related to cell cycle and positive regulation of cell proliferation seen in our data.

It is of interest that many dysregulated genes in our data have been reported by previous microarray studies carried out on intestinal mucosa samples, such as genes for S100 family proteins (*S100A8* and *S100A9*) reported by Lawrence *et al*<sup>[7]</sup> and Noble *et al*<sup>[12]</sup>. In addition, another calcium binding protein, *S100A12*, has been demonstrated to be a novel noninvasive marker for IBD with high sensitivity and specificity<sup>[28]</sup>; this gene also showed upregulation in our data. Up-regulation of *CEACAM1* has been reported by Costello *et al*<sup>[8]</sup> and Dooley *et al*<sup>[26]</sup>. The up-regulation of another CEACAM family gene in our data, *CEACAM6*, has not been reported by any microarray study but has been found acting as a receptor for certain bacteria<sup>[29]</sup>.

Although there is little overlap between the individual genes identified compare to those other microarray studies due to different microarray platforms, specimens, and race, all the studies point to the involvement of one major biological process: immune and inflammatory response. GO enrichment analysis found several high-enrichment terms linked to immunity and inflammation, including immune response (33 genes), inflammatory response (17 genes), innate immune response (9 genes). The fact that the immune system and inflammatory mechanisms are activated in IBD is also well known<sup>[30]</sup>. It is not surprising that a general up-regulation of immune and inflammatory response genes is a feature of the intestine due to the nonsterile environment, but many up-regulated genes appear in PBMCs that had been ruled out by excluding patients with infections, malignancies, autoimmune disorders and so on that may impact the gene expression of PBMCs consistent with its role in the pathogenesis of UC. Evidently, not all of those genes involved in immune and inflammatory categories are UC specific. Other inflammatory conditions such as arthritis, dermatitis and atherosclerosis share common immunologic/inflamma-

tory mechanisms, although in different organ systems. Therefore, it is of importance to identify the profile of inflammatory genes that are differentially expressed in UC and compare it with those that characterize other inflammatory diseases. Although a crossover of some of these mechanisms is expected, it is highly plausible that differences between the diseases will also become apparent. Such differences have great potential as better diagnostic and prognostic biomarkers for these conditions.

NK cells and NKT cells are effector lymphocytes of the innate immune system and can limit or exacerbate immune responses<sup>[31]</sup>. Labeled peripheral blood granulocytes and monocytes have been shown to accumulate rapidly to the diseased intestine in IBD patients<sup>[15]</sup>, leading to increased numbers of NKT cells accumulating in the lamina propria<sup>[32]</sup>. This movement is correlated with the disease activity<sup>[14]</sup>. NK cells and NKT cells can contribute to tissue damage not only by their direct cytotoxic effects on the targets, but also by secretion of inflammatory cytokines, and those cytokines can enhance their cytotoxicity and attract much more leukocyte aggregation to release more chemokines that perpetuate and amplify this vicious circle<sup>[33,34]</sup>. Considering cell cycling is essential to induce tolerance and generate effector T cells<sup>[35]</sup>, a faster cycle will thus lead to an increased capacity for cellular expansion. It is tempting to speculate that up-regulated genes positively modulate proliferation of NK cells, NKT cells and cell cycling, playing a vital role in putting PMBCs as a source of effector immune cells in the inflamed intestine and magnifying the function of innate immunity and breaking the immune homeostasis. Therefore, it is a promising target to restrain the activation and proliferation of NK and NKT cells for treatment in active patients. However, the action of microflora and other factors which can activate these cells is complex and obscure. Accordingly, *ELF4* and *MEFV*, which are overexpressed genes from the categories that regulate the proliferation of NK and NKT cells, might be a focus of interest.

Studies have suggested that *ELF4* is a key transcriptional regulator of innate immune cells, affecting the number and function of NK cells and NKT development<sup>[36]</sup>. In a murine experiment<sup>[36]</sup>, the number of NKT cells was significantly reduced in the *ELF4*-deficient thymus. Similarly, NK cell development and function is impaired, with a 60% reduction in the number of NK cells in *ELF4* knock-out spleens and defects in their function. Another gene, *MEFV*, responsible for familial Mediterranean fever (FMF), also may contribute to the onset of IBD. One epidemiologic investigation of non-Askenazi Jews indicated that FMF patients have a high incidence and severity of both Crohn's disease (CD) and UC<sup>[37]</sup>, indicating the potential modifying effect of *MEFV* expressed in certain inflammatory diseases. The expression of *MEFV* was found to be significantly higher in the colonic mucosa of colitis mice models (trinitrobenzenesulfonic acid and dextran sodium sulfate) and CD and severe UC patients<sup>[38]</sup>. Apart from *MEFV* and *ELF4*, upregulated genes involved in categories that relate to cell growth and proliferation, including positive regulation of

cell proliferation, cell division, cell proliferation, also deserve further exploration.

Chromosome instability and microsatellite instability (MSI) have been proven to be associated with UC<sup>[39]</sup>, and further contribute to the carcinogenesis of UC-associated neoplasms<sup>[40]</sup>. Increased production of free radicals may impair cellular and DNA proteins and decrease the ability of the cell to repair DNA damage prior to replication. We have identified increased expression of transcripts involved in DNA repair in PBMCs (response to DNA damage stimulus, DNA repair, DNA recombination, double-strand break repair *via* homologous recombination, DNA replication, and positive regulation of DNA repair). Those categories positively regulating DNA repair seem to act as a defense role against carcinogenesis in UC, but opposite to this conjecture, a recent study observed that increased activity of base excision repair enzymes can contribute to increasing of MSI in inflamed UC mucosa<sup>[41]</sup>. Another study identified increased transcription involved in DNA repair in UC-associated colorectal cancer biopsies compared to UC mucosa<sup>[42]</sup>. In other words, increased expression of those categories involved in DNA repair may be injurious to the stability of the genome and may even increase neoplastic risk. Watanabe *et al.*<sup>[43]</sup> have identified 40 genes which are involved in categories such as in cell cycle, cell proliferation, and receptor activity to predict the development of cancer and/or dysplasia in UC with sensitivity and specificity of 100% and 83.7%, respectively. The dysregulated genes involved in categories of cell cycle and DNA repair identified in our study deserve in-depth studies to investigate the possibility of non-invasive markers for predicting the development risk of cancer in UC patients.

In conclusion, this study identified genome-wide gene expression profiles of PBMCs in UC, and GO analysis highlighted the significance of several categories related to the immune and inflammatory response, cell cycle and proliferation and DNA repair. The gene enrichment analysis provides greater understanding of the processes that may be involved in UC.

## COMMENTS

### Background

Evidence indicates that peripheral blood immune cells play a vital role in the pathogenesis of ulcerative colitis (UC). Microarray transcript profiling has the promise to clarify the molecular processes involved in the etiopathogenesis of UC.

### Research frontiers

Microarrays represent important tools to identify biomarkers of disease; biomarkers can be constituted by gene expression patterns or clusters of genes. Systems biology tools can find some useful information hidden behind the lists of differentially expressed genes at significant levels.

### Innovations and breakthroughs

In contrast to most studies utilizing intestinal tissue, the authors adopted more accessible specimens and carried out the research in Chinese UC patients for the first time.

### Applications

This study further elucidated the biological process that may lead to the pathogenesis of UC.

### Terminology

The gene ontology project is a major bioinformatics initiative to unify the repre-

sentation of gene and gene product attributes across all species.

### Peer review

The authors found some dysregulated genes in peripheral blood mononuclear cells of UC and further demonstrated biological processes represented by those differentially expressed genes. This is a very important study which could unravel the mechanisms of UC. The scientific content of the manuscript is original and novel.

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E- Editor Li JY



## Varicella zoster meningitis complicating combined anti-tumor necrosis factor and corticosteroid therapy in Crohn's disease

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Author contributions: Ma C, Walters B and Fedorak RN contributed to the manuscript writing and revision of the manuscript. Supported by Abbott Canada to Fedorak RN

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Received: January 3, 2013 Revised: March 21, 2013

Accepted: April 3, 2013

Published online: June 7, 2013

### Abstract

Opportunistic viral infections are a well-recognized complication of anti-tumor necrosis factor (TNF) therapy for inflammatory bowel disease (IBD). Cases of severe or atypical varicella zoster virus infection, both primary and latent reactivation, have been described in association with immunosuppression of Crohn's disease (CD) patients. However, central nervous system varicella zoster virus infections have been rarely described, and there are no previous reports of varicella zoster virus meningitis associated with anti-TNF therapy among the CD population. Here, we present the case of a 40-year-old male with severe ileocecal-CD who developed a reactivation of dermatomal herpes zoster after treatment with prednisone and adalimumab. The reactivation presented as debilitating varicella zoster virus meningitis, which was not completely resolved despite aggressive antiviral therapy with prolonged intravenous acyclovir and subsequent oral valacyclovir. This is the first reported case of opportunistic central nervous system varicella zoster infection complicating anti-TNF therapy in the CD population. This paper also reviews the literature on varicella zoster virus infections of immunosuppressed IBD patients and the importance of vaccination prior to initiation of

anti-TNF therapy.

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**Key words:** Varicella zoster virus; Meningitis; Crohn's disease; Adalimumab; Infliximab; corticosteroids; Anti-tumor necrosis factor

**Core tip:** Opportunistic viral infections can complicate anti-tumor necrosis factor (TNF) therapy for inflammatory bowel disease (IBD). Central nervous system varicella zoster virus (VZV) infections associated with the use of anti-TNF therapy have not been previously described in Crohn's disease patients. We present the first reported case of VZV meningitis in a 40-year-old male with Crohn's disease who developed reactivation dermatomal herpes zoster and VZV meningitis after treatment with adalimumab and prednisone. Despite aggressive antiviral therapy, he had significant morbidity, highlighting the risk of opportunistic viral infections in this population and the importance of vaccination before anti-TNF therapy.

Ma C, Walters B, Fedorak RN. Varicella zoster meningitis complicating combined anti-tumor necrosis factor and corticosteroid therapy in Crohn's disease. *World J Gastroenterol* 2013; 19(21): 3347-3351 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3347.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3347>

### INTRODUCTION

Biologic therapies which target tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), including infliximab, adalimumab, and certolizumab pegol, are increasingly common in the management of inflammatory bowel disease (IBD); however, their use is associated with opportunistic infections<sup>[1,2]</sup>. For Crohn's disease (CD) patients, infection risk is further

increased by combination treatment with immunosuppressants such as corticosteroids, methotrexate, azathioprine, or 6-mercaptopurine (6-MP)<sup>[3,4]</sup>.

As with the other herpes viruses, varicella zoster virus (VZV) infection risk is high for IBD patients<sup>[5,6]</sup>. VZV, an alpha-herpes virus, causes a primary infection (varicella/chickenpox), but the virus can also be reactivated from a latent state in which it sequesters in the dorsal root ganglia (herpes zoster/shingles)<sup>[7]</sup>. Evidence from the rheumatologic literature suggests an association between VZV and TNF inhibitors. Indeed, a large prospective cohort of 3266 rheumatoid arthritis patients on anti-TNF therapy found an adjusted hazard ratio for VZV of 1.82 (95%CI: 1.05-3.15), and these VZV cases were often severe enough to necessitate hospitalization<sup>[8]</sup>.

While cutaneous VZV is common, neurological VZV is rare; presentations include cerebellar ataxia, myelitis, radiculitis, Ramsay-Hunt syndrome, and meningitis or encephalitis<sup>[9]</sup>. VZV meningitis in association with anti-TNF therapy for Crohn's disease has not previously been reported. Here, we present the case of a 40-year-old male with CD who developed debilitating VZV meningitis while being treated with adalimumab and prednisone. The literature on VZV among anti-TNF immunosuppressed CD patients and on pre-treatment vaccination is reviewed in the Discussion which follows.

## CASE REPORT

A 40-year-old male presented to hospital with a four day history of increasing headaches. He had been diagnosed with medically refractory ileocecal CD in 2006. Initial treatment with azathioprine had to be discontinued because of acute pancreatitis. In 2007, he underwent an ileal resection and hemicolectomy; however, the disease recurred at the anastomosis by 2008. Despite treatment with post-operative mesalamine, disease activity persisted, and he began taking oral prednisone at 20 mg/d in September 2008. The disease became steroid-refractory, and infliximab 400 mg IV q8 weekly was started in 2009. With initiation of infliximab, the patient was able to wean off prednisone for a period of 8 mo. However, after one year, an allergic reaction to infliximab prompted a switch to adalimumab 40 mg SC q2 weekly at 25 mo prior to presentation. About 6 mo after the initiation of adalimumab, the patient experienced a disease flare and was restarted on prednisone. He experienced difficulties with weaning from the prednisone and was taking 15 mg *po* daily on presentation.

Four days prior to presentation, the patient developed insidious onset but constant bifrontal, progressively worsening headaches with photophobia. While the patient was experiencing unmeasured fever and generalized malaise, there was no history of neck pain, focal neurological deficits, seizures, or confusion. He had no recent infectious contacts or travel history. Though he had a history of childhood chickenpox, he had experienced no recent reactivation and he had not received a herpes zoster vac-

ination.

Two days prior to presentation, the patient developed increasing left upper quadrant abdominal pain, radiating to his back. The initial examination revealed voluntary guarding but no rash. Shortly after admission, the patient developed a vesicular maculopapular rash in the left T7 dermatome corresponding to the area of pain.

A detailed neurological examination demonstrated no focal motor or sensory deficits. Cranial nerve testing results were normal. Fundoscopy did not reveal papilledema. There was no nuchal rigidity; both Brudzinski's and Kernig's signs were negative, but jolt accentuation was positive.

Diagnostic investigations revealed an elevated white blood cell count of  $14 \times 10^9/L$ . Computer tomography of the head was unremarkable. Lumbar puncture was performed: the cerebrospinal fluid (CSF) revealed an elevated protein level [0.76 g/L, (normal range 0.15-0.45 g/L)], normal glucose [3.1 mmol/L, (normal range 2.2-4.4 mmol/L)], and a marked lymphocytic pleocytosis ( $391 \times 10^6$  WBCs with 98% lymphocytes). CSF polymerase chain reaction was subsequently positive for VZV. After consultation with the Infectious Disease specialist, we prescribed treatment for VZV meningitis: one month of intravenous acyclovir (10 mg/kg q8 h). Adalimumab was discontinued but, given the patient's severe CD, prednisone, 20 mg/d, was started. The patient has been unable to taper off this dose of prednisone.

Unfortunately, the patient's post-discharge course has been difficult. He continued to experience debilitating residual symptoms of post-meningitis syndrome, including intermittent headaches and cognitive slowing, and was unable to return to work 3 mo post-discharge. Given his ongoing symptoms and continuing immunosuppression, he was treated with an additional course of suppressive valacyclovir 1000 mg *po* daily for 3 mo.

## DISCUSSION

Although VZV reactivation in response to anti-TNF therapy has been described in the literature, central nervous system involvement is rare. This is the first reported case of VZV meningitis in a CD patient taking adalimumab, and it highlights the risk of atypical and severe VZV infection among immunosuppressed patients. As the long-term sequelae of central nervous system VZV can be debilitating, even with early detection and antiviral therapy, preventative strategies including vaccination are very important for this population.

VZV infection risk for IBD patients is high; a review of six global trials of adalimumab (CHARM, CARE, CLASSIC, GAIN, CHOICE, M04-729) involving 3160 CD patients found 46 cases of VZV, six of which required hospitalization<sup>[10]</sup>. Furthermore, severe disseminated and fatal VZV infections have been experienced by IBD patients on immunosuppression with steroids, thiopurines and anti-TNF therapy<sup>[11-14]</sup>. In one case, VZV caused fatal hepatic failure and disseminated intravascular

coagulation shortly after infliximab initiation<sup>[15]</sup>. As in the currently reported case, the VZV infection risk attributable to anti-TNF agents is confounded by combination immunosuppression with prednisone and adalimumab. Evidence from the prospective TREAT registry suggests corticosteroids are an especially strong independent risk factor for serious infection (OR = 2.21, 95%CI: 1.46-3.34)<sup>[3]</sup> and VZV reactivation among IBD patients taking corticosteroids is well-described. Marehbian *et al*<sup>[16]</sup> retrospectively evaluated 22310 CD patients and reported a zoster hazard ratio of 3.11 (95%CI: 1.57-6.17) if patients were on corticosteroids. The risk was even higher among patients on combination immunosuppression therapy. Similar findings have been corroborated by other authors<sup>[17]</sup>. For instance, Cullen *et al*<sup>[18]</sup> recently reviewed nine cases of primary VZV related to anti-TNF therapy for CD patients. As in the current case, all were taking concomitant immunosuppressive or corticosteroid therapy. Cases of severe disseminated VZV have been reported among IBD patients on steroid therapy alone<sup>[11,12]</sup>, while central nervous system VZV has been primarily described for immunosuppressive conditions such as HIV/AIDS or malignancies. Nonetheless, steroid therapy is recognized as a risk factor<sup>[7,19]</sup>, with several case reports describing varicella encephalitis or meningitis associated with corticosteroids in other patient populations<sup>[20-22]</sup>. In our case report, the clinical picture is complex; likely both adalimumab and prednisone contributed to this patient's increased susceptibility to VZV meningitis.

In the CD population, only two previous cases of central nervous system VZV infection have been reported. Cullen *et al*<sup>[18]</sup> described a case of meningoradiculitis in a 49-year-old male taking 6-MP; despite three weeks of acyclovir and discontinuation of 6-MP, his neurological deficits persisted. Salmon-Ceron *et al*<sup>[23]</sup> also identified one case of radiculitis in a CD patient on adalimumab in the large French RATIO registry documenting > 50000 patient-years of anti-TNF exposure; additional details were not provided. No cases of VZV meningitis or encephalitis in CD patients on anti-TNF therapy have been reported until now, but at least two cases have been identified in the rheumatology literature. One case is of a 38-year-old female with psoriatic arthritis treated with one year of adalimumab who developed VZV encephalitis that resolved with acyclovir<sup>[24]</sup>. The other case was of a patient on methotrexate and adalimumab; treatment details were not reported<sup>[25]</sup>.

Currently, there is no evidence on which to base a decision as to whether anti-TNF therapy should be restarted after VZV infection. Some authors have suggested that, in cases of mild, confined zoster, biologics can be restarted after complete lesion resolution<sup>[8,26]</sup>. However, discontinuation has been advocated in cases of severe or disseminated VZV<sup>[27]</sup>. Discontinuation, however, poses a therapeutic dilemma for IBD patients when step-up management strategies have been employed and biologic agents initiated only after failure of other immunosuppressants. In these cases, few options exist for non-

steroid maintenance therapy. In our case, adalimumab was discontinued but prednisone could not be further tapered off despite the possibility of its contributing to his VZV infection. In contrast, some experts have advocated for the use of steroid therapy as an anti-inflammatory in the management of central nervous system VZV infections<sup>[19]</sup>. Future management strategies in this setting may include consideration for granulocyte-macrophage colony-stimulating factor (sargramostim), but this is not yet an approved indication in our jurisdiction and would be accessible only through investigational trial.

Given both the likelihood of VZV infection and the seriousness of its potential sequelae, it seems obvious that CD patients should be vaccinated prior to initiation of steroid, immunosuppressive, or anti-TNF therapy. The varicella vaccine (VARIVAX<sup>®</sup>, PROQUAD<sup>®</sup>, Merck and Co., Inc)<sup>[28]</sup> and the herpes zoster vaccine (ZOSTAVAX<sup>®</sup>, Merck and Co., Inc)<sup>[29]</sup> are effective in reducing the incidence of VZV. Strong evidence from the Shingles Prevention Study Group, which evaluated > 38000 patients, demonstrated reduced herpes zoster incidence for vaccination (VZV dropped from 11.1 in the placebo group to 5.4 cases per 1000 person-years in vaccine treated patients)<sup>[30]</sup>, and the vaccine was safe and generally well-tolerated<sup>[31]</sup>. However, this particular study excluded immunocompromised patients due to the risk of iatrogenic infection from the live, attenuated virus in the vaccine. For IBD patients without a history of chickenpox, shingles, or previous vaccination, the 2009 European Crohn's and Colitis Organization guidelines, which are based on expert consensus opinion, recommend routine immunization with VZV vaccine at least three weeks prior to the onset of immunomodulation<sup>[32]</sup>. Nonetheless, routine immunization is not universal in clinical practice. Survey-based evidence suggests that < 50% of susceptible patients actually receive immunization<sup>[33]</sup>. At a minimum, the physician should discuss VZV prevention, including immunization, with the patient, and serology should be performed to confirm immunity if there is no documented history of past varicella infection<sup>[32]</sup>.

Even among IBD patients who have been previously vaccinated, evidence that post-vaccination immunity wanes over time argues for providing a second "catch-up" dose to adult patients. A retrospective review of 1080 breakthrough varicella cases found the annual rate increased from 1.6 (95%CI: 1.2-2.0) cases per 1000 person-years within 1 year of vaccination to 58.2 (95%CI: 36.0-94.0) cases per 1000 person-years 9 years post-vaccination<sup>[34]</sup>. Evidence in the pediatric literature also suggests that a two-dose vaccination regimen significantly decreases the risk of varicella infection<sup>[35]</sup>. Thus, immunosuppressed IBD patients, who are at higher risk for VZV infection, would benefit from a second vaccination in adulthood.

Vaccination timing presents another challenge in the case of IBD. Patients on other immunosuppressants or those started on rescue anti-TNF may not previously have had immunity evaluations, and the vaccine is con-

traindicated for patients already on immunosuppression therapy. This difficulty emphasizes the need for assessment of immunity at the time of CD diagnosis. In other patient populations, temporary 2-4 wk immunosuppression withdrawal to allow safe vaccination has been advocated<sup>[36]</sup>. However, such a temporary withdrawal of treatment is not usually feasible in the case of patients at risk of CD relapse. For susceptible CD patients already on anti-TNF agents, there may not be an ideal strategy for VZV prevention.

In conclusion, this paper presents the first reported case of VZV meningitis occurring opportunistically in association with adalimumab and corticosteroid therapy for CD. This case highlights this population's risk of severe, atypical opportunistic infections, the need for early recognition of VZV and aggressive management with antiviral therapy, and the potential confounders of this clinical picture, especially concomitant immunosuppression.

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**P- Reviewers** Annese V, Gaya DR, Gurvits GE, Keshavarzian A  
**S- Editor** Zhai HH **L- Editor** A **E- Editor** Ma S



## Endoscopic management of an esophagopericardial fistula after radiofrequency ablation for atrial fibrillation

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Author contributions: All authors contributed to conception and design of this case report; Quénéhervé L and Musquer N wrote the draft and the final manuscript; all authors revised and approved the final version of the manuscript.

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Received: January 4, 2013 Revised: February 19, 2013

Accepted: March 6, 2013

Published online: June 7, 2013

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**Key words:** Fistula; Esophageal stent; Radiofrequency ablation; Endoscopy; Complication; Atrial fibrillation

**Core tip:** We report the successful endoscopic management of an esophagopericardial fistula induced by radiofrequency ablation for atrial fibrillation. Esophagopericardial fistulas are a complication of great significance, being extremely difficult to manage and having a very high mortality rate. In this case, the patient was treated with a fully covered esophageal stent combined with surgical pericardial drainage. It is important to be aware of the increasing incidence of post-radiofrequency esophageal complications for cardiologic applications. Furthermore, the possibility to place endoscopic stents as a potential therapeutic option for the occurrence of post-radiofrequency esophageal fistula is extremely important as part of a treatment plan for this complication.

### Abstract

A case is reported of a 76-year-old man with a past history of atrial fibrillation. A radiofrequency ablation procedure was suggested following several failed cardioversion attempts. However, an esophagopericardial fistula complicated the procedure. This life-threatening complication was successfully managed using both the placement of a covered esophageal stent and surgical pericardial and mediastinal drainage. In fact, no persisting fistula could be detected when the esophageal stent was removed seven weeks later. Atrioesophageal and esophagopericardial fistulas are two of the most severe complications associated with cardiologic radiofrequency ablation procedures. They are responsible for majority of the deaths associated with this procedure. Despite the extremely high morbimortality associated with cardiothoracic surgery in such conditions, this treatment is the gold-standard for the management of such complications. This case report emphasizes the importance and efficacy of the endoscopic approach as part of a multidisciplinary management approach to this serious adverse event following radiofrequency ablation for atrial fibrillation.

Quénéhervé L, Musquer N, Léauté F, Coron E. Endoscopic management of an esophagopericardial fistula after radiofrequency ablation for atrial fibrillation. *World J Gastroenterol* 2013; 19(21): 3352-3353 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3352.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3352>

### INTRODUCTION

With the increasing use of radiofrequency ablation (RFA) for the treatment of atrial fibrillation, cardiologists have to face the consequences of minor and major complications related to this procedure. One class of the most severe types of adverse events occurring during RFA is cardioesophageal fistulas, including atrioesophageal fistulas (AEF) and esophagopericardial fistulas<sup>[1]</sup>. Growing evidence supporting the efficacy and safety of conserva-

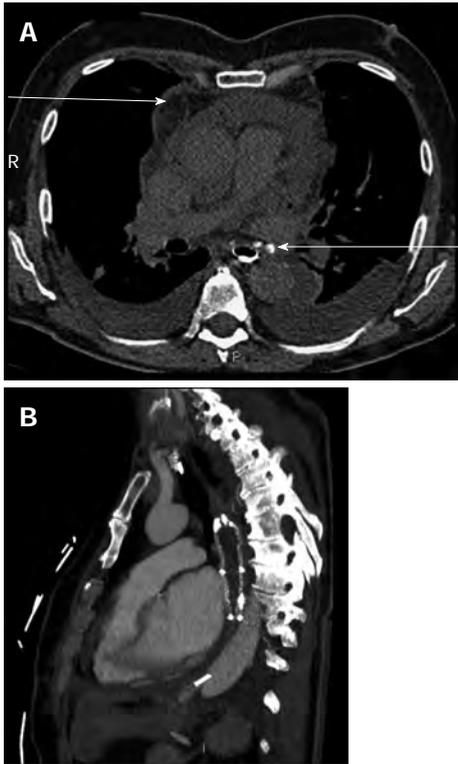


Figure 1 Computed tomography-scan. A: Computed tomography (CT)-scan with esophageal opacification. Pericardial effusion (upper arrow) and esophagopericardial fistula (lower arrow) were both present; B: CT-scan performed after esophageal stent placement and surgical drainage. Pericardial effusion was no longer present following esophageal stent insertion.

tive management has made digestive endoscopy one of the most important therapeutic options in the multidisciplinary treatment of such complications<sup>[2]</sup>.

## CASE REPORT

A 76-year-old man was admitted for chest pain. He had a medical history of chronic atrial fibrillation previously treated by several unsuccessful cardioversions. He finally underwent a radiofrequency ablation procedure. Although the electrocardiogram results showed evidence of pericarditis, no pericardial effusion was detected by echocardiography. However, a computed tomography scan revealed the presence of pleural effusion as well as pneumopericardium (Figure 1A).

A computed tomography-scan with esophageal opacification confirmed the presence of esophagopericardial fistula (OPF) and pericardial effusion. A combined surgical and endoscopic approach was performed,

consisting of surgical pericardial and mediastinal drainage immediately followed by the placement of an 8 cm fully covered self expanding metallic esophageal stent (Hanarostent Esophagus; MI Tech, Seoul, Korea) under both fluoroscopic and endoscopic (EG 530; Fujinon, Tokyo, Japan) guidance (Figure 1B). Seven weeks later, the stent was successfully removed during an upper gastrointestinal endoscopy, which also did not show persisting fistula. The patient was admitted for additional time due to multiple complications unrelated to the OPF, but was discharged 2 mo following his admission.

## DISCUSSION

Two types of cardioesophageal fistulas are AEF and OPF. AEF is a rare but dreadful complication of radiofrequency ablation with an overall mortality rate above 70%<sup>[1]</sup>. Currently, the best management option for AEF is surgery because of the risk of air embolism in case of an endoscopic approach<sup>[3]</sup>. In contrast, OPF can be managed either using conventional surgical or pericardioscopic drainage of the pericardium<sup>[2]</sup>. In addition, as in the patient cited here, endoscopic stenting has been used as a temporary treatment to seal off the fistula in cases of OPF<sup>[4]</sup>. Indeed, the ability for a gastroenterologist to diagnose AEF or OPF as potential complications of this increasingly common procedure is of pivotal importance to offer prompt treatment of the patient and multidisciplinary management of the condition.

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P- Reviewer Garg P S- Editor Gou SX L- Editor A  
E- Editor Li JY



## Malignant solitary fibrous tumor involving the liver

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Received: December 13, 2012 Revised: March 30, 2013

Accepted: April 27, 2013

Published online: June 7, 2013

such tumors in the literature, little can be said about the benefit of adjuvant therapy and prognosis for the rare cases with malignant histological findings.

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**Key words:** Malignant; Solitary fibrous tumor; Liver; Extrathoracic; Mesenchymal neoplasm; Surgical resection

**Core tip:** Solitary fibrous tumors are predominantly benign and most commonly found in the thoracic cavity and pleura, but occasionally show malignant characteristics and occur in extrathoracic organs. A malignant solitary fibrous tumor involving the liver that was diagnosed in a 62-year-old woman and treated by surgical resection is reported here. Solitary fibrous tumor is very rare in a hepatic location, and surgery is the mainstay of treatment. Due to limited reports of such tumors in the literature, little can be said about the benefit of adjuvant therapy and prognosis for the rare cases with malignant histological findings.

### Abstract

Solitary fibrous tumors are predominantly benign and are most commonly found in the thoracic cavity and pleura; while reports exist in the literature of malignant solitary fibrous tumors and those located in extrathoracic organs, these cases are considered extremely rare. Herein, a case is reported of a malignant solitary fibrous tumor involving the liver that was diagnosed and treated in a 62-year-old woman. The patient presented with complaints of upper abdominal pain and unintentional weight loss. Computed tomography scan of the abdomen revealed a remarkably large mass, measuring 15 cm × 10 cm × 20 cm, which appeared to be unrelated to any particular organ. The intraoperative finding of a wide communication with the left liver suggested hepatic origin, and served as an indicator for tumor resection *via* left hemihepatectomy. The diagnosis of solitary fibrous tumor and its malignant nature was confirmed by histological and immunohistochemical examination of the resected tissues. Hepatic solitary fibrous tumor is very rare, and surgery remains the mainstay of treatment. Due to limited reports of

Jakob M, Schneider M, Hoeller I, Laffer U, Kaderli R. Malignant solitary fibrous tumor involving the liver. *World J Gastroenterol* 2013; 19(21): 3354-3357 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3354.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3354>

### INTRODUCTION

Solitary fibrous tumor (SFT) is a rare mesenchymal neoplasm. Whereas most of the SFTs reported in the literature have occurred in the thoracic cavity and pleura, cases of SFTs involving extrathoracic organs exist<sup>[1-3]</sup>. The majority of SFTs are benign and patients commonly present as asymptomatic; however, excessive size or involvement of vital structures may lead to paraneoplastic and local symptoms<sup>[1,2,4,5]</sup>. Surgery remains the treatment of choice, and little is known about the benefits of adjuvant therapy for those rare cases with histological findings that indi-



Figure 1 Computed tomography scan of the abdomen showing a well-circumscribed, solitary, large mass (15 cm × 10 cm × 20 cm) adjacent to the left liver lobe. The claw sign of hepatic tissue suggests intrahepatic localization. Notable features include hypervascularity, heterogeneous enhancement, and smooth margins.

cate malignancy<sup>[6]</sup>. Herein, we report a very rare case of a malignant SFT involving the liver.

## CASE REPORT

A 62-year-old woman presented at our hospital with complaints of upper abdominal pain persisting for several days and unintended weight loss that had occurred over the past month approximately. The patient's surgical history included resection of the transverse colon in 1984 to remove an adenocarcinoma, for which the results of follow-up examinations were unremarkable.

Physical examination upon admission revealed cachexia and a palpable large, firm mass in the epigastrium region that was roughly the size of a coconut. Laboratory tests showed normal complete blood cell count and slight thrombocytosis (525 g/L *vs* normal range: 150-400 g/L), as well as normal levels of all liver function markers except for C-reactive protein, which was elevated (29 mg/L *vs* normal range: 0-5 mg/L).

Computed tomography (CT) scan revealed a remarkably large single circumscribed intraabdominal tumor (Figure 1) without evidence of lymphatic metastases; the imaging analysis provided no indication of tumor relation to any particular organ. The presence of distant metastases was excluded by findings from additional CT scanning of the thoracic cavity. A median laparotomy was performed. The intraoperative findings of a wide communication between the tumor and the left liver and absence of penetration into adjacent organs suggested hepatic origin, and a left hemihepatectomy was carried out. The gross specimen was a large, lobulated, sharply demarcated tumor with obvious intratumoral necrotic areas (Figure 2).

Histological examination revealed the specimen to be a well-circumscribed mesenchymal tumor adjacent to the hepatic parenchyma and capsule. The tumor was composed of short spindly and ovoid cells arranged in a random pattern amidst a variable collagenous background with hemangiopericytoma-like thin-walled ectatic blood



Figure 2 Gross specimen is a bulky neoplasm attached to the liver capsule.

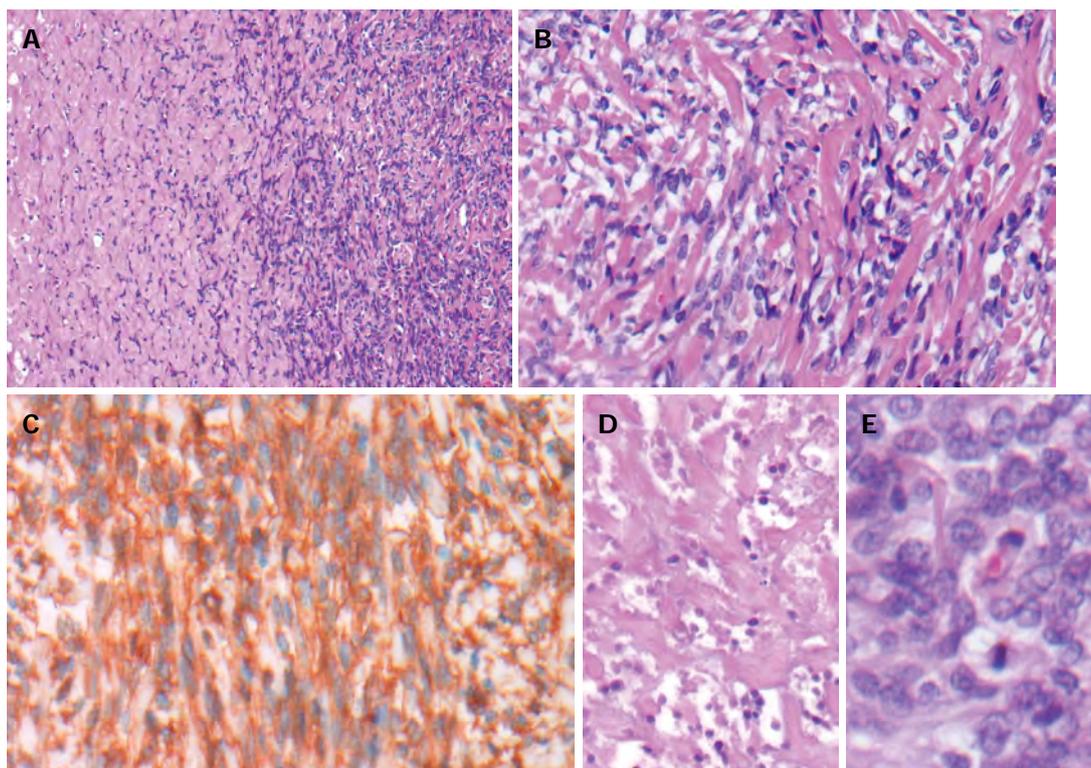
vessels (Figure 3). In addition, areas of high cellularity with nuclear crowding, cytological atypia, tumor necrosis, and increased mitotic rate (up to six mitotic figures per 10 high-power fields) were observed. Immunohistochemical analysis showed positivity for CD34, CD99 and Bcl-2, and negativity for cytokeratin, CD117, desmin, S-100 and actin. The diagnosis was made of a malignant SFT involving the liver.

The postoperative course was uneventful, although the recovery duration was slightly protracted because of the patient's poor nutritional status.

## DISCUSSION

SFTs are most commonly found in the thoracic cavity and pleura, but have also been reported in various extrathoracic organs, including the upper respiratory tract, orbits, soft tissue, abdomen, and breast<sup>[1-3]</sup>. Very rarely do SFTs involve the liver, and the incidence of these tumors is unknown<sup>[7]</sup>. The majority of hepatic SFT diagnoses have been in adults (mean age: 57.5 years) and there appears to be a strong bias towards the female sex (2:1)<sup>[4]</sup>. While most cases present as asymptomatic, some cases, such as our patient described herein, show abdominal fullness and a palpable mass<sup>[5]</sup>. Other symptoms that have been described include paraneoplastic hypoglycemia, fatigue, and weight loss<sup>[4]</sup>. From a biochemical perspective, the association of neoplasms with thrombocytosis and elevated C-reactive protein level is well known<sup>[8,9]</sup>, and the presence of a tumor may be suspected when these findings are present. However, definitive diagnosis of SFT is based on a characteristic panel of histological and immunohistochemical features. Histologically, SFT is composed of spindly to ovoid cells, possibly of fibroblastic origin, which are arranged in a haphazard "patternless" pattern and intimately intertwined by collagen fibers of various thicknesses, and associated with numerous hemangiopericytoma-like dilated thin-walled blood vessels. Immunohistochemically, SFT is positive for CD34, CD99 and Bcl-2<sup>[10]</sup>. The diagnosis of SFT was made in the present case according to these characteristic immunohistochemical findings and histological features.

Whereas most of the SFTs reported in the literature



**Figure 3** Histological findings indicative of malignant solitary fibrous tumor. A: Hypo- and hypercellular patternless growth patterns [hematoxylin and eosin (HE), × 40]; B: Short spindly to ovoid tumor cells in a collagenous background (HE, × 200); C: Immunohistochemical staining with positive detection of CD34 (HE, × 200); D: Multifocal areas of necrosis (HE, × 200); E: Hypercellularity, cytologic atypia, and increased mitotic activity (HE, × 400).

have been benign (> 80%), their malignant potential is largely unknown<sup>[2]</sup>. About 10%-15% of SFTs behave aggressively. Although no strict correlation has been found between the SFTs' morphological features and malignancy, the current World Health Organization (WHO) classification criteria of soft tissue tumors is used to identify malignant SFT; these criteria include hypercellularity, cytological atypia, tumor necrosis, high mitotic rate (four or more mitotic figures per 10 high-power fields), and/or infiltrative margins<sup>[10,11]</sup>. The resected tumor specimen from our case showed features of high cellularity with nuclear crowding, moderate to marked cellular atypia, up to six mitotic figures per 10 high-power fields and tumor necrosis, fulfilling the WHO criteria for malignant SFT. The imaging features previously reported for other benign and malignant SFTs involving the liver seem to overlap, and there is no distinctive radiological criterion for diagnosing malignancy, excepting the presence of distant metastases<sup>[12]</sup>.

Malignant SFTs involving the liver are currently treated by surgical resection, with the aim of obtaining a margin-negative specimen<sup>[6]</sup>. Because the number of cases reported to date is small, evidence of the benefit of adjuvant therapy is lacking and its use remains controversial<sup>[2,13]</sup>. As the biological behavior of these tumors and the patients' prognosis after treatment have not been well defined<sup>[5]</sup>, careful follow-up is mandatory.

In conclusion, malignant SFT involving the liver is very rare. Nevertheless, it should be considered as a po-

tential differential diagnosis when a single large hepatic mass is present. Definitive diagnosis is based on histopathological and immunohistochemical findings, and surgery remains the mainstay of treatment. The rarity of these cases and limited reports in the literature preclude any significant speculation regarding the benefit of adjuvant therapy and patient prognosis.

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P- Reviewer Xian L S- Editor Zhai HH L- Editor A  
E- Editor Li JY



## Synchronous pancreatic solid pseudopapillary neoplasm and intraductal papillary mucinous neoplasm

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Received: January 15, 2013 Revised: March 1, 2013

Accepted: March 15, 2013

Published online: June 7, 2013

### Abstract

Solid pseudopapillary neoplasm (SPN) is a rare and low-grade malignant pancreatic neoplasm composed of poorly cohesive monomorphic neoplastic cells forming solid and pseudopapillary structures with frequent hemorrhagic-cystic degeneration. Intraductal papillary mucinous neoplasm (IPMN) is a pancreatic exocrine tumor composed of intraductal papillary growth of mucin containing neoplastic cells in the main pancreatic duct or its major branches. In the case presented here, a 53-year-old, Japanese man was found to have multiple

cystic lesions and dilatation of the main pancreatic duct in the neck of the pancreas. Histological examination revealed a main-duct and branch-duct type IPMN, of the gastric-type, involving the neck of the pancreas, associated with a 0.5 cm SPN in the caudal side of the IPMN. We diagnosed this case as synchronous SPN and IPMN. As far as we know, only one other case of synchronous SPN and IPMN has been reported. Both the present case and the previously reported case showed abnormal nuclear expression of  $\beta$ -catenin in SPN, whereas IPMN showed no abnormal nuclear expression. These results suggest that  $\beta$ -catenin abnormality is not a common pathogenetic factor of synchronous SPN and IPMN.

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**Key words:** Intraductal papillary mucinous neoplasms; Solid pseudopapillary neoplasms; Pancreatic neoplasms; Synchronous neoplasms; Pancreas; Beta-catenin

**Core tip:** We report the second case of synchronous solid pseudopapillary neoplasm (SPN) and intraductal papillary mucinous neoplasm (IPMN) of the pancreas in a 53-year-old Japanese man. IPMN was classified as a "combined" type due to the involvement of both the main and branch ducts, and it showed a gastric subtype with low-grade dysplasia. Adjacent to the caudal side of IPMN, a 0.5-cm solid SPN was present. The SPN showed abnormal nuclear expression of  $\beta$ -catenin in SPN, whereas IPMN showed no abnormal nuclear expression. This result suggested that  $\beta$ -catenin abnormality is not a common pathogenetic factor of synchronous SPN or IPMN.

Hirabayashi K, Zamboni G, Ito H, Ogawa M, Kawaguchi Y, Yamashita T, Nakagohri T, Nakamura N. Synchronous pancreatic solid pseudopapillary neoplasm and intraductal papillary mucin-

nous neoplasm. *World J Gastroenterol* 2013; 19(21): 3358-3363 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3358.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3358>

## INTRODUCTION

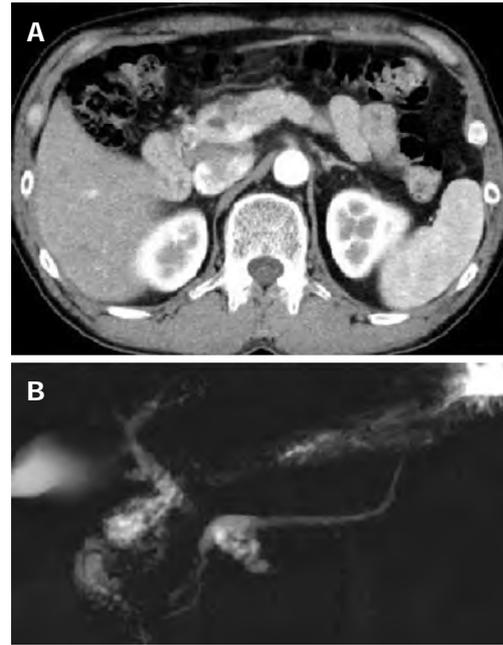
Solid pseudopapillary neoplasm (SPN) is a rare and low-grade malignant pancreatic neoplasm composed of poorly cohesive, monomorphic cells forming solid and pseudopapillary structures with frequent hemorrhagic-cystic degeneration, which predominantly occurs in young women. Immunohistochemically, SPN usually expresses vimentin,  $\alpha$ -1-antitrypsin, CD56, progesterone receptor, CD10, and nuclear/cytoplasmic  $\beta$ -catenin<sup>[1-3]</sup>.

Intraductal papillary mucinous neoplasm (IPMN) is a macroscopic pancreatic exocrine tumour that grows primarily within the main pancreatic duct or its major branches, and mainly occurs in older men. IPMNs are composed of intraductal papillary growth of mucin-containing neoplastic cells that are classified into 4 major subgroups: gastric, intestinal, pancreatobiliary, and oncocytic types<sup>[4,5]</sup>. Gastric-type IPMN consists of innocuous, tall columnar cells that resemble gastric foveolar epithelium and express mucin (MUC) 5AC, but are negative for MUC1, MUC2, and CDX2<sup>[4,5]</sup>. Intestinal-type IPMN consists of neoplastic cells that resemble intestinal villous neoplasms with MUC2, MUC5AC, and CDX2 expression, but without MUC1 expression<sup>[4,5]</sup>. Pancreatobiliary-type IPMN consists of thin, branching papillae with high-grade dysplasia and is at least focally positive for MUC1 and MUC5AC, but is negative for MUC2 or CDX2<sup>[4,5]</sup>. Oncocytic-type IPMN is characterised by complex and arborizing papillae lined by cuboidal cells with granular eosinophilic cytoplasm, which usually express MUC5AC and MUC6, but are negative for MUC2 and CDX2<sup>[4,5]</sup>.

Although IPMNs have been reported to occur synchronously with a variety of primary pancreatic neoplasms such as pancreatic ductal adenocarcinomas, endocrine neoplasms, or serous cyst adenomas<sup>[6-9]</sup>, the synchronous occurrence of SPN with other pancreatic neoplasms has only been reported in a single case report associated with an IPMN<sup>[10]</sup>. In the current study, we present the histological and clinical features of a second case of synchronous “early” SPN and IPMN, with a review of the literature.

## CASE REPORT

A 53-year-old Japanese man with multiple pancreatic cystic lesions, which were detected at a local hospital, was referred to the Tokai University Hospital for further examination. Upon inspection, he had no complaints, and no abnormal physical findings were reported. Laboratory data, including tumour markers, carcinoembryonic antigen, or carbohydrate antigen 19-9, showed no abnormal findings. Computed tomography showed multiple cystic



**Figure 1** Computed tomography and magnetic resonance cholangiopancreatography imaging. A: Multiple cystic lesions measuring 23 mm are indicated in the neck of the pancreas; B: Multiple cystic dilations of pancreatic branch duct connecting to the dilated main pancreatic duct are indicated in the neck of the pancreas.

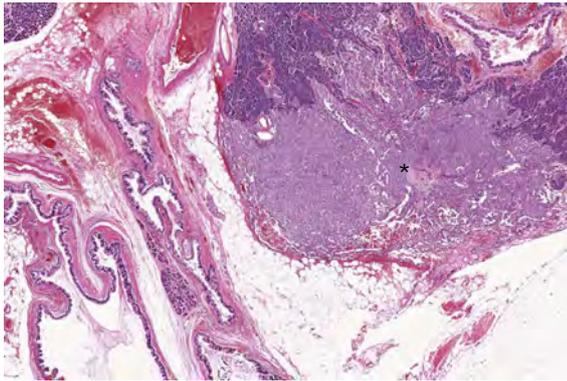
lesions measuring 2.3 cm in the neck of the pancreas (Figure 1A). Magnetic resonance cholangiopancreatography imaging showed multiple cystic dilations of the pancreatic branch ducts connecting to the dilated main pancreatic duct in the neck of the pancreas (Figure 1B). Endoscopic retrograde pancreaticography showed a dilated main pancreatic duct in the neck of the pancreas, measuring 6 mm in diameter, with multiple nodular defects. Distal pancreatectomy and splenectomy were performed. At present, the patient is alive and well 25 mo after the operation.

### Tissues

The resected specimen was fixed in 10% formalin, routinely embedded in paraffin, sectioned at 4- $\mu$ m, and stained with haematoxylin and eosin.

### Immunohistochemistry

The sections were deparaffinised in xylene and rehydrated in a graded ethanol series. The primary antibodies and their dilutions and sources are summarized in Table 1. If necessary, antigen retrieval was performed by autoclaving the samples for 15 min in a sodium citrate buffer at pH 6.0 ( $\beta$ -catenin, CD56, chromogranin A, E-cadherin, MUC1, MUC2, MUC5AC, MUC6, vimentin, and Ki67) or by treatment with 0.1% trypsin at 37 °C for 30 min (synaptophysin). The antibodies were detected using EnVision Plus (Dako, Glostrup, Denmark; for  $\beta$ -catenin, CD56, chromogranin A, E-cadherin, MUC1, MUC2, MUC5AC, MUC6, vimentin, synaptophysin, and Ki67), or Horseradish Peroxidase-conjugated polyclonal goat anti-rabbit



**Figure 2** Microscopic findings (low-power view). A small and irregularly shaped solid lesion of solid pseudopapillary neoplasm (asterisk) can be observed near the cystically dilated branch ducts of intraductal papillary mucinous neoplasm (hematoxylin and eosin stain, × 10).

immunoglobulin (Dako; for alpha-1-antitrypsin), with 3,3'-diaminobenzidine as the chromogen. Assays for pancytokeratin, progesterone receptor (PR), and CD10 were performed using Ventana Benchmark XT (Ventana Medical Systems, Tucson, AZ, United States) equipped with the Ultra View Detection Kit. Antigen retrieval was performed by treatment with CC1 (Ventana; for PR and CD10) or protease 1 (Ventana; for pancytokeratin).

On gross examination, the distal pancreatectomy specimen showed a dilatation of the main pancreatic duct, with multiple cystic dilatations of the branch ducts, filled with mucus and containing papillae, in the neck of the pancreas. A whitish and solid lesion measuring 0.5 cm × 0.3 cm in size, located adjacent to the caudal side of the cystically dilated branch ducts, was identified.

Microscopically, 2 distinct primary pancreatic neoplasms were present (Figure 2): (1) a typical central and branch-duct type IPMN, with the cyst lined by tall and pale columnar cells with only low-grade dysplasia, and pyloric-gland adenoma-like papillary projections (Figure 3A). Immunohistochemically, the neoplastic cells showed gastric-type phenotype with MUC5AC (Figure 3B) and MUC6 positivity, and MUC1 and MUC2 negativity. β-catenin immunostaining showed membranous expression, but did not show abnormal nuclear expression (Figure 3C). On the basis of current World Health Organization (WHO) criteria<sup>[4]</sup>, we diagnosed the patient with gastric-type IPMN with low-grade dysplasia; and (2) a small, SPN, adjacent to the IPMN, which showed an ill-defined demarcation, with focal extension to the pancreatic parenchyma. The tumor showed a prevalent solid pattern, with focal degenerative changes and rough pseudopapillary structures (Figure 3D). The neoplastic cells had eosinophilic cytoplasm and small, round nuclei with finely dispersed chromatin. We failed to find perineural infiltration, peripancreatic invasion, or calcification. Immunohistochemically, the neoplastic cells expressed CD56, vimentin, alpha-1-antitrypsin, CD10, progesterone receptor (Figure 3E), nuclear/cytoplasmic β-catenin (Figure 3F), focal positivity for synaptophysin and negativity

**Table 1** Immunohistochemical findings

|                        | Clone               | Source  | Dilution   | IPMN | SPN   |
|------------------------|---------------------|---------|------------|------|-------|
| Pancytokeratin         | AE1, AE3, and PCK26 | Ventana | Prediluted | (+)  | (-)   |
| Alpha-1 anti-trypsin   | Polyclonal          | Dako    | 1:100      | (-)  | (+)   |
| CD10                   | 56C6                | Leica   | 1:50       | (-)  | (+)   |
| CD56                   | 1B6                 | Leica   | 1:30       | (-)  | (+)   |
| MUC1                   | Ma695               | Leica   | 1:50       | (-)  | (-)   |
| MUC2                   | Ccp58               | Leica   | 1:100      | (-)  | (-)   |
| MUC5AC                 | CLH2                | Leica   | 1:100      | (+)  | (-)   |
| MUC6                   | CLH5                | Leica   | 1:100      | (+)  | (-)   |
| Vimentin               | V9                  | Dako    | 1:100      | (-)  | (+)   |
| Synaptophysin          | 27G12               | Leica   | 1:50       | (-)  | (-/+) |
| Chromogranin A         | DAK-A3              | Dako    | 1:20       | (-)  | (-)   |
| β-catenin <sup>1</sup> | 17C2                | Leica   | 1:100      | (-)  | (+)   |
| E-cadherin             | 36B5                | Leica   | 1:20       | (+)  | (-)   |
| Progesterone receptor  | 1E2                 | Ventana | Prediluted | (-)  | (+)   |
| Ki67                   | MIB1                | Dako    | 1:50       | < 1% | < 1%  |

<sup>1</sup>Nuclear labelling. Ventana Medical Systems, Tucson, AZ, United States; Dako, Glostrup, Denmark; Leica Microsystems, Newcastle upon Tyne, United Kingdom. +: Positive; -: Negative; -/+ : Focal positive; MUC: Mucin; IPMN: Intraductal papillary mucinous neoplasm; SPN: Solid pseudopapillary neoplasm.

for chromogranin A, and E-cadherin and cytokeratin AE1 and 3. The immunohistochemical profiles of the 2 neoplasms are summarized in Table 1.

As our results show, the SPN was located near the IPMN. However, neither transition nor collision between IPMN and SPN were identified. Therefore, we diagnosed the present case as synchronous SPN and IPMN of the pancreas. The surgical margins were free of both types of tumors. No lymph node metastasis was identified.

## DISCUSSION

We reported a case of synchronous small, radiologically undetected SPN, in a patient operated on for a radiologically evident IPMN of the pancreas. To our knowledge, this association has only been previously reported by Imamura *et al*<sup>[10]</sup>. They reported a patient who presented with a 3-cm, solid SPN in the head of the pancreas that invaded the mesocolon with a synchronous IPMN constituted by 2 multilocular cystic lesions in the head of the pancreas. In other words, the 2 cases showed mirror clinicopathologic traits.

In general, SPN occurs predominantly in young women (91%) and is rare in men (9%)<sup>[11]</sup>. Interestingly, however, both our case and Imamura's occurred in men<sup>[10]</sup>. Several authors have reported the clinicopathologic correlations between genders and SPN. Takahashi *et al*<sup>[12]</sup> reported that the cases of SPN in men tend to exhibit solid components without prominent degenerative changes. Tien *et al*<sup>[13]</sup> reported no significant differences in patient age, size, neoplasm location, or malignancy rate between the genders in SPN. By contrast, Machado *et al*<sup>[14]</sup> reported that cases of SPN in men are more aggressive and that the patients are older. According to the literature review of 1014 SPN patients (women, 877; men, 137)



According to the current WHO classification<sup>[4]</sup>, the IPMN in the present case should be classified as low-grade dysplasia. Imamura *et al*<sup>[10]</sup> diagnosed the IPMN component in their case as intraductal papillary mucinous adenoma (IPMA). Under the current WHO classification guidelines, IPMA is now designated as IPMN with low-grade dysplasia<sup>[4]</sup>. Imamura *et al*<sup>[10]</sup> did not mention the subtype of IPMN applicable to their case. We assume that it might have been a gastric type, based on the features of their figures and the MUC5AC positivity, and MUC1 and MUC2 negativity.

In both our case and Imamura's case, the SPNs were adjacent to the IPMNs, without any transition or collision between the 2 neoplasms<sup>[10]</sup>.

The occurrence of SPN and IPMN offers an interesting opportunity to investigate the role of  $\beta$ -catenin gene mutation in the pathogenesis of both lesions. In fact, almost all SPNs harbour somatic point mutations in exon 3 of CTNNB1, the gene that encodes  $\beta$ -catenin, which results in abnormal nuclear expression of  $\beta$ -catenin on immunohistochemistry<sup>[1]</sup>. The role of  $\beta$ -catenin gene mutations in IPMN is more controversial.  $\beta$ -catenin nuclear protein accumulation has been reported in one patient with familial adenomatous polyposis<sup>[16]</sup> and in a small series of IPMNs. In the latter study, 7 of 18 IPMNs indicated abnormal  $\beta$ -catenin nuclear protein accumulation (1 of 8 adenomas, 2 of 3 borderline, and 4 of 7 carcinomas)<sup>[17]</sup>. Both the present case and Imamura *et al*<sup>[10]</sup> showed abnormal nuclear expression of  $\beta$ -catenin in SPN, whereas IPMN showed only membranous expression, but no abnormal nuclear expression. These results suggest that, at least in these 2 patients, there is no correlation between the  $\beta$ -catenin abnormalities, exclusively found in SPNs, and the development of synchronous IPMNs.

In conclusion, we present a second case of synchronous SPN and IPMN of the pancreas. Unlike SPNs that usually occur in adolescent girls or young women, the 2 synchronous SPNs and IPMNs occurred in old, male patients (53 and 66 years, respectively). It is still unknown whether SPN or IPMN neoplasms occur independently or are in some way linked. From the comparison of the 2 cases, it appears that the  $\beta$ -catenin gene abnormalities play a role exclusively in the development of SPN, whereas a different molecular pathogenesis has to be considered for the IPMN counterpart.

## ACKNOWLEDGMENTS

The authors thank Mr. Hiroyuki Oyamada (Division of Diagnostic Pathology, Tokai University Hospital) for his valuable technical assistance.

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**P- Reviewers** Lee KT, Schlitter AM **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Li JY



## Foreign body retained in liver long after gauze packing

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Supported by Grants from the Technology Program of the Science Technology Department of Zhejiang Province, No. 2011C37087; and the National Natural Science Foundation of China, No. 81000065

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Received: March 10, 2013 Revised: April 17, 2013

Accepted: May 16, 2013

Published online: June 7, 2013

### Abstract

This case report describes a foreign body retained in the liver long after perihepatic gauze packing. A 64-year-old female patient had suffered a rib fracture and liver rupture during a traffic accident in 1973. She discovered a mass in her right hypochondrium. Her hepatic ultrasonography showed a round mass (20.3 cm × 17.3 cm × 16.0 cm in size) with fluid echogenicity in the right lobe of her liver, and a hepatic cystic-solid mass (19.7 cm × 18.5 cm × 15.6 cm in size) was identified in an abdominal computerized tomography scan. Several pieces of gauze were extracted, and brown

pus from the hepatic mass was suctioned during her exploratory laparotomy. Histology documented gauze remnants with necrotic material inclusions and fibrotic capsules. To our knowledge, this patient's case represents the longest time for which a foreign body has been retained in the liver. In addition, we conducted a comprehensive literature review of foreign bodies retained in the liver. Foreign bodies may be introduced into the liver via penetrating trauma, surgical procedures or the ingestion of foreign bodies (which then migrate from the gut). Thus, they can be classified into the following three categories: penetrating, medical and migrated foreign bodies. The details of the case are thoroughly described.

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**Key words:** Liver; Foreign bodies; Foreign-body migration; Foreign-body reaction; Surgical sponges

**Core tip:** We reported a case of foreign body retained in liver 39 years post perihepatic gauze packing. To our knowledge, this seems to be the longest time and first report of gossypiboma post gauze packing in literature. We classified foreign bodies retained in liver as 3 categories: penetrating, medical and migrated foreign bodies. Also, we presented a comprehensive review, which may greatly help a surgeon make clinical decision when patients presented with hepatic mass.

Xu J, Wang H, Song ZW, Shen MD, Shi SH, Zhang W, Zhang M, Zheng SS. Foreign body retained in liver long after gauze packing. *World J Gastroenterol* 2013; 19(21): 3364-3368 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3364.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3364>

### INTRODUCTION

Various foreign bodies retained in parts of the human body (such as the abdominal cavity and intrathoracic and

pericardial spaces) as a result of surgery, trauma or exposure to chemical substances (such as pigment or suture materials) have previously been described<sup>[1]</sup>. However, reports related to the liver are rare and had not been well-reviewed. In this report, we describe a case of a retained foreign body in the liver (gossypiboma) in which surgical gauze used for perihepatic packing had been retained for 39 years after an abdominal hepatorrhaphy following a traffic accident.

## CASE REPORT

A 64-year-old female patient was admitted to our department. She complained of slight intermittent chest pain and dyspnea on exertion for approximately 20 d associated with poor appetite and weight loss. A mass was present in her epigastrium and right hypochondrium. She has lived in a region in which schistosomiasis is epidemic and has had well-controlled hypertension with regular anti-hypertensive therapy for the last five years. She suffered rib fractures and liver rupture in a traffic accident 39 years ago and recovered post-laparotomy. Her general examination and laboratory parameters, including liver function tests, were within the normal limits. Upon abdominal examination, a large-sized vertical midline incision scar was identified, and a mass with restricted mobility and a smooth surface was palpable. Hepatic ultrasonography showed a round mass (20.3 cm × 17.3 cm × 16.0 cm in size) with fluid echogenicity in the right lobe of her liver. A hepatic cystic-solid mass (19.7 cm × 18.5 cm × 15.6 cm in size) was identified with the aid of an abdominal computerized tomography (CT) scan (Figure 1). Liver textiloma was highly suspected.

The patient was scheduled for an exploratory laparotomy under general anesthesia. After the abdomen was opened, a very large cyst was found in the right lobe of her liver protruding into the right chest cavity. All appropriate precautions were taken to prevent the cyst from rupturing. Thin, brown pus was suctioned after anhydrous alcohol was repeatedly infused into the cyst, and several pieces of gauze were found after the cyst was opened (Figure 2). Pus samples were collected in sterile syringes and sent for culture and sensitivity analyses. The surgical gauze was removed, and peritoneal lavage was performed. A double-lumen tube was used to drain the remaining fluid in the cyst and another rubber tube was inserted in hepatorenal recess. The abdomen was then closed after obtaining exact counts of sponges and instruments and taking all necessary precautions.

The culture of the pus was sterile. The histological analysis documented gauze remnants with necrotic material inclusions and a fibrotic capsule with old, calcified schistosome eggs that was attached to the foreign body granuloma. The postoperative period was uneventful; the patient was discharged with the double-lumen drainage tube in place and advised to return for follow-up visits. The double-lumen tube was extracted two months later, and the patient now lives a normal life.

## DISCUSSION

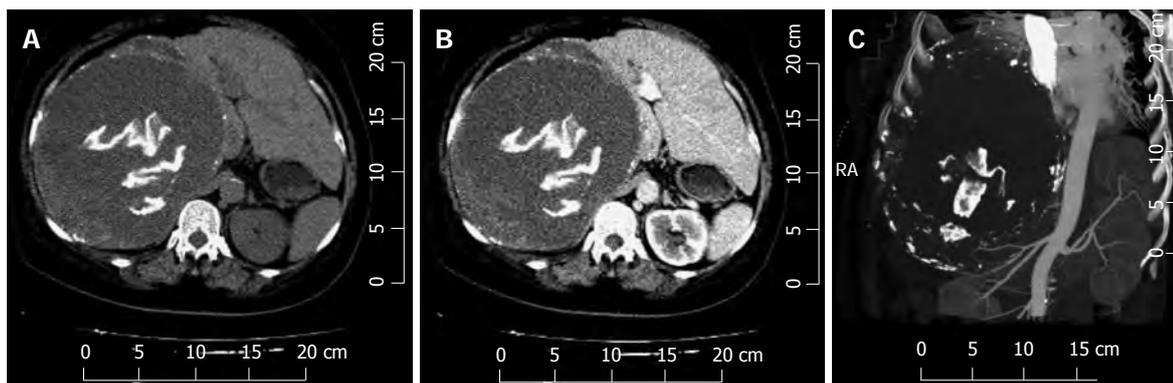
Foreign bodies may be introduced into the liver via penetrating trauma, surgical procedures or the ingestion of foreign bodies (which later migrate from the gut). Thus, these foreign bodies can be classified into the following three categories: penetrating, medical and migrated foreign bodies.

### *Penetrating foreign bodies*

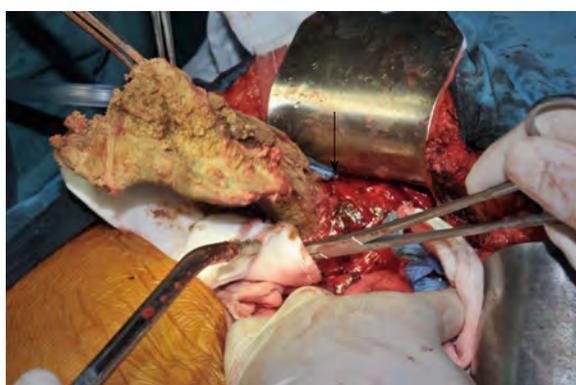
Penetrating foreign bodies, such as bullets, shell fragments, glass and wood, can become lodged in the liver via gunshot or stab wounds<sup>[2-7]</sup>. Although gunshot or stab injuries may not introduce penetrating foreign bodies into the liver<sup>[2,3]</sup>, they remain the most common causes. An immediate assessment with ultrasound or CT after a blunt or penetrating abdominal injury remains the gold standard method for identifying bleeding and foreign bodies<sup>[4]</sup>. Liver is the most commonly injured solid organ in blunt or penetrating abdominal or thoracic trauma, however, liver wounds associated with penetrating foreign bodies that are retained in the liver are rare<sup>[5]</sup>. Botoi *et al*<sup>[6]</sup> reported six cases of liver shotgun wounds that all resulted in metal foreign bodies being retained in the liver. The patients were all male, and most were young. Hunting rifles were involved in three of the cases. The wounds were complex in five of the six cases, and most of the injuries affected the right liver lobe. Hepatectomy was used in 40% of the cases<sup>[6]</sup>. Hepatic penetrating foreign bodies are surgical emergencies that should prompt prophylactic extraction through laparotomy; however, the tissue injury caused by the penetrating liver trauma and not the penetrating foreign bodies themselves require surgery<sup>[4,5]</sup>. Some penetrating foreign bodies, such as bullets, produce no clinical symptoms for many years.

Laparotomy remains the standard treatment for penetrating organ injuries with retained foreign bodies; however, non-operative management<sup>[7]</sup> is preferred for stable patients, especially those with penetrating organ injuries without retained foreign bodies. In the appropriate trauma center environment, selective non-operative management of penetrating abdominal solid organ injuries, especially liver injuries, has a high success rate and a low complication rate. Even high-grade liver injuries do not preclude non-operative management<sup>[8]</sup>.

Major complex surgical procedures, such as an anatomical resection, an irregular hepatotomy with direct suture and resectional debridement or atriocaval shunting, are thought to be indicated in the emergency setting in specialist units<sup>[7,8]</sup>. Fast and effective surgical damage control procedures (*i.e.*, temporary gauze packing and mesh wrapping of the fragmented liver with absorbable mesh (mesh hepatorrhaphy) combined with ipsilateral ligation of the bleeding vessel) are safe and effective for treating severe liver injuries, especially in non-specialist centers or in remote, rural settings. Interventional radiological techniques are becoming more widely used to more effectively manage severe liver injuries, particularly in patients



**Figure 1** Computed tomography images of the abdomino-thoracic region showing a cystic mass in the liver with filiform calcification inside and a small amount of pleural effusion. A: Plain; B: Contrast-enhanced; C: Reconstructive. RA: Right anterior.



**Figure 2** Surgical gauze (arrow) and the cyst wall (held with vessel forceps) in the right liver lobe following the evacuation of pus.

who are managed non-operatively or have been stabilized using perihepatic packing<sup>[8]</sup>. Liver transplantation for trauma is extremely rare; although this type of transplantation has been attempted<sup>[9]</sup>, it is very limited to date.

Although temporary perihepatic gauze packing was not used for decades, packing has been reintroduced because more aggressive attempts at controlling hemorrhaging without temporary packing failed to improve results. Packing is also recommended for inexperienced surgeons to control and stabilize patients before they are transferred to a tertiary center<sup>[4,5,7,8]</sup>. The patient in this case study suffered from blunt trauma in a traffic accident. Although the surgical details are obscure due to the passing of 39 years, perihepatic gauze packing saved the patient's life, and she lived a normal life for 39 years. Mesh hepatorrhaphy may control bleeding without many of the adverse effects of packing<sup>[4,7,8]</sup>.

### Migrated foreign bodies

Migrated foreign bodies are sharp objects that perforate the alimentary tract and migrate to the liver<sup>[10]</sup>. Ingested foreign bodies with sharp pointed end include sewing needles, dental plates, fish bones, chicken bones and office supplies. Foreign bodies are commonly ingested by elderly individuals, children, psychiatric patients, prison inmates, alcoholics, certain professionals (*e.g.*, carpenters

and dressmakers) and individuals wearing dentures<sup>[10-20]</sup>.

The migration of foreign bodies from the gut into the liver is often a slow process that encourages an inflammatory and fibrotic reaction that results in adhesion<sup>[11,12]</sup>. Clinical manifestations, such as perforation, bleeding and bowel obstruction, often do not occur with migrated foreign bodies<sup>[13]</sup>. Perforation of the gastrointestinal tract by a foreign body typically occurs at sites at which a physiological narrowing of the stomach<sup>[14,15]</sup> or duodenum<sup>[16,17]</sup> is present. Ingestion history is not often considered in the initial diagnosis<sup>[13-17]</sup>. Migrated foreign bodies in the liver often cause pyogenic liver abscesses or pseudotumors<sup>[18]</sup>.

Patients who present with a liver abscess or a pseudotumor may have a foreign body granuloma, which may be difficult to distinguish from a tumor upon radiological examination. Right upper-quadrant pain and cholangitis<sup>[11,12]</sup> are often the first symptoms, which may be mild initially but increase over time. An inflammatory mass mimicking a liver abscess or a pseudotumor that looks like hepatocellular carcinoma or cholangiocarcinoma may cause systemic symptoms, such as fevers and chills, anorexia, weight loss, abdominal pain, nausea and vomiting<sup>[12,13]</sup>. Abdominal ultrasonography and CT can reveal a hepatic abscess with a linear calcified foreign body and gas (which is often hyperechoic in ultrasonography and highly dense in the CT<sup>[18,19]</sup>) and are very helpful for establishing the correct diagnosis. A CT scan can demonstrate the location of the foreign body clearly and accurately, which may assist the clinician in selecting the appropriate treatment plan.

Conservative management has been described for asymptomatic patients with a stable, uncomplicated migrated foreign body<sup>[11,12,16]</sup>; however, in an emergency, a hepatic migrated foreign body should be extracted through laparotomy or laparoscopy<sup>[17,18]</sup> to avoid complications, such as hemorrhaging or the formation of an abscess or a fistula. Other strategies for removing the foreign body include endoscopy<sup>[19]</sup> and percutaneous abscess drainage under ultrasonographic guidance<sup>[20]</sup>.

### Medical foreign bodies

Medical foreign bodies in the liver include surgical objects,

such as surgical gauze (or gossypiboma/textiloma)<sup>[21-23]</sup>, surgical clips<sup>[24,25]</sup>, medical sutures<sup>[23,26]</sup>, shrapnel splinters<sup>[27]</sup> and T-tubes<sup>[28]</sup>, which are retained in the liver parenchyma or biliary tracts after surgical operations. These bodies can be grouped into two main categories: bodies in the biliary tract and bodies in the liver parenchyma.

Gossypibomas account for most of the malpractice claims for retained foreign bodies. They are most frequently observed in obese patients or patients in unstable conditions; during emergency surgeries, long operations or surgical procedures that include unexpected changes; and after laparoscopic interventions<sup>[29,30]</sup>. Gossypibomas in the liver parenchyma or the biliary tracts commonly follow cholecystectomy or liver surgery<sup>[28-30]</sup>. In our case, the patient underwent an emergent hepatorrhaphy that included perihepatic packing with surgical gauze. Typically, surgical gauze should be extracted when hemorrhaging stops; however, the gauze in the liver of our patient was not extracted and caused gossypiboma.

The symptoms of gossypiboma are usually non-specific and may appear years after surgery. Pain, irritation, a palpable mass, anorexia, fever and weight loss are the common signs and symptoms of gossypibomas in the liver parenchyma; however, some patients were asymptomatic or presented with foreign body granulomas mimicking liver metastasis<sup>[1]</sup>. The signs and symptoms of foreign bodies in the biliary tracts include jaundice and elevated liver enzymes<sup>[30]</sup>. The patient in this case complained of slight intermittent chest pain and dyspnea upon exertion, poor appetite and weight loss. The mass was palpable, but jaundice was absent.

Eguchi *et al*<sup>[27]</sup> reported a case of a shrapnel splinter in the common bile duct (CBD) that had migrated from the right thoracic cavity 36 years after the initial injury. It was serially documented that the shrapnel had migrated toward the diaphragm and then burrowed into the liver, settling in the CBD and causing obstructive jaundice. This timeframe represents the longest time reported in the literature that a foreign body remained in liver. In our case, the retained gauze was extracted 39 years later. To our knowledge, this is the longest time that a medical foreign body has been retained in the abdomen.

The most common detection methods are CT, radiography and ultrasound<sup>[22]</sup>. The most impressive imaging findings of gossypiboma are curved or banded radio-opaque lines on a plain radiograph. The ultrasound usually shows a well-defined mass with a wavy internal echogenic focus, a hypoechoic rim and a strong posterior shadow<sup>[31]</sup>. On a CT, a gossypiboma may manifest as a cystic lesion with an internal spongiform appearance with hyperdense capsules, concentric layering and mottled shadows as bubbles or mottled mural calcifications<sup>[32]</sup>. Magnetic resonance imaging pictures of gossypiboma in the abdomen and the pelvis reflect a well-defined mass with a peripheral wall of a low signal intensity on T1- and T2-weighted imaging, and T1-weighted imaging shows whorled stripes in the central portion and peripheral wall enhancement after intravenous gadolinium admin-

istration<sup>[33]</sup>. In our case, the patient's hepatic ultrasound showed a round mass with fluid echogenicity in the right liver lobe. The plain (Figure 1A), contrast-enhanced (Figure 1B) and reconstructive (Figure 1C) abdominal CT scans showed a cystic mass in the liver with filiform calcification inside, mottled mural calcifications and a small amount of pleural effusion.

Early diagnosis may be difficult, and the removal of a medical foreign body is crucial for preventing serious complications and medicolegal issues. The strategies for removing a medical foreign body include laparoscopy and laparotomy for foreign bodies in the liver parenchyma or in the CBD. If endoscopic retrograde cholangiopancreatogram is futile, percutaneous abscess drainage under ultrasonographic guidance will be useful<sup>[23]</sup>. The patient described in this case study underwent an exploratory laparotomy. The surgical gauze in her right liver was extracted, and double-lumen drainage was performed.

The retention of medical foreign bodies can be prevented with simple precautions, such as keeping a thorough pack count. The use of radiologically tagged materials is recommended, and radiofrequency chip identification with a barcode scanner will hopefully decrease the incidence of retained medical foreign bodies<sup>[34,35]</sup>.

In conclusion, retained foreign bodies in the liver can be classified into three categories: penetrating, medical and migrated foreign bodies. Any patient who presents with a hepatic mass and has a history of liver injury, surgery or an occult infection of unknown cause should arouse suspicion of a retained foreign body.

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**P- Reviewers** de Campos FF, Falconi M, Tovey FI  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Li JY



## Is the American Association for the Study of Liver Diseases recommendation for hepatocellular carcinoma screening a cul-de-sac?

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Received: October 26, 2012 Revised: March 12, 2013

Accepted: March 15, 2013

Published online: June 7, 2013

test, but is a public health issue: a national program is needed to ensure minimal participation, quality controls and evaluation of the results to improve the process.

Braillon A. Is the American Association for the Study of Liver Diseases recommendation for hepatocellular carcinoma screening a cul-de-sac? *World J Gastroenterol* 2013; 19(21): 3369-3370 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i21/3369.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i21.3369>

### Abstract

The American Association for the Study of Liver Diseases just confirmed a grade I recommendation for hepatocellular carcinoma (HCC) screening despite growing controversy. Why should HCC be an exception in the long list of other cancers where the feasibility and the efficacy of screening were investigated by randomized trials? Only 12.0% of United States patients are screened, a fact that precludes efficacy, and there are no relevant figures on the benefit-risk ratio. The ethics of belief is a treacherous reef. Screening is not just performing a test, but is a public health issue: a national program is needed to ensure minimal participation, quality controls and evaluation of the results to improve the process. There are also serious concerns regarding undisclosed potential conflicts of interest.

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**Key words:** Hepatocellular carcinoma; Screening; Public health

**Core tip:** Why should hepatocellular carcinoma be an exception in the long list of other cancers where the feasibility and the efficacy of screening were investigated by randomized trials? The ethics of belief is a treacherous reef. Screening is not just performing a

### TO THE EDITOR

A recent editorial from the American Association for the Study of Liver Diseases (AASLD) Practice Guidelines Committee on hepatocellular carcinoma (HCC) screening deserves comment<sup>[1]</sup>.

This recommendation for HCC screening, maintains a grade I (best evidence from randomized trials) despite the fact that it should have been downgraded to a grade III (experts statement) considering rising controversy<sup>[2,3]</sup>. Indeed, only two randomized trials are available, both from China, one is negative, the other is positive but has several major flaws<sup>[2]</sup>. In developed countries, only observational studies are available, concluding that screening improves survival despite raw data which consistently show that screened patients die younger than non-screened patients (length of time and lead time biases)<sup>[3,4]</sup>.

This is not a Byzantine comment on Evidence Medicine: this highest grade in the recommendation with flawed evidence blocks advances, is counterproductive and breaches patients' rights.

The grade I level blocks further advances as randomized controlled trials (RCT) are no longer acceptable. Moreover, the recommendation tries to justify why a RCT is not feasible. Among other arguments, it cites an Austra-

lian survey which showed that 90% of patients would refuse participation in a RCT, preferring to undergo screening (see 29 in 1). This did not provide evidence that a trial is not possible, it only showed that the patients did not receive balanced and unbiased information about screening. Randomized controlled trials are always difficult, however, why should HCC be an exception in the long list of other cancers where the feasibility and efficacy of screening were investigated by randomized trials? For example, French and United States urologists, whose associations have strongly promoted prostate cancer screening for a long time, did not hesitate to recruit patients for large multi-national trials. Everyone must support the trial recently submitted to the VA Cooperative Studies Program.

The maintenance of the grade I level cannot mask the fact that the recommendation has not been implemented. Only 12.0% of United States patients are screened, a fact that precludes efficacy<sup>[5]</sup>. Indeed, there are no relevant figures on screening to inform patients about the benefit-risk ratio, a pre-request for compliance. The benefit may be very limited, e.g., the 5-year HCC risk is 1.9% in patients with alcoholic cirrhosis<sup>[6]</sup>. With regard to harm, overdiagnosis is the inevitable trade-off involved in early cancer detection. Cancer overdiagnosis means that some cancers never progress or progress slowly and the patient dies of other causes before symptoms are apparent. Such patients, as patients with false-positive results, do not benefit from diagnostic procedures and unnecessary treatments, and can only be harmed by them<sup>[7]</sup>. The magnitude of overdiagnosis is estimated to be about 25% in mammographically-detected breast cancers and 60% in prostate-specific antigen-detected prostate cancers<sup>[7]</sup>. No data are available for HCC screening, and stating that “the risk of this is felt to be small” is not the best way to reassure patients<sup>[1]</sup>. Moreover, both the European Association for the Study of the Liver and AASLD noninvasive recall strategies for nodules of 10-30 mm in the cirrhotic liver, based on the vascular pattern, have a false-negative rate of approximately 20%<sup>[8]</sup>. Ultrasound screening is far from being a simple and non-invasive procedure, because the next step findings are frequently discordant on both computed tomography and magnetic resonance imaging, supporting the use of biopsy for the diagnosis of small HCCs<sup>[9,10]</sup>.

The ethics of belief is a treacherous reef when practiced at the bedside, and is without hope for improvement and progress. Screening is not just performing a blood test or a radiological exam, it is a complex issue which is beyond the scope of any clinical disciplines. It is a public health issue: a national program is needed to ensure minimal participation, quality controls (even more mandatory here as the algorithms are complex for recall strategies), and evaluation of the results to improve the process. Moreover, although on the rise, HCC is only the ninth leading cause of cancer deaths in the United States and has to compete with other priorities in a cost-constrained economy<sup>[11]</sup>.

Finally, the conflict of interest section is puzzling<sup>[1]</sup>.

It contrasts with a previous statement where both authors of the recommendation stated they served on the Speaker’s Bureau of Bayer<sup>[12]</sup>. Bayer markets sorafenib, the \$5000/mo drug for advanced HCC, which is now being investigated for the prevention of early HCC recurrence after local ablation (NCT01126645). Early HCC could be a huge market for Bayer. Moreover, AASLD which edits *Hepatology* receives grants from Bayer for its practice guidelines program and Bayer also held three different booth spaces during the last AASLD meeting. (see <http://www.aasld.org/practiceguidelines/Pages/ArchivePracticeGuidelines.aspx> and <http://www.aasld.org/lm2012/2012/exhibits/Pages/currentexhibitors.aspx>).

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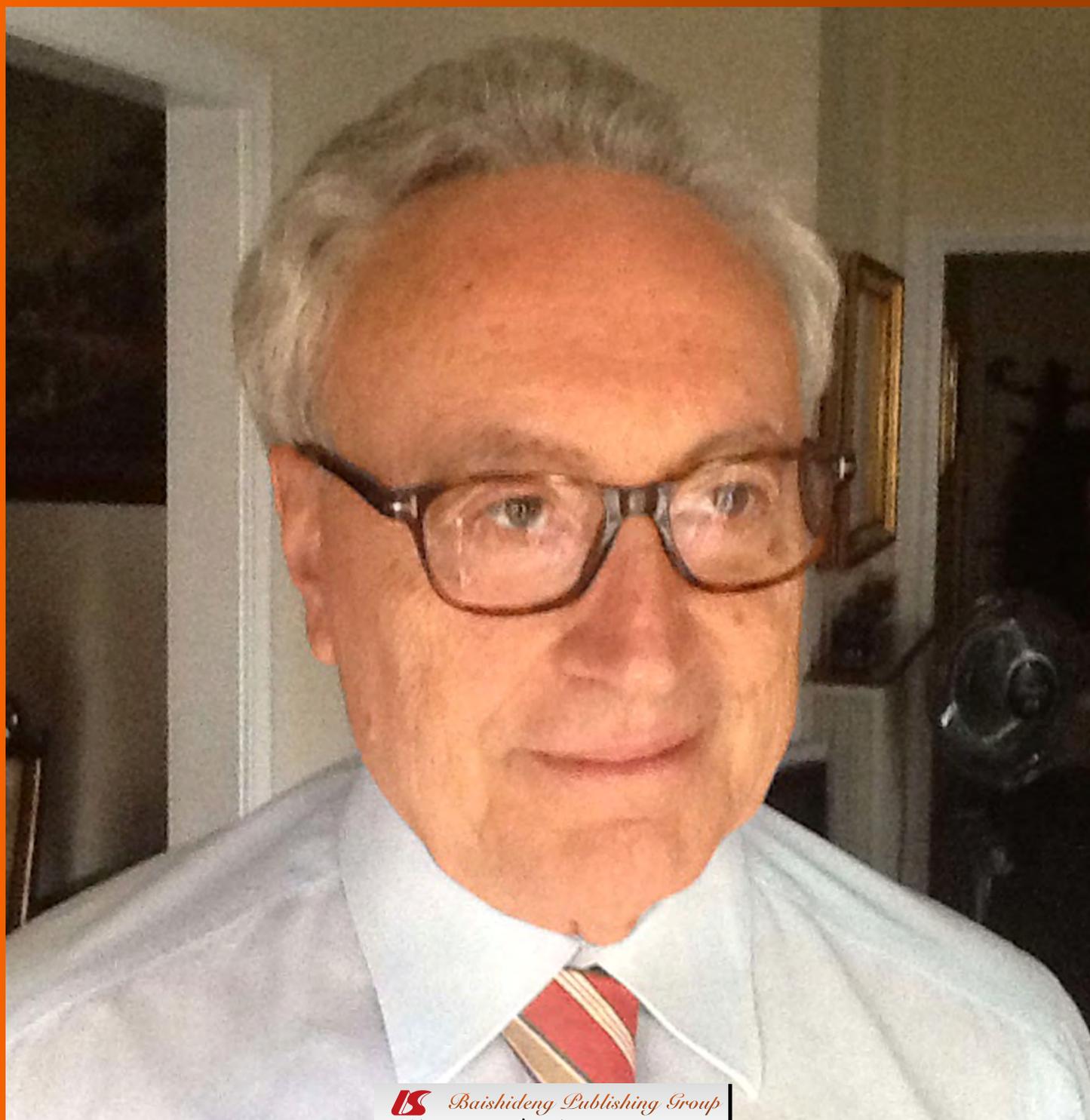
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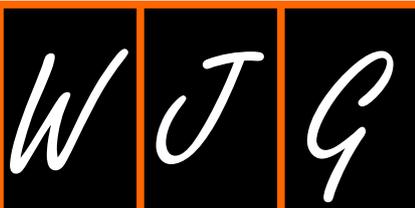
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# World Journal of *Gastroenterology*

*World J Gastroenterol* 2013 June 14; 19(22): 3371-3530





|                         |             |   |
|-------------------------|-------------|---|
| <b>FIELD OF VISION</b>  | <b>3371</b> | Controversy over the use of intraoperative blood salvage autotransfusion during liver transplantation for hepatocellular carcinoma patients<br><i>Zhai B, Sun XY</i>  |
| <b>REVIEW</b>           | <b>3375</b> | What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome?<br><i>Tarantino G, Finelli C</i>  |
|                         | <b>3385</b> | Thinking outside the liver: Induced pluripotent stem cells for hepatic applications<br><i>Subba Rao M, Sasikala M, Reddy DN</i>   |
| <b>MINIREVIEWS</b>      | <b>3397</b> | Endoscopic ultrasound-guided ethanol ablation therapy for tumors<br><i>Zhang WY, Li ZS, Jin ZD</i>  |
| <b>ORIGINAL ARTICLE</b> | <b>3404</b> | Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India<br><i>Kumari R, Ahuja V, Paul J</i>   |
|                         | <b>3415</b> | Disruption of interstitial cells of Cajal networks after massive small bowel resection<br><i>Chen J, Du L, Xiao YT, Cai W</i>   |
|                         | <b>3423</b> | Human liver tissue metabolic profiling research on hepatitis B virus-related hepatocellular carcinoma<br><i>Liu SY, Zhang RL, Kang H, Fan ZJ, Du Z</i>  |
| <b>BRIEF ARTICLE</b>    | <b>3433</b> | Polysomnographic sleep aspects in liver cirrhosis: A case control study<br><i>Teodoro VV, Júnior MAB, Lucchesi LM, Cavignolli D, de Mello MT, Kondo M, Tufik S</i>  |
|                         | <b>3439</b> | Deep sedation during gastrointestinal endoscopy: Propofol-fentanyl and midazolam-fentanyl regimens<br><i>Lera dos Santos ME, Maluf-Filho F, Chaves DM, Matuguma SE, Ide E, Luz GO, de Souza TF, Pessorusso FCS, de Moura EGH, Sakai P</i> |
|                         | <b>3447</b> | Endoscopic gastrojejunostomy with a natural orifice transluminal endoscopic surgery technique<br><i>Song TJ, Seo DW, Kim SH, Park DH, Lee SS, Lee SK, Kim MH</i>  |

- 3453** Incidental focal colorectal <sup>18</sup>F-fluorodeoxyglucose uptake on positron emission tomography/computed tomography  
*Cho SH, Kim SW, Kim WC, Park JM, Yoo IR, Kim SH, Oh ST*
- 3459** Perceptions about preventing hepatocellular carcinoma among patients with chronic hepatitis in Taiwan  
*Chen YW, Liu CC, Perng DS*
- 3466** Rockall score in predicting outcomes of elderly patients with acute upper gastrointestinal bleeding  
*Wang CY, Qin J, Wang J, Sun CY, Cao T, Zhu DD*
- 3473** Combination treatment with comprehensive cryoablation and immunotherapy in metastatic hepatocellular cancer  
*Niu LZ, Li JL, Zeng JY, Mu F, Liao MT, Yao F, Li L, Liu CY, Chen JB, Zuo JS, Xu KC*
- 3481** Efficacy of combined therapy in patients with hepatitis B virus-related decompensated cirrhosis  
*Lv GC, Yao JM, Yang YD, Zheng L, Sheng JF, Chen Y, Li LJ*
- 3487** Role of nesfatin-1 in a rat model of visceral hypersensitivity  
*Jia FY, Li XL, Li TN, Wu J, Xie BY, Lin L*
- 3494** Psychometrics of chronic liver disease questionnaire in Chinese chronic hepatitis B patients  
*Zhou KN, Zhang M, Wu Q, Ji ZH, Zhang XM, Zhuang GH*
- CASE REPORT**
- 3502** Nodular regenerative hyperplasia related portal hypertension in a patient with hypogammaglobulinaemia  
*Lal BK, Stanley A*
- 3505** Mini-loop ligation of a bleeding duodenal Dieulafoy's lesion  
*Gomerčić Palčić M, Ljubičić N*
- 3508** Colonic mucormycosis presented with ischemic colitis in a liver transplant recipient  
*Do GW, Jung SW, Jun JB, Seo JH, Nah YW*
- 3512** Transarterial embolization of metastatic mediastinal hepatocellular carcinoma  
*Chen CC, Yeh HZ, Chang CS, Ko CW, Lien HC, Wu CY, Hung SW*

- 3517** A long adult intussusception secondary to transverse colon cancer  
*Xu XQ, Hong T, Liu W, Zheng CJ, He XD, Li BL*
- 3520** Hemolymphangioma: A rare differential diagnosis of cystic-solid or cystic tumors of the pancreas  
*Dong F, Zheng Y, Wu JJ, Fu YB, Jin K, Chao M*
- 3524** Hepatoid adenocarcinoma of the extrahepatic duct  
*Wang Y, Liu YY, Han GP*

- LETTERS TO THE EDITOR 3528** Reducing risk of transjugular intrahepatic portosystemic shunt using ultrasound guided single needle pass  
*Leong S, Kok HK, Govender P, Torreggiani W*

**APPENDIX** I-VI Instructions to authors

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**INDEXING/ABSTRACTING** *World Journal of Gastroenterology* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16951 (7/74); Current Articles: 677 (1/74); and Eigenfactor® Score: 0.06035 (5/74).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 23/F, Lucky Plaza, 315-321 Lockhart Road, Wanchai, Hong Kong, China

Fax: +852-65557188  
Telephone: +852-31779906  
E-mail: [bpgoffice@wjgnet.com](mailto:bpgoffice@wjgnet.com)  
<http://www.wjgnet.com>

**PUBLICATION DATE**  
June 14, 2013

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## Controversy over the use of intraoperative blood salvage autotransfusion during liver transplantation for hepatocellular carcinoma patients

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Received: April 23, 2013 Revised: May 2, 2013

Accepted: May 9, 2013

Published online: June 14, 2013

### Abstract

Intraoperative blood salvage autotransfusion (IBSA) is used in various surgical procedures. However, because of the risk of reinfusion of salvaged blood contaminated by tumor cells, the use of IBSA in hepatocellular carcinoma (HCC) patients undergoing liver transplantation (LT) is controversial. The critical points include whether tumor cells can be cleared by IBSA, whether IBSA increases the risk of recurrence or metastasis, and what are the indications for IBSA. Moreover, is it warranted to take the risk of tumor dissemination by using IBSA to avoid allogeneic blood transfusion? Do the remaining tumor cells after additional filtration by leukocyte depletion filters still possess potential tumorigenicity? Does IBSA always work well? We have reviewed the literature and tried to address these questions. The available data indicate that IBSA is safe in LT for HCC, but randomized, controlled and prospective trials are urgently required to clarify the uncertainty.

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**Key words:** Intraoperative blood salvage autotransfu-

sion; Liver transplantation; Hepatocellular carcinoma; Leukocyte depletion filters; Allogeneic blood transfusion

**Core tip:** The use of intraoperative blood salvage autotransfusion (IBSA) in hepatocellular carcinoma (HCC) patients undergoing liver transplantation is controversial as it may reinfuse salvaged blood contaminated by tumor cells. In this article, we reviewed the relevant literature and tried to address the critical questions about IBSA. The available data indicate that IBSA is safe in liver transplantation for HCC, but randomized, controlled and prospective trials are urgently required to clarify the uncertainty.

Zhai B, Sun XY. Controversy over the use of intraoperative blood salvage autotransfusion during liver transplantation for hepatocellular carcinoma patients. *World J Gastroenterol* 2013; 19(22): 3371-3374 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3371.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3371>

### COMMENTARY ON HOT TOPICS

We have read with great interest the recent article by Akbulut *et al*<sup>[1]</sup> describing the effects of intraoperative blood salvage autotransfusion (IBSA) during liver transplantation (LT) in patients with hepatocellular carcinoma (HCC) on tumor recurrence or metastasis, and would strongly recommend it to the readers.

HCC is often associated with chronic hepatitis B and C, particularly in East and Southeast Asia, Middle and Western Africa, and Northern and Eastern Europe, where as high as 85% of HCC patients simultaneously suffer from liver cirrhosis<sup>[2]</sup>. In view of the impaired liver function, elevated portal pressure and increased collateral circulation in these patients, LT has been recommended

as an optimal treatment for HCC as the tumor burden and the underlying liver disease are resolved at the same time<sup>[3]</sup>. However, the decreased synthesis of coagulation factors, elevated portal pressure and increased collateral circulation all increase the risk of hemorrhage during surgery. Intraoperative hemorrhage is currently recognized as a mortality risk and massive blood transfusion is necessary during LT<sup>[4]</sup>.

As a result of the underlying risk of transfusion of banked blood, IBSA has been used in various surgery procedures<sup>[5,6]</sup>. With this technique, blood lost during surgery is recovered and processed through a pump system called cell saver, then transfused back into the patient<sup>[7]</sup>. This requires a system that suctions the wound, separates the blood cells from the other blood products and debris, washes the cells, and returns them to the patient<sup>[1]</sup>. It is estimated that IBSA reduces the intraoperative blood requirement by up to 60%<sup>[1]</sup>. The main complication is dilutional or disseminated coagulopathy<sup>[8,9]</sup>. Another rare complication is pulmonary injury probably linked to leukoagglutinins and transient hemoglobinuria<sup>[10]</sup>. At present, the risk of complications during IBSA has declined due to technical advances, and IBSA has significantly lower adverse events than allogeneic blood transfusion<sup>[11]</sup>. However, the use of IBSA in surgical oncology involving LT in HCC patients is controversial, as it may result in reinfusion of salvaged blood contaminated by tumor cells<sup>[11]</sup>. To date, a few studies have investigated the effects of IBSA system on tumor recurrence in HCC patients undergoing LT<sup>[4,6,11]</sup>.

### Can tumor cells be filtered away by IBSA?

The process of IBSA involves collection of blood, filtration, washing, and reinfusion. The use of IBSA for cancer patients is always performed with caution as blood collected from the operating site is at a high risk of contamination with tumor cells. Tumor cells are detected in 91%-93% of blood samples from surgical sites during various cancer surgical procedures including liver resection for liver metastasis<sup>[12,13]</sup>. During LT for HCC patients, the detection rate of tumor cells in samples from surgical sites was as high as 62.5%<sup>[6]</sup>. Moreover, Hansen *et al*<sup>[13]</sup> has reported that in one-third of cases examined, tumor cells in blood collected from surgical sites showed proliferative capacity by forming cell colonies, invasive capacity by passing the collagen coated membrane *in vitro*, and one cell line displayed tumorigenicity *in vivo*, indicating the underlying hazards of salvaged blood.

The crucial consideration is whether IBSA can effectively filter out the tumor cells. Obviously, the use of IBSA alone is not satisfactory, as tumor cells are detected in 62%-91.2% of blood samples after filtration<sup>[6,11,14,15]</sup>. Leukocyte depletion filters (LDF) with smaller-diameter (3-8  $\mu\text{m}$ ) holes have been recommended to further clear away tumor cells from the collected blood. The high efficacy of LDF in removing tumor cells from blood collected from surgical sites has been reported in patients with HCC and prostate, bladder, lung, breast, endometrial, cervical and ovarian cancers<sup>[11,14-18]</sup>. Catling *et al*<sup>[15]</sup>

reported that LDF removed all the tumor cells in 91.2% of positive samples after LDF with IBSA. Considering the difference in diameters of tumor cells, Liang *et al*<sup>[6]</sup> further compared the positive cell rates by using the IBSA system with and without LDF in HCC patients undergoing LT, and the results showed that only 25% of the positive samples became negative after cell saver processing, while after additional filtration by LDF, only 2 out of 20 patients whose tumors were unexpectedly ruptured during surgery had the collected blood positive for  $\alpha$ -fetoprotein mRNA, and one of these patients was still positive after the second LDF<sup>[6]</sup>. These data indicate that LDF can render IBSA more efficient in eliminating tumor cells from blood collected from surgical sites, and thus reduce the risk of tumor cell reinfusion.

### Does IBSA increase the risk of recurrence or metastasis?

Although more evidence supports application of IBSA in surgical oncology, the fear of reinfusing tumor cells always troubles surgeons. However, in fact, a case report was the only evidence supporting the opinion up to now<sup>[19]</sup>. In 1975, a patient died from diffuse metastasis 4 wk after pneumonectomy. Because of the blood salvaged during surgery and tumor cells detected in salvaged blood, the metastasis was ascribed to the autotransfusion of blood<sup>[19]</sup>. Although the American Medical Association issued an alert about the use of IBSA in cancer surgery in 1986<sup>[11,13]</sup>, some organizations, including the National Institute of Clinical Excellence, the Association of Anaesthetists of Great Britain and Ireland, the Obstetric Anaesthetists Association, the American College of Obstetricians and Gynecologists, and the British Confidential Enquiry of Maternal and Child Health have developed guidelines to support the use of IBSA alone or in combination with LDF in cancer surgery<sup>[11,15,20,21]</sup>.

Notwithstanding the above facts, clinical investigations to clarify the correlation of IBSA with tumor recurrence or metastasis have been carefully conducted in the past few decades. The currently available results have failed to show that IBSA increases the risk of recurrence or metastasis; on the contrary, equivalent or even better outcomes have been reported in patients with various cancers who received IBSA during surgery<sup>[5,6,12,22-25]</sup>.

A study aimed at evaluating the long-term safety of IBSA in hepatectomy for HCC was conducted by Hirano and collaborators<sup>[22]</sup>. Significantly higher 10-year cumulative overall survival and disease-free survival rates were demonstrated in the patients receiving IBSA, particularly patients with stage I / II HCC, but the differences in cumulative survival and cancer-free survival rates of patients with stage III/IV HCC were not significant from those who did not receive IBSA<sup>[22]</sup>. Another study reported similar cumulative overall survival and recurrence rates in the IBSA group and IBSA-free group, but IBSA reduced the mean volume of infused allogeneic blood<sup>[26]</sup>.

To date, three studies have investigated the use of IBSA in LT for HCC<sup>[4,6,11]</sup>. One was to evaluate the efficiency of additional LDF in eliminating tumor cells from IBSA<sup>[6]</sup>; the other two investigated whether IBSA

increased the risk of recurrence or metastasis<sup>[4,11]</sup>. In the study by Muscari *et al*<sup>[4]</sup>, among the 47 HCC patients undergoing LT, 31 patients received IBSA while the other 16 did not. During a mean 34-mo follow-up period, both groups showed similar recurrence rates (6.4% in the IBSA group *vs* 6.3% in the IBSA-free group). In another study, Foltys *et al*<sup>[11]</sup> reported a similar recurrence rate and 5-year survival rate in the IBSA group of 40 patients compared with the IBSA-free group of 96 patients during a mean follow-up period of 1015 d. However, because the two studies lacked a randomized design, heterogeneities existed in age<sup>[11]</sup>, Child score<sup>[4,11]</sup>, the percentage of severe portal hypertension<sup>[4]</sup> and pre-treatment with transarterial chemoembolization<sup>[11]</sup>. In the study by Akbulut *et al*<sup>[11]</sup>, recurrence, overall survival and disease-free survival rates were comparable in the IBSA and non-IBSA groups, which were similar in age, gender, body mass index, underlying disease, donor type, graft-to-recipient weight ratio, Child-Pugh and model for end-stage liver disease scores, number of tumors, tumor size, alpha-fetoprotein level, Milan and University of California San Francisco (UCSF) criteria, tumor differentiation, macrovascular invasion, or median hospital stay.

Without results from prospective, randomized and controlled clinical trials in a large number of subjects, it is difficult at this stage to judge whether the use of IBSA during LT for HCC is more beneficial or not. However, the current available data may at least indicate that IBSA does not increase the risk of recurrence or metastasis.

### What are the indications of IBSA?

Although the current studies partially reduced the fear of the theoretical risk of IBSA during cancer surgery, there are still a series of problems urgently requiring attention.

First, is it justified to take the risk of tumor dissemination during IBSA to avoid or reduce allogeneic blood transfusion? Allogeneic blood transfusion is not a cost-effective method as it is associated with increased risk of transfusion reactions and transfusion-transmitted infections, and induction of immunosuppression<sup>[27]</sup>. In hepatectomy for HCC, autologous blood transfusion has shown benefits in simulating liver synthetic function<sup>[28]</sup>. Moreover, allogeneic blood transfusion also increased the tumor recurrence rate by nearly 2-fold in a dose-dependent manner<sup>[28]</sup>. From the prevailing evidence, it was concluded that the correlation of cancer recurrence and allogeneic blood carried more weight than the theoretical risk of utilizing blood salvage in cancer surgery<sup>[28]</sup>.

Second, do the remaining tumor cells after additional filtration by LDF still possess potential carcinogenicity? It is difficult to answer this question because of lacking of detailed and systemic studies, although the answer has been speculated by some authors<sup>[20,28]</sup>. Only 0.01%–0.000001% of circulating tumor cells have the potential to form metastatic lesions<sup>[20,25,28]</sup>. However, the effect of LDF in eliminating tumor cells is also limited<sup>[25]</sup>. In patients undergoing LT for HCC, a 10% remnant rate

of tumor cells was reported by Liang *et al*<sup>[6]</sup>. However, another fact is that tumor cells ubiquitously exist in circulating blood of cancer patients<sup>[28]</sup>. Although the theoretical risk of the remnant tumor cells after reinfusion has not been verified<sup>[5,6]</sup>, the correlation of circulating tumor cells and poor prognosis has been proved in various cancers<sup>[25,28]</sup>. In the study by Akbulut *et al*<sup>[11]</sup>, more than 50% of patients were beyond the Milan and UCSF criteria in the IBSA group, but the metastasis rate in the IBSA group was similar to that in the non-IBSA group. Based on the above facts, it is difficult to distinguish whether the recurrence or metastasis is caused by the reinfusion or circulating tumor cells, but the risk indeed exists. There is the point of view that if tumor cells are already in circulation, is there any significance to adding a few more<sup>[20,28]</sup>?

Third, does IBSA always work well? Although the scavenging capacity of LDF is far beyond the amount of tumor cells remaining in the reinfusion blood<sup>[6,13]</sup>, the remnant tumor cells appeared in all salvaged blood samples from the HCC patients with ruptured tumors<sup>[6]</sup>. Moreover, IBSA showed less benefit for patients with stage III/IV HCC than for those with stage I/II HCC, when compared respectively with corresponding patients without IBSA<sup>[22]</sup>. The results warn against the application of IBSA in patients with more tumor cells in salvaged blood, such as patients with ruptured tumors or advanced HCC, which may exceed the filtering capacity of IBSA.

In conclusion, IBSA is a safe procedure of blood salvage in LT for HCC based on the available evidence to date. However, well-designed, randomized, controlled, prospective trials are urgently required to clarify the existing concerns.

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P- Reviewer Dehghani SM S- Editor Zhai HH  
L- Editor Cant MR E- Editor Zhang DN



## What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome?

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Received: November 8, 2012 Revised: January 16, 2013

Accepted: January 23, 2013

Published online: June 14, 2013

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**Key words:** Non-alcoholic fatty liver disease; Metabolic syndrome; Nonalcoholic steatohepatitis; Ultrasonography; Criteria

Tarantino G, Finelli C. What about non-alcoholic fatty liver disease as a new criterion to define metabolic syndrome? *World J Gastroenterol* 2013; 19(22): 3375-3384 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3375.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3375>

### Abstract

Non-alcoholic fatty liver disease (NAFLD) is currently not a component of the diagnostic criteria for metabolic syndrome (MetS); however, the development of NAFLD has some common mechanisms with the development of MetS, as they share the pathophysiologic basis of insulin resistance. It is also recognized that NAFLD is the hepatic manifestation of MetS. To define MetS, the presence of at least three of the proposed criteria is required, and sometimes it is sufficient to have only one laboratory value, modified by diet or drugs, for the classification of MetS. Ultrasonographically-detected NAFLD (US-NAFLD) is more stable, only changing during the middle- to long-term. Although controversies over MetS continue, and considering that abdominal ultrasonography for diagnosing NAFLD has high specificity and guidelines to modify the natural course of NAFLD by diet composition or lifestyle have not yet been established, why should we not introduce US-NAFLD as a new criterion to define MetS?

### INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is characterized by the accumulation of fat in the liver when it exceeds 5%-10% of its weight<sup>[1]</sup>. In addition to leading to major histopathological alterations, it may be associated with elevated liver enzymes and abnormal liver function, ranging from steatosis to steatohepatitis, fibrosis, and cirrhosis<sup>[2,3]</sup>. Although diagnosed worldwide, its prevalence varies, reaching approximately 20%-30% in western countries<sup>[4]</sup>. In the United States, where 25% of the adult population is obese, the disease occurs in more than two-thirds of these individuals and in more than 90% of class III obese individuals<sup>[5]</sup>. It is estimated that 2% to 3% of the population has nonalcoholic steatohepatitis (NASH)<sup>[6,7]</sup>.

Approximately 74%-90% of patients who undergo liver biopsy show alterations due to triacylglycerol accumulation<sup>[8]</sup>. The disease is highly prevalent (88.7%) in obese patients undergoing bariatric surgery<sup>[9]</sup>, and the likelihood of developing steatohepatitis is increased in class III obesity, with 15%-20% of these patients diagnosed with NASH<sup>[10]</sup>.

Some studies have shown an increased prevalence and higher incidence of cardiovascular disease (CVD) in individuals with NAFLD. These studies have shown hepatic

**Table 1 National Cholesterol Education Programme Adult Treatment Panel III - 2001/American Heart Association - 2005 metabolic syndrome (diagnosis: 3 of 5)**

| Risk factor                       | Defining level (AHA 2005) |
|-----------------------------------|---------------------------|
| Abdominal obesity (waist, inches) |                           |
| Men                               | > 40                      |
| Women                             | > 35                      |
| Triglycerides (mg/dL)             | 150 (or Med)              |
| HDL-C (mg/dL)                     |                           |
| Men                               | < 40 (or Med)             |
| Women                             | < 50                      |
| BP (mmHg)                         | 130/85 (or Med)           |
| Fasting glucose (mg/dL)           | 110 (100)                 |

AHA: American Heart Association; HDL-C: High-density lipoprotein-cholesterol; BP: Blood pressure; Med: Medium.

steatosis as an independent risk factor for the development of this disease<sup>[11,12]</sup>.

Metabolic syndrome (MetS), which involves the combination of risk factors for CVD such as insulin resistance, abdominal fat, dyslipidaemia, glucose intolerance, and hypertension, has often been associated with more severe liver abnormalities<sup>[13]</sup>.

NAFLD is now considered to be the hepatic component of the MetS<sup>[14,15]</sup>.

Conventional radiology studies used in the diagnosis of fatty liver include ultrasonography (US), computed tomography, and magnetic resonance imaging. Other than these radiological studies, there are no sensitive and low invasive screening methods for NAFLD. Alanine aminotransferase (ALT) > 30 IU/L is usually used as the cut off level for screening NAFLD<sup>[16,17]</sup>. This threshold had a sensitivity of 0.92 for detecting the fatty-fibrotic pattern proven by ultrasound among obese children<sup>[18]</sup>. However, ALT was within normal levels in 69% of those who had increased liver fat<sup>[19]</sup>. Similarly, in the Dallas Heart Study, 79% of the subjects with a fatty liver (liver fat content > 5.6%) had normal serum ALT<sup>[20]</sup>. This implies that a normal ALT does not exclude steatosis. Aspartate aminotransferase and gamma glutamyltransferase also correlate with liver fat content independent of obesity<sup>[21]</sup>, but are even less sensitive than serum ALT.

It is well known that NAFLD mirrors insulin resistance, and patients with NAFLD tend to have the abnormal components of the MetS. However, the target for correctly detecting MetS has not yet been met.

## METABOLIC SYNDROME: DEFINITION, IMPORTANCE AND PATHOPHYSIOLOGY

### Definition

MetS identifies patients at increased risk of developing CVD and type 2 diabetes mellitus. As it is a clustering of different risk factors, and its pathogenesis is not well understood, this has given rise to the development of multiple concurrent definitions. Central obesity and insulin resistance are acknowledged as important causative

**Table 2 International Diabetes Federation: Definitions of metabolic syndrome**

| Central obesity plus any two of the following four factors |  |
|--|--|
| Raised triglyceride  | 150 mg/dL, or specific treatment for this lipid abnormality  |
| Reduced HDL-C  | < 40 mg/dL in men and < 50 mg/dL in women, or specific treatment for this lipid abnormality  |
| Raised BP  | Systolic BP 130 or diastolic BP 85 mmHg, or treatment of previously diagnosed hypertension   |
| Raised FPG   | 100 mg/dL, or previously diagnosed type 2 diabetes. If above 100 mg/dL, OGTT is strongly recommended but is not necessary to define presence of the syndrome |

OGTT: Oral glucose tolerance test; FPG: Fasting plasma glucose; HDL-C: High-density lipoprotein-cholesterol; BP: Blood pressure.

factors<sup>[22-24]</sup>, together with other associated conditions, including physical inactivity<sup>[25]</sup>, ageing<sup>[26]</sup> and hormonal imbalance<sup>[27,28]</sup> such as polycystic ovary syndrome or testosterone insufficiency.

The concept of clustering of risk factors was first described by Reaven<sup>[29]</sup>, when the term “insulin resistance syndrome” was conceived. However, as the mechanisms underlying the link to CVD risk factors remain uncertain and insulin resistance is not easily measured in clinical practice, the more recent consensus, *e.g.*, 2001 National Cholesterol Education Programme (NCEP) Adult Treatment Panel III (ATP III)<sup>[30]</sup> (Table 1) and the 2006 International Diabetes Federation (IDF) criteria<sup>[31]</sup> (Table 2), favours focusing on other clinical parameters that are easier to measure. It is, therefore, imperative to bear in mind that as the newer criteria do not include insulin resistance as one of its diagnostic criteria, individuals diagnosed as having the syndrome using these criteria may not necessarily be insulin resistant. This is in contrast to the 1999 World Health Organization (WHO)<sup>[32]</sup> and the 1999 European Group for the Study of Insulin Resistance<sup>[33]</sup> criteria, which emphasise insulin resistance. The IDF consensus, however, takes into account the importance of gender and ethnic differences in predicting early cardiovascular risk and may indeed be a better predictor for risk in women<sup>[34]</sup> and specific ethnic groups, *e.g.*, South Asians (Indians), who appear to be more susceptible to the development of MetS at waist circumferences below that of the NCEP/ATP III cutpoints<sup>[35]</sup> (Table 3). It is also worth noting that the NCEP/ATP III criteria were revised in 2004, where the threshold for fasting glucose was lowered to  $\geq 100$  mg/dL (5.6 mmol/L) in concordance with American Diabetes Association criteria for impaired fasting glucose (IFG)<sup>[36]</sup>. Hence, in view of the various diagnostic criteria used, care will need to be exercised when interpreting clinical studies related to MetS.

### Importance

MetS continues to be highly prevalent and contributes to a rapidly growing problem globally. About 40% of adults in the US population are estimated to have MetS by the

**Table 3** Central obesity defined according to the International Diabetes Federation

| Country/ethnic group  | WC              |
|---|-----------------|
| Europids (In the United States, the NCEP-ATP III values <sup>1</sup> are likely to continue to be used for clinical purposes) |                 |
| Male  | ≥ 94 cm (37 in) |
| Female  | ≥ 80 cm (32 in) |
| South Asians (based on a Chinese, Malay, and Asian-Indian population)   |                 |
| Male  | ≥ 90 cm (35 in) |
| Female  | ≥ 80 cm (32 in) |
| Chinese   |                 |
| Male  | ≥ 90 cm (35 in) |
| Female  | ≥ 80 cm (32 in) |
| Japanese  |                 |
| Male  | ≥ 85 cm (34 in) |
| Female  | ≥ 90 cm (32 in) |

<sup>1</sup>102 cm, 40 in, male; 88 cm, 35 in, female. NCEP: National Cholesterol Education Programme; WC: Waist circumference; ATP: Adult Treatment Panel.

age of 60 years<sup>[26,37]</sup>. At least one-fourth of the adult European population may have MetS<sup>[38-40]</sup>, with a similar prevalence in Latin America<sup>[41]</sup>. MetS is also considered an emerging epidemic in developing Asian countries, including Singapore, China, Japan and South Korea, with a prevalence of 8%-13% in men and 2%-18% in women, depending on the population and definitions used<sup>[42-44]</sup>.

### Pathophysiology

There have been several proposed hypotheses for the development of MetS. One such widely quoted hypothesis suggests that adipose tissue dysfunction is the underlying cause, resulting in abnormal metabolism of free fatty acids and the release of adipocytokines which are responsible for the observed inflammatory changes and insulin resistance<sup>[45-47]</sup>. Adipose tissue is in itself an endocrine organ that is metabolically active, rather than purely an energy storage organ<sup>[48-51]</sup>. Adiponectin is secreted exclusively by adipocytes in adipose tissue, and low levels in individuals have consistently predicted the presence of MetS and CVD risk<sup>[52-56]</sup>. In fact, adiponectin can be measured reliably in a clinical setting; its circulating values do not undergo diurnal fluctuation as much as other markers such as insulin, glucose or triglycerides, and only a small amount is required for its measurement, making this a potentially suitable biomarker for MetS<sup>[57,58]</sup>. Resistin<sup>[23,59,60]</sup> and visfatin<sup>[61-64]</sup> are the other adipocytokines implicated in the pathogenesis of MetS.

An alternative proposed aetiology suggests an underlying state of chronic, low-grade inflammation<sup>[65-67]</sup>, leading to endothelial dysfunction and the release of inflammatory cytokines, which induce insulin resistance in adipose tissue and muscle<sup>[67,68]</sup>. Indeed, insulin-resistant individuals manifest evidence of low-grade inflammation even without an increase in total body fat<sup>[69]</sup>.

Excess visceral fat accumulation may be causally related to the features of insulin resistance, but might also be a marker of dysfunctional adipose tissue which is unable to

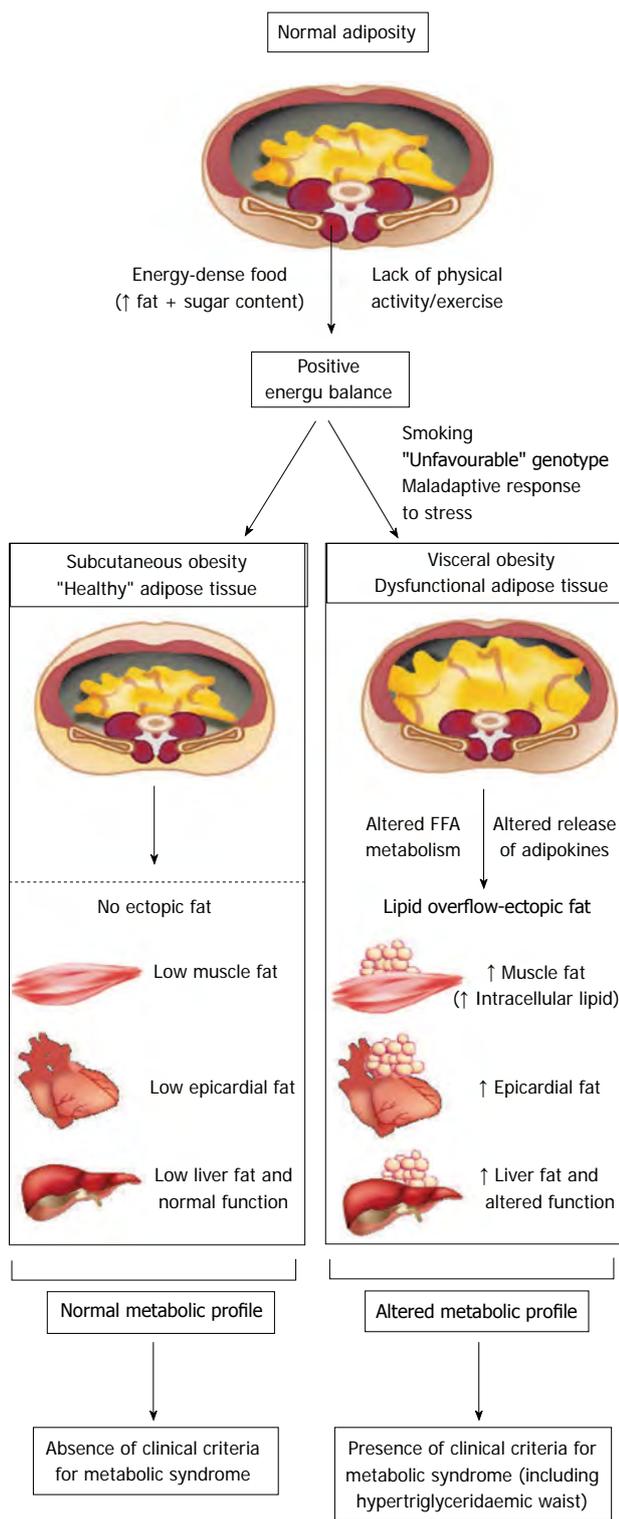
appropriately store the energy excess (Figure 1). According to this model, the body's ability to cope with the surplus of calories (resulting from excess caloric consumption, a sedentary lifestyle or, as is often the case, a combination of both factors) might, ultimately, determine the individual's susceptibility to developing MetS. There is evidence to suggest that if the extra energy is channelled into insulin-sensitive subcutaneous adipose tissue, the individual, although in positive energy balance, will be protected against the development of MetS. However, in cases in which adipose tissue is absent, deficient or insulin resistant with a limited ability to store the energy excess, the triacylglycerol surplus will be deposited at undesirable sites such as the liver, the heart, the skeletal muscle and in visceral adipose tissue - a phenomenon described as ectopic fat deposition. Factors associated with the preferential accumulation of visceral fat and with features of insulin resistance include, among others, smoking, the well documented genetic susceptibility to visceral obesity<sup>[70]</sup> and a neuroendocrine profile related to a maladaptive response to stress<sup>[71]</sup>. The resulting metabolic consequences of this "defect" in energy partitioning include visceral obesity, insulin resistance, an atherogenic dyslipidaemia and a pro-thrombotic, inflammatory profile. These are defining features of MetS.

This constellation of abnormalities can be detected by the clinical criteria for MetS, the two simplest being the simultaneous presence of increased waist girth and fasting triacylglycerol levels, a condition that has been described as "hypertriglyceridaemic waist"<sup>[72]</sup>.

It is noteworthy to stress that the identification of other risk factors might improve knowledge on the pathogenesis of NAFLD and open the way to new therapeutic approaches<sup>[73-75]</sup>. The debate surrounding the mechanisms inducing or favouring the presence/severity of NAFLD continues. In fact, some investigators have identified factors other than MetS to be associated with NAFLD<sup>[76-78]</sup>.

### RECENT CONTROVERSY

Although the MetS has existed in various forms and definitions for more than eight decades, only in the past 5 years has real controversy about its definition and significance emerged<sup>[79,80]</sup>. The main controversy is that the syndrome has had too many definitions and there is a lack of clarity about its role and value in clinical practice<sup>[81]</sup>. It is fair to say, with exceptions<sup>[82]</sup>, that most of the published reports indicate that the syndrome does not predict cardiovascular events or disease progression any better than the sum of its components<sup>[3,83,84]</sup>. The relative value in predicting type 2 diabetes remains uncertain<sup>[85]</sup>. The controversy, however, drove the need for a single global definition. Thus, came the initiative of the IDF and the American Heart Association/National Heart, Lung, and Blood Institute, joined by the World Heart Federation, International Atherosclerosis Society, and International Association for the Study of Obesity<sup>[79]</sup> to develop one unified definition<sup>[86]</sup>.



**Figure 1** The lipid overflow-ectopic fat model. FFA: Free fatty acid.

The main difference between the NCEP ATP III (National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults)<sup>[87]</sup> and the IDF definitions<sup>[88]</sup> was that the IDF had a threshold value for waist circumference as obligatory. As a major step in consensus, this obligation has been reversed so that we now have a platform

for standardised reporting in epidemiological and clinical research<sup>[81]</sup>. Yet, because the relation between waist circumference and CVD and diabetes risk differs globally, the definition for the expanded waist circumference remains unsettled. In the meantime, national or regional cutpoints for waist circumference can be used.

Insulin resistance continues to explain most, if not all of the MetS. In fact, no other mechanisms have emerged that come close to justifying the individual components or their clustering. Evidence now indicates that the MetS begins with excess central adiposity<sup>[89]</sup>. When  $\beta$ -cell function is responsive, hyperinsulinaemia results but fasting and postprandial glycaemia often remain normal for years. However, in those genetically predisposed, defects in insulin secretion and IFG and/or impaired glucose tolerance follow<sup>[90]</sup>.

Most controversial has been the mechanism of hypertension under the tent of insulin resistance. However, not only are the effects of insulin on sodium reabsorption and sympathetic nervous system activation maintained despite insulin resistance, but increases in angiotensinogen, resistin, and leptin secretion from adipose tissue have also been implicated in the pathophysiology of hypertension in the syndrome<sup>[91]</sup>. Moreover, insulin resistance is closely associated with abnormalities in nitric oxide (NO) bioavailability and reduced phosphatidylinositol 3-kinase/protein kinase B signalling in the vascular wall, both of which have a crucial role in mobilisation of endothelial progenitor cells from bone marrow<sup>[91]</sup>. Not only do higher levels of free fatty acids directly reduce NO-dependent vasodilatation, but insulin resistance itself also results in structural or functional damage to the endothelium and apoptosis<sup>[91]</sup>. Reparative processes that regenerate injured endothelium might be increased by agents such as peroxisome proliferator-activated receptor  $\gamma$  agonists that enhance insulin sensitivity, an effect mediated by endothelial progenitor cells<sup>[92]</sup>.

Genetic predisposition also relates to the MetS. A recent study found that a polymorphism in the multi-PDZ domain-containing adaptor protein, a protein that regulates the high-density lipoprotein-receptor scavenger-receptor type B class 1, was associated with the MetS<sup>[93]</sup>. Shift work, sleep deprivation, and bright-light exposure at night also relate to increased adiposity and prevalence of the MetS; clock genes are expressed in adipose tissue, and both their levels of expression and their genetic variants correlate with different components of the syndrome<sup>[94]</sup>.

Another area of recent interest is vitamin D. Increasing evidence indicates that vitamin D deficiency is associated with the risk of CVD. Particularly relevant is a study that examined the association of serum vitamin D concentrations with risk factors for CVD in US adolescents<sup>[95]</sup>.

The hypothesis that the MetS is an outgrowth of insulin resistance provides a strategy for management. Weight loss often reduces insulin resistance; and caloric restriction, weight-loss drugs, and bariatric surgery have been proved to be effective.

Although long-term weight reduction through dietary and pharmacological means is theoretically possible, most dietary and weight-loss drug studies have only continued for a few years. In contrast, in one 10-year follow-up after bariatric surgery<sup>[96]</sup>, weight loss of 25% and improvement in the MetS were achieved; total mortality was also reduced. Even in the absence of weight loss, long-term physical activity, as measured by cardiorespiratory fitness, prevents the MetS<sup>[97]</sup>, reduces cancer incidence and related mortality, and all-cause mortality<sup>[98]</sup>.

Finally, one class of drugs that reduces insulin resistance and many of the components of the syndrome is the thiazolidinediones. These drugs act mainly in adipose tissue to favourably modify secretions of products that contribute to the pathophysiology of the MetS, including free fatty acids and adipocytokines. The major effect of thiazolidinediones is on dysglycaemia, which accounts for their use in the treatment of diabetes, yet the class as a whole has anti-inflammatory effects. At present, however, drug therapy for the MetS largely requires separate agents for the treatment of dysglycaemia, dyslipidaemia, and hypertension<sup>[99]</sup>.

The MetS is a widely accepted concept that identifies the centrally obese patient with increased risk for CVD and diabetes. A global definition has now been proposed, insights into aetiology and mechanisms have been furthered, and, despite the controversies, lifestyle interventions remain the primary therapy. After lifestyle, residual risk for CVD needs to be treated with appropriate drugs.

## US AND NAFLD

US is currently the most common method for screening asymptomatic patients with elevated liver enzymes and suspected NAFLD<sup>[100]</sup>. US findings of fatty liver include hepatomegaly, diffuse increases in the echogenicity of the liver parenchyma, and vascular blunting.

Nonsteatotic hepatic parenchyma exhibits an echotexture similar to that of renal parenchyma, but becomes “brighter” when infiltrated with fat<sup>[101]</sup>. This hepatorenal contrast can be used to detect hepatosteatosi<sup>[102,103]</sup>. However, bright liver contrast associated with fibrosis is discussed in the literature<sup>[103]</sup>. US is easily performed and has a low cost, however, it also has limitations. It is operator dependent and subject to significant intra- and inter-observer variability<sup>[104]</sup>. It is impossible for US to provide quantitative information about the degree of fat accumulation. The sensitivity of US to detect steatosis decreases with a degree of fat infiltration less than 30%<sup>[105]</sup>. In obese patients, sensitivity lower than 40% has been reported in the detection of hepatosteatosi<sup>[106]</sup>. Finally, US has failed to prove efficacious in the detection of inflammation and fibrosis, therefore, it cannot be utilized to diagnose NASH and hepatic fibrosis<sup>[107]</sup>. However, in a recent study, Iijima *et al.*<sup>[108]</sup> used an ultrasound contrast agent (Levovist; Sherling, Berlin, Germany) to distinguish between simple steatosis and NASH. Levovist contains galactose and palmitic acid and is taken up by hepatocytes<sup>[109]</sup>. These

moieties participate in sugar and fat metabolism<sup>[110]</sup>. The uptake of Levovist was observed to significantly decrease in NASH patients, thus correlating with fibrosis rather than steatosis<sup>[108]</sup>. Larger studies are needed to evaluate the use of contrast US in the diagnosis of NASH characterised by inflammation and fibrosis, although there is a no absolute consensus in separating NASH from simple fatty liver as two distinct entities.

## FUTURE REMARKS

The MetS is associated with abdominal obesity and its criteria include waist circumference<sup>[30,31]</sup>. In addition, NAFLD has been reported to be associated with abdominal obesity<sup>[110]</sup>.

The presence of multiple metabolic disorders such as diabetes mellitus, obesity, dyslipidaemia and hypertension is associated with a potentially progressive, severe liver disease<sup>[14,111-115]</sup>. Previous reports demonstrated that the prevalence of NAFLD increased to 10%-80% in individuals with obesity, 35%-90% in individuals with type 2 diabetes mellitus, 30%-56% in individuals with hypertension, and 26%-58% in individuals with dyslipidaemia<sup>[116-119]</sup>.

It is clinically critical that a large number of patients with NAFLD were not diagnosed with the MetS, when we used today’s definition of the MetS<sup>[120]</sup>. Why not change the approach and use the presence of NAFLD as a new criterion for detecting the MetS?

Recently, it was shown that ultrasonographically-detected NAFLD (US-NAFLD) is an independent predictor for identifying patients with insulin resistance in non-obese, non-diabetic middle-aged Asian adults<sup>[121]</sup>. Therefore, US-NAFLD may identify individuals with insulin resistance that cannot be identified by MetS in this population<sup>[121]</sup>.

On this basis, we believe that this suggestion, *i.e.*, the inclusion of NAFLD could help initiate weight control at the “earliest possible time” in the progression of disease, *i.e.*, obesity/MetS, which means diagnosing NAFLD earlier rather than later, using the simplest method possible, *i.e.*, at US<sup>[110]</sup>.

## CONCLUSION

NAFLD is highly prevalent and is considered the hepatic component of the MetS. The WHO, the NCEP-ATP III and the IDF have different criteria to define MetS. The MetS is associated with NAFLD, with the WHO definition being the best to determine its presence, probably due to the inclusion of insulin resistance as a main component. Unification of criteria is needed to adequately compare the prevalence of MetS and its relationship with NAFLD in different population, however, this is very hard task.

Further study will be needed to verify whether the inclusion of steatosis in the panel of MetS indicators will improve the predictive power of cardiovascular risk bet-

ter than the current MetS criteria.

To define MetS, the presence of at least three of the proposed criteria is required, however, sometimes it is sufficient to have only one laboratory value, modified by diet or drugs, for the classification. US-NAFLD detection is more stable, and changes in the middle-to-long term. Although the controversy surrounding the utility of the MetS continue, considering that abdominal US in the diagnosis of NAFLD has a sensitivity of 91.7% and a specificity of 100%<sup>[122]</sup> and guidelines to modify the natural course of NAFLD by diet composition or lifestyle have not been established<sup>[123]</sup>, why should we not introduce US-NAFLD as a new criterion to define MetS?

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**P- Reviewers** Alberto P, Alessandro G **S- Editor** Jiang L  
**L- Editor** Webster JR **E- Editor** Li JY



## Thinking outside the liver: Induced pluripotent stem cells for hepatic applications

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Received: August 15, 2011 Revised: December 6, 2011

Accepted: December 15, 2011

Published online: June 14, 2013

tions. Further, we discuss the location and detection of liver stem cells and their role in liver regeneration. Although tumor formation and genetic mutations are a cause of concern, iPSCs still form a promising source for clinical applications.

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**Key words:** Liver stem cells; Hepatocytes; Disease modeling; Drug toxicity; Clinical applications; Patient-specific induced pluripotent stem cell-derived hepatocytes

Subba Rao M, Sasikala M, Reddy DN. Thinking outside the liver: Induced pluripotent stem cells for hepatic applications. *World J Gastroenterol* 2013; 19(22): 3385-3396 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3385.htm>  
DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3385>

### Abstract

The discovery of induced pluripotent stem cells (iPSCs) unraveled a mystery in stem cell research, after identification of four re-programming factors for generating pluripotent stem cells without the need of embryos. This breakthrough in generating iPSCs from somatic cells has overcome the ethical issues and immune rejection involved in the use of human embryonic stem cells. Hence, iPSCs form a great potential source for developing disease models, drug toxicity screening and cell-based therapies. These cells have the potential to differentiate into desired cell types, including hepatocytes, under *in vitro* as well as under *in vivo* conditions given the proper microenvironment. iPSC-derived hepatocytes could be useful as an unlimited source, which can be utilized in disease modeling, drug toxicity testing and producing autologous cell therapies that would avoid immune rejection and enable correction of gene defects prior to cell transplantation. In this review, we discuss the induction methods, role of reprogramming factors, and characterization of iPSCs, along with hepatocyte differentiation from iPSCs and potential applica-

### INTRODUCTION

Patients suffering from chronic end-stage liver disease are currently receiving inadequate treatment due to the lack of organ donors for transplantation<sup>[1]</sup>. Alternatively, cell-based therapies are gaining importance as supportive therapy. Hepatocytes (adult, fetal) and liver stem cells form promising sources for cellular therapies in the treatment of liver diseases. However, inadequate proliferation, ethical issues and scanty numbers limit their applicability<sup>[2-5]</sup>. Therefore, it is essential to think outside the liver in favor of generating hepatocytes for drug screening, disease modeling and cell therapy applications. Identification of four reprogramming transcription factors revolutionized stem cell research in generating induced pluripotent stem cells (iPSCs). iPSCs generated from somatic cells can be utilized not only for cell-based therapies, but also for disease modeling and drug toxicity screening. Patient-specific iPSCs can be generated by reprogramming and differentiating somatic cells from the patient into the desired cell type. Key advantages of iPSCs over current transplantation approaches are that

they form an unlimited potential source and are patient-specific. In addition, the possibility of correcting genetic defects in liver diseases is currently under investigation<sup>[6]</sup>.

The identification of patient-specific pluripotent stem cells has long been an important goal for scientists working in the field of stem cells. In 2006, Takahashi *et al*<sup>[7]</sup> first reported that forced expression of four transcription factors [octamer-binding transcription factor (Oct) 3/4, SRY box-containing gene 2 (Sox2), Kruppel-like factor 4 (Klf4) and c-Myc] reprogrammed mouse somatic fibroblasts into embryonic stem cell (ESC)-like colonies, which were termed iPSCs. Later, human induced pluripotent stem cells (hiPSCs) were generated from embryonic, neonatal and adult fibroblasts<sup>[8-10]</sup>. In addition, derivation of patient-specific iPSCs for various diseases/disorders has also been reported<sup>[11-15]</sup>. Recently, several groups have investigated the possibilities of disease modeling using patient-derived iPSCs<sup>[6,16-23]</sup>. Apart from all these applications, hepatocytes derived from iPSCs will have larger implications in drug toxicity studies. Before iPSCs, several approaches were used to reprogram the differentiated cells to a pluripotent state. In the beginning, patient-specific human embryonic stem cells (hESCs) were derived using somatic cell nuclear transfer or therapeutic cloning. This technique requires the introduction of a nucleus from an adult donor cell into an enucleated oocyte to generate a nuclear transfer embryo. The objective of this technique is to produce pluripotent hESCs that carry the nuclear genome of the patient and then induce them to differentiate into cells which may be transplanted back into the patient<sup>[24-28]</sup>. Another method is the fusion of fibroblasts with ESCs<sup>[29,30]</sup>. However, the therapeutic application of either approach has been experiencing both ethical and technical difficulties, summarized in Table 1.

## METHODS TO GENERATE iPSCs

It was demonstrated that somatic cells can be re-programmed into pluripotent stem cells by ectopic expression of four transcription factors, namely Oct4, Klf4, Sox2, and c-Myc, using four independent retroviral vectors<sup>[7]</sup>. This achievement revolutionized stem cell research. Initially, iPSCs were derived from somatic cells by the retroviral or lentiviral transduction of transcription factors in which transgenes are randomly inserted into the genome of the hosts. Such integration of transgenes has the risk of tumorigenicity<sup>[31]</sup>. Later, trials to omit transgenic insertion of c-Myc resulted in low reprogramming efficiency and did not eliminate the risk of tumor formation<sup>[32]</sup>, as overexpression of Oct3/4 and Klf4 can also cause tumor formation<sup>[33]</sup>. In Table 2, we have summarized the advantages and disadvantages of various strategies used for inducing iPSCs generation<sup>[7,8,9,32,34-49]</sup>. Furthermore, combining all four factors (Oct4, Klf4, Sox2, and c-Myc) into a single vector allowed derivation of iPSCs with a single lentiviral stem cell cassette containing a loxP sequence in the long terminal repeat (LTR)<sup>[43]</sup>. Following this, transgenes were removed using Cre-mediated excision. Although it left an incomplete LTR in the iPS genome, this method mini-

mized the genomic alteration<sup>[44]</sup>. A transposon system encoding a reprogramming cassette has also been used for iPSC induction. The transduction of a plasmid-based transposon vector can integrate into the host genome with the help of transposase, and induces iPSC colony formation. The re-expression of the transposase after the establishment of iPSCs recognizes the terminal repeat of the integrated transposon vector, and excises it from the genome. The excision of the transposon does not leave a footprint in most cases, so it maintains the original endogenous sequences<sup>[45,46,50,51]</sup>. Several techniques have been used for obtaining transgene-free iPSCs.

The first integration-free iPSCs were generated from adult mouse hepatocytes using non integrating adenoviral vectors. However, this required repeated transduction to maintain transgene expression<sup>[34,38]</sup>. Another technique used is transduction with the Sendai virus, an RNA virus, to deliver the reprogramming factors<sup>[35]</sup>. The Sendai virus does not integrate into the genome, but working with this system requires more than 15 passages to eliminate viral transgene expression. This complexity limits the general use of this method<sup>[48]</sup>. Transient transfection of plasmids, episome-based DNA vectors and minicircle vectors has been used to generate transgene-free iPSCs. Mouse embryonic fibroblasts were reprogrammed by repeated transfection with two plasmid constructs carrying the reprogramming factors; the first plasmid expressed c-Myc, while the second expressed the other three factors Oct4, Klf4 and Sox2<sup>[36]</sup>. Furthermore, experiments with non-integrating episomal vectors have also been successful in iPSC generation<sup>[16]</sup>. Similarly, minicircle vectors lack the bacterial origin of replication and antibiotic resistance gene and offer higher transfection efficiencies and more prolonged transgene expression as compared to regular plasmids<sup>[52]</sup>. Moreover, iPSCs have been established by the direct delivery of recombinant reprogramming proteins<sup>[38]</sup> and small molecules<sup>[39]</sup>. More recently, one research group has utilized synthetic mRNA molecules to reprogram human fibroblasts to pluripotency and stimulate them into myogenic cells<sup>[32]</sup>. However, reprogramming using modified RNAs is technically difficult, sensitive to reagents and requires labor-intensive procedures. The efficiency of iPSC induction using transgene-free methods is lower than that with retrovirus vectors, possibly due to low transduction efficiency and unstable expression. Therefore, it is essential to develop methods that require less time and have higher efficiency of reprogramming involving viral and transgene-free techniques to generate iPSCs.

## REPROGRAMMING FACTORS

Takahashi *et al*<sup>[7]</sup> used a combination of four nuclear reprogramming factors, such as Oct4, Sox2, c-Myc and Klf4, for generating iPSCs from mice and reported an efficiency of 0.02%. Simultaneously, the Thomson group used a slightly different combination of factors, namely Oct4, Sox2, Nanog and Lin28, to reprogram human somatic cells at a similar efficiency (0.02%)<sup>[9]</sup>. Subsequently,

**Table 1** New approaches to reprogramming of differentiated cells to a pluripotent state

| Method  | Results of reprogramming   | Drawbacks   | Ref.    |
|---|--|---|---------|
| Transfer of the nucleus from a somatic cell to an enucleated oocyte   | The somatic cell nucleus is reprogrammed in the oocyte, and a whole organism develops as a result. Patient-specific hESCs can be derived | Low efficiency. Developmental abnormalities in cloned animals. Ethical and legal restrictions | [24-28] |
| Fusion of ESCs with differentiated cells  | Hybrids of differentiated cells and ESCs display all properties of pluripotent cells   | Cell hybrids lack a normal diploid chromosome set   | [29,30] |
| Reprogramming of somatic cells to a pluripotent state can be generated by the ectopic expression of 4 transcription factors, Oct4, Klf4, Sox2 and c-Myc | Somatic cells regain a pluripotent state and become similar in properties to ESCs  | Low efficiency of iPSC derivation. Viral integration. Tumor formation                         | [7]     |

ESCs: Embryonic stem cells; hESCs: Human embryonic stem cells; iPSC: Induced pluripotent stem cell; Oct: Octamer-binding transcription factor; Sox2: SRY box-containing gene 2; Klf4: Kruppel-like factor 4.

**Table 2** Various induction methods to generate induced pluripotent stem cells

| Methods                         | Advantages   | Disadvantages                                    | Ref.           |
|---------------------------------|--|--|----------------|
| Retroviral vectors              | High efficiency                                    | Genome integration, dividing target cells needed | [7-9,32,41,42] |
| Lentiviral vectors              | High efficiency, target cells need not be dividing | Genome integration                               | [47-49]        |
| Lentiviral vectors with Cre/Lox | High efficiency                                    | Minimize genomic integration                     | [43,44]        |
| Piggyback transposon            | Precise deletion is possible                       | Minimize genomic integration, laborious          | [45,46]        |
| Viral vectors                   | No genome integration                              | Low efficiency                                   | [34-37]        |
| Adenoviral vectors              |  |  |                |
| Sendai vectors                  |  |  |                |
| DNA vectors                     |  |  |                |
| Plasmid vectors                 |  |  |                |
| Episomal vectors                |  |  |                |
| Minicircle vectors              |  |  |                |
| Protein transduction            | No genome integration                              | Low efficiency                                   | [38]           |
| Small molecules                 | No genetic modification                            | Low efficiency                                   | [39]           |
| Synthetic mRNA                  | No genetic modification, high efficiency           | Multiple rounds of transfection are needed       | [40]           |

researchers have started to identify new reprogramming factors and usage of minimum factors for generating safe iPSCs. iPSCs have been established by 3 transcriptional factors without c-Myc (Oct3/4, Klf4, SOX2) at an efficiency of 0.002%<sup>[53,54]</sup>. It was also shown that using only Oct4 and Klf4 was enough to reprogram murine NSCs at an efficiency of 0.11%<sup>[55]</sup>. More recently, the forced expression of Oct4 alone was shown sufficient to reprogram murine NSCs, at a low efficiency of 0.014%<sup>[56]</sup>. However, the efficiency of iPSC generation has been significantly reduced with usage of minimum factors for generating safe iPSCs. The *Oct4*, *Sox2*, and *Nanog* genes code for transcription factors that activate the genes and signaling pathways responsible for the establishment and maintenance of the pluripotent state and repress the genes responsible for differentiation<sup>[57,58]</sup>. Others have reported that the expression of *Oct4* and *Sox2* genes is absolutely essential for iPSC generation. In addition, the products of the *Nanog*, *c-Myc*, *Klf4* and *Lin28* genes seem to act as catalysts which accelerate the reprogramming<sup>[59]</sup>. In Table 3, we have summarized the role of various reprogramming factors for iPSC generation<sup>[60-66]</sup>.

Recently, molecules have been used in combination with reprogramming factors to improve the efficiency of iPSC generation, including cotransduction of the catalytic subunit of human telomerase, human telomerase reverse transcriptase, along with SV40 large T antigen, or the

repression of the *Ink4a/Arf* locus (encoding cell cycle-dependent kinase inhibitors), or repression of the p53/p21 pathway. These efforts have led to dramatic increases in the efficiency of reprogramming<sup>[10,67-69]</sup>.

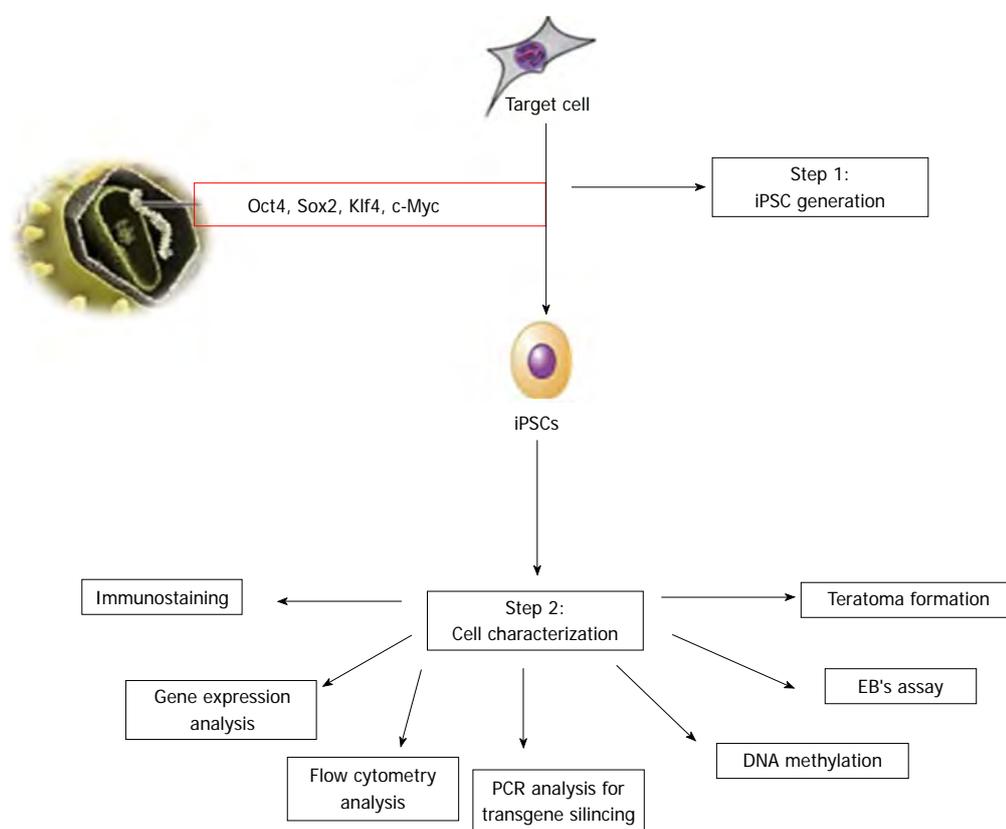
## CHARACTERIZATION OF iPSCs

The hiPSCs generated can be characterized for their pluripotency, as shown in Figure 1. In addition, assessment of their epigenetic status, silencing of transgene expression and DNA fingerprinting need to be established for confirmation. Assessment of pluripotency of iPSCs can be performed by checking the expression of protein and genes of Oct4, Sox2, Nanog, as well as for SSEA-1 (mouse) or SSEA-3/-4 and TRA-1-60/-81 (human) using flow cytometry, immunocytochemistry and reverse transcription-polymerase chain reaction (PCR) methods<sup>[70]</sup>. The pluripotent nature of iPSCs is routinely tested by two methods. The first is to determine the *in vitro* differentiation ability of iPSCs, where iPSCs can be allowed to differentiate spontaneously *in vitro* to form embryoid bodies. These embryoid bodies can be assessed for three embryonic germ layers, *i.e.*, mesoderm, endoderm and ectoderm. The second is to determine the *in vivo* differentiation ability of iPSCs<sup>[71]</sup>, where iPSCs can be injected into adult immune-deficient mice (SCID mice). In the host animal, injected iPSCs can form tumors called teratomas.

**Table 3 Role of reprogramming factors for induced pluripotent stem cell generation**

| Reprogramming factors | Description                            | Function  | Ref.    |
|-----------------------|--|---|---------|
| Oct4                  | Octamer binding transcription factor 4 | This transcription factor plays a role in embryonic development, especially during early embryogenesis, and it is necessary for embryonic stem cell pluripotency  | [7]     |
| Sox2                  | SRY box 2                              | In embryonic stem cells, Sox2 and Oct3/4 often co-occupy target genes, including own promoters. These proteins cooperate regulatory feedback loops to maintain pluripotency   | [60]    |
| Klf4                  | Kruppel-like factor 4                  | This transcription factor plays a role in upregulation of pluripotency gene <i>Nanog</i> and the modification of chromatin structure to facilitate the binding of Oct3/4 and Sox2 to their sequences. Klf4 itself is an oncogenic factor. This gene is over expressed in a variety of tumor types associated with advanced cancer | [61-63] |
| c-Myc                 | Proto oncogene protein                 | An oncogene that induces global histone acetylation, allowing Oct3/4 and Sox2 to bind to their specific target loci   | [60,63] |
| Nanog                 | Homeo box transcription factor         | A transcription factor critically involved with self-renewal of undifferentiated embryonic stem cells   | [64]    |
| Lin28                 | RNA binding protein Lin28              | The <i>Lin28</i> gene codes for an RNA-binding protein that selectively blocks the processing of microRNAs of the let-7 family, and possibly certain other microRNAs in ESCs, to prevent their differentiation  | [65,66] |

ESCs: Embryonic stem cells; Oct: Octamer-binding transcription factor; Sox2: SRY box-containing gene 2; Klf4: Kruppel-like factor 4.



**Figure 1** Flow diagram of generation and characterization of human induced pluripotent stem cells. Induced pluripotent stem cells (iPSCs) are derived through the introduction of stem cell factors into fibroblasts. After that, assessment of pluripotency of iPSCs can be studied by expression of protein and genes using various techniques such as immunocytochemistry, flow cytometry and reverse transcription-polymerase chain reaction (PCR) methods, respectively. *In vitro* and *in vivo* differentiation ability of iPSCs can be studied by embryoid body assay (EB assay) and teratoma formation assay, respectively. In addition, PCR analysis is required to demonstrate silencing of transgene expression in iPSCs and DNA methylation to confirm reprogramming of somatic cells. Oct: Octamer-binding transcription factor; Sox2: SRY box-containing gene 2; Klf4: Kruppel-like factor 4.

In addition to pluripotency assessment, it is important to confirm the silencing of exogenous transgene expression. PCR analysis can be used to demonstrate silencing of retro/lentiviral transgene expression using virus-specific primers<sup>[70]</sup>. Further, DNA fingerprinting can be performed to confirm iPSCs are genetically matched to

their parental somatic cells. DNA methylation analysis of the *Oct4*, *Sox2* and *Nanog* promoter regions using bisulfite sequencing can be used to reveal the different epigenetic states of the cells. Thus, the methylation status of promoter regions of pluripotency genes confirms successful reprogramming<sup>[70]</sup>.

**Table 4** Differentiation protocols for induced pluripotent stem cell-derived hepatocytes

| Ref.                                   | Species | Differentiation protocol  | Remarks   |
|--|---------|---|---|
| Sullivan <i>et al.</i> <sup>[78]</sup> | Human   | Activin A, Wnt3a (3 d), Activin A (2 d), DMSO (3 d), HGF, OSM (6 d)                           | Generated functional hepatocyte-like cells from human-iPSCs   |
| Song <i>et al.</i> <sup>[79]</sup>     | Human   | Activin A (3 d), FGF4, BMP-2 (4 d), HGF, KGF (6 d), OSM, Dex (5 d) then OSM, Dex, N2B27 (3 d) | iPSCs had fewer expressed liver-enriched genes compared with human hepatocytes                          |
| Si-Tayeb <i>et al.</i> <sup>[80]</sup> | Human   | Activin A (5 d), bFGF, BMP-4 (5 d), HGF (5 d), OSM (5 d)                                      | Transplanted hepatocyte-like cells into the lobe of newborn mice and demonstrated homing of donor cells |
| Liu <i>et al.</i> <sup>[81]</sup>      | Human   | Activin A (5 d), FGF4, HGF (5 d), Single Quotes (lonza), FGF4, HGF, OSM, Dex (10 d)           | Human hepatocyte-derived iPSCs are able to differentiate into functional hepatocytes                    |
| Takata <i>et al.</i> <sup>[82]</sup>   | Human   | Activin A (3 d), HGF (5 d), OSM (5 d)   | Generated hepatocyte-like cells from iPSCs using three growth factors in a short time                   |
| Gai <i>et al.</i> <sup>[83]</sup>      | Mouse   | Activin A, Wnt3 (6 d), bFGF, DMSO (3 d), HGF, DMSO (9 d), HGF, OSM, DMSO (7 d)                | Generated hepatocytes from iPSCs  |
| Iwamuro <i>et al.</i> <sup>[84]</sup>  | Mouse   | Activin A, bFGF (3 d), HGF (5 d)  | Generated hepatocyte-like cells from iPSCs  |

iPSCs: Induced pluripotent stem cells; DMSO: Dimethyl sulfoxide; HGF: Hepatocyte growth factor; OSM: Oncostatin M; Dex: Dexamethasone; FGF4: Fibroblast growth factor-4; BMP: Bone morphogenetic protein; KGF: Keratinocyte growth factor; bFGF: Basic fibroblast growth factor.

## GENERATION OF HEPATOCYTES FROM iPSCs

To date, many protocols have been used to differentiate iPSCs into desired cell types. However, different iPSC lines have different outcomes under identical culture conditions. iPSC lines have a propensity to produce certain lineages or cell types when allowed to differentiate spontaneously, indicating that choosing a proper clone is also essential in differentiating iPSCs into a specific lineage<sup>[72-74]</sup>. A major issue in differentiation is to obtain hepatocytes from pluripotent stem cells that have an adult phenotype, and which stably express liver-like functions and reflect those *in vivo* functions<sup>[75]</sup>. Recently, a number of protocols have been developed to derive hepatocytes from hiPSCs. These protocols for hepatocyte generation are hampered by inefficient differentiation and maturation that lead to low yield and heterogeneous cell populations in cultures<sup>[76]</sup>. Recently, a homogenous population of hepatocytes from pluripotent stem cells has been isolated by sorting for surface asialoglycoprotein receptor marker; however, these enriched cells are found to retain immature fetal liver characteristics<sup>[77]</sup>. In Table 4, we have summarized various protocols used to differentiate hepatocytes from iPSCs<sup>[78-84]</sup>. Even after enriching the hepatocytes from culture prior to transplantation, the risk of teratoma formation may arise due to the presence of a few undifferentiated iPSCs. Therefore, further enriching hepatocytes using negative selection against pluripotent cells could be useful to avoid teratoma formation. Figure 2 summarizes the strategy on differentiation of human iPSCs into hepatocytes. Figure 3 depicts the hepatocytes generated from hiPSCs in our laboratory.

## POTENTIAL APPLICATIONS OF iPSC-DERIVED HEPATOCYTES

iPSCs represent a promising source of hepatocytes for a wide range of applications, including disease modeling, drug toxicity testing and cell transplantation (Figure 4).

### Disease modeling

iPSCs represent a novel tool for *in vitro* disease modeling. Traditionally, researchers rely on animal models, hepatic immortalized cell lines, or short-lived primary hepatocyte cultures to understand the mechanisms and pathogenesis of diseases and testing of drug candidates<sup>[85-87]</sup>. Each of these has limitations in functionality, reproducibility and availability. Disease-specific iPSCs derived from patients suffering from specific diseases may provide a more relevant model system because their properties closely resemble those found in the patient's own system, without the need for genetic manipulation. Several groups have successfully derived a wide range of iPSCs from patients with diseases<sup>[88]</sup> and inherited liver diseases<sup>[21]</sup>. These cells can be used as models to study the pathogenesis, disease mechanism(s) and possible cure for liver disorders. Therefore, human iPSC-derived hepatocytes could generate more accurate predictions of human physiological responses than animal models. iPSC-derived hepatocytes will overcome these limitations and provide a reliable source of highly reproducible and readily available human hepatocytes for disease modeling in pre-clinical drug development.

### Drug toxicity screening

Hepatotoxicity is the most common side effect of new candidate drugs under clinical trial, and is the leading cause of post approval drug recalls; for example, bromfenac and troglitazone<sup>[89]</sup>. The development of liver toxicity screening technologies utilizing iPSC-derived hepatocytes would allow investigation into the effects of single nucleotide polymorphisms on drug metabolism and toxicity<sup>[90]</sup>. An example of this is warfarin, a drug for which polymorphisms in cytochrome P-450 2C9 create problems with obtaining an appropriate pharmacotherapeutic range<sup>[91]</sup>. iPSC-derived hepatocytes could remain viable in culture for several months, enabling the assessment of acute and chronic toxicity of drugs due to their pluripotent ability. Drug toxicity assays will be performed in petri dishes which require small amounts of compound for a hepatic cytotoxicity profile. Terminally differentiated he-

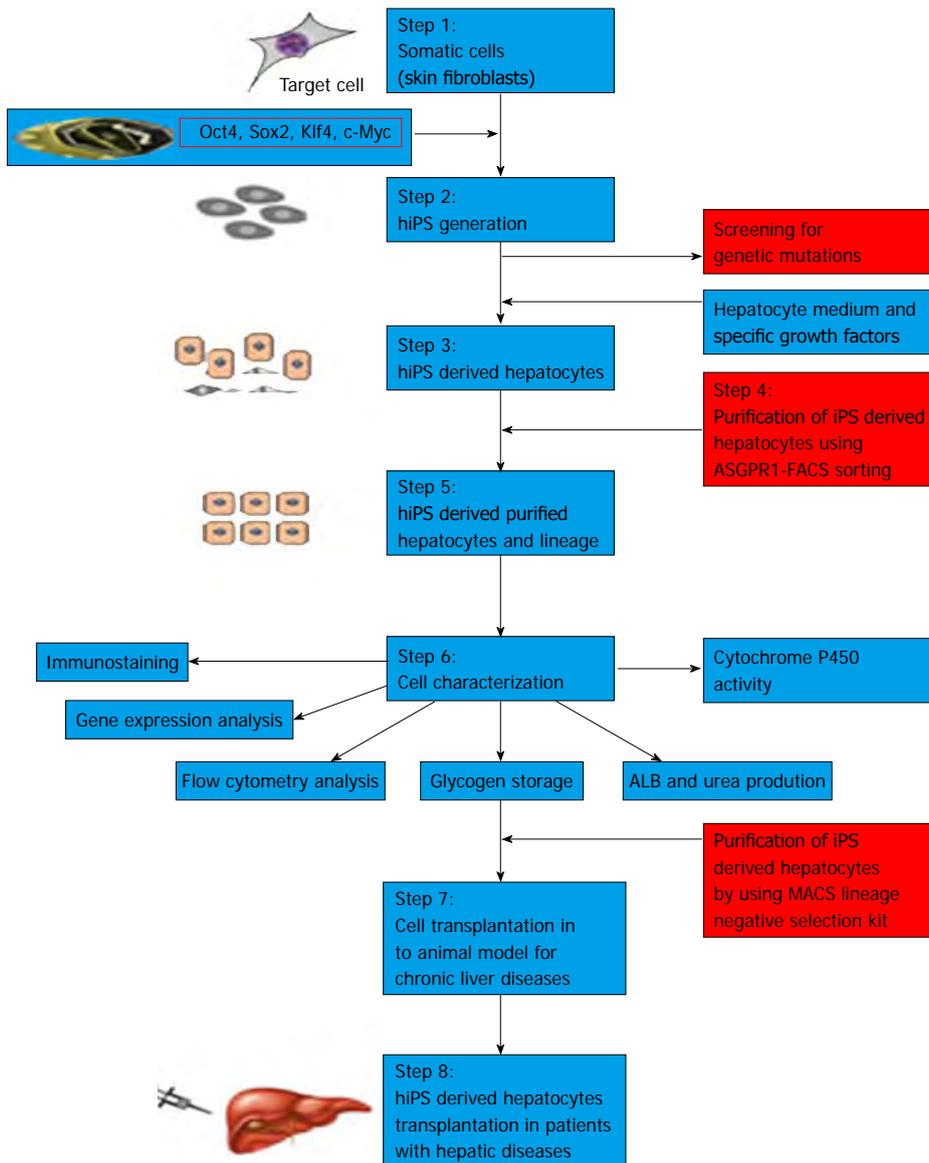


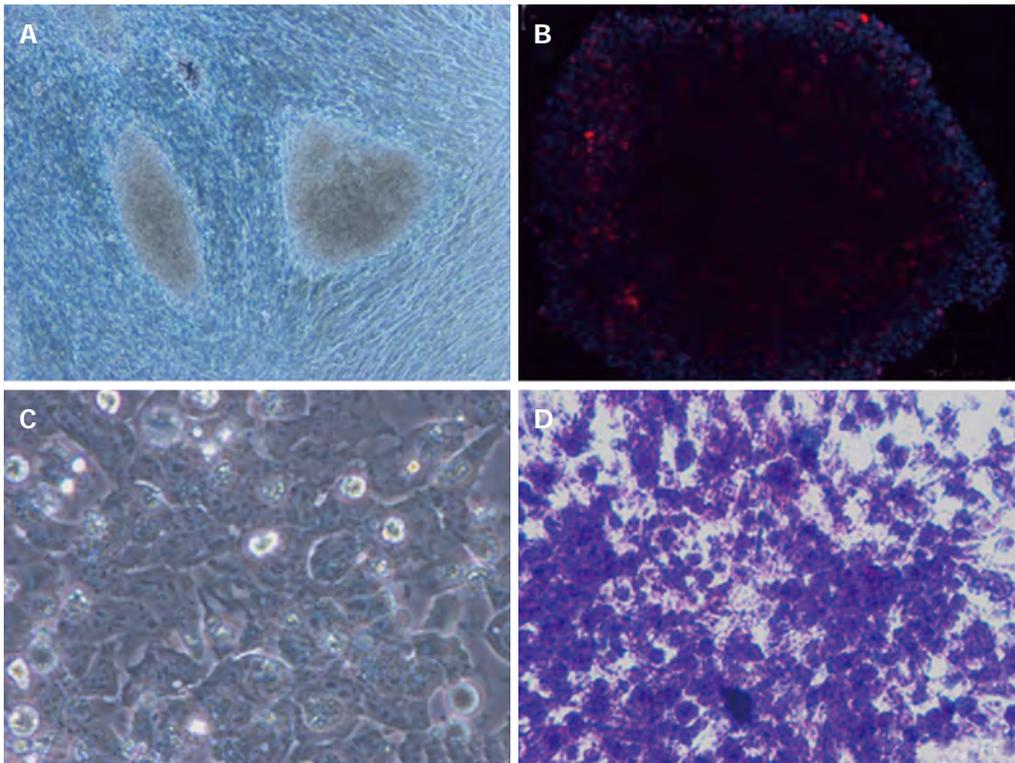
Figure 2 Flow diagram showing the strategy for human induced pluripotent stem cell-derived hepatocyte clinical applications. Steps 1 and 2, human induced pluripotent stem cells (hiPSCs) are generated from somatic cells using reprogramming techniques and screened for mutations; Step 3, hiPSCs are differentiated into hepatocytes using specific growth factors and medium; Step 4, enrichment of hiPSC-derived hepatocytes; Steps 5 and 6, characterization of enriched iPSC-derived hepatocytes for protein expression, gene expression and functional assays. Before clinical transplantation, hiPSC-derived hepatocytes are enriched again using negative selection against pluripotent cells to avoid teratoma formation; Step 7, transplantation of enriched hiPSC-derived hepatocytes into chronic liver failure animal model; Step 8, hiPSC-derived enriched hepatocytes could be transplanted into liver disease patients. Oct: Octamer-binding transcription factor; Sox2: SRY box-containing gene 2; Klf4: Kruppel-like factor 4; ASGPR: Asialoglycoprotein receptor; FACS: Fluorescence activated cell sorting; MACS: Magnetic-activated cell sorting; ALB: Albumin.

patocytes with cytochrome P-3A4 functional activity and scale-up of iPSC-derived hepatocytes will help in pharmaceutical industry drug toxicity applications.

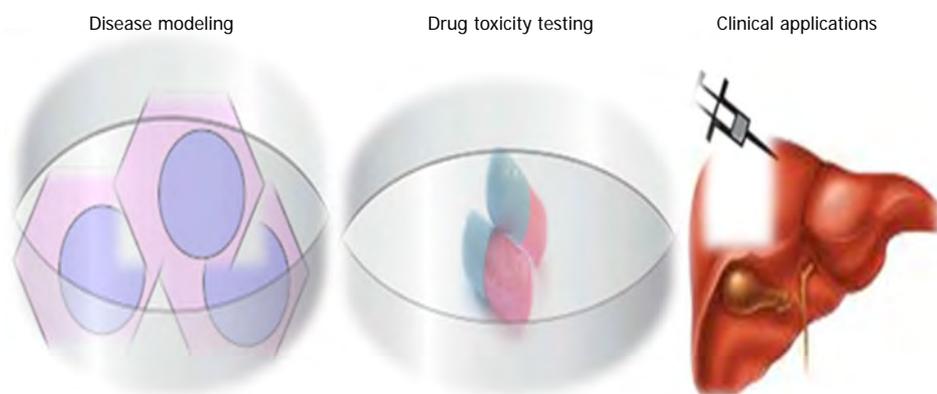
**Patient-specific iPSC-derived hepatocytes for cell transplantation**

Liver transplantation represents the only way to treat patients suffering from chronic liver failure, but this is associated with numerous problems, including shortage of donors, high cost, rejection and complications. Transplantation of hepatocytes derived from hiPSCs could represent an alternative cell source for liver failure and inborn liver diseases. The important issue is the generation of safe and functional cell types for therapy. Indeed,

the cell sources of iPSCs influence the safety of the established iPSCs. It has been demonstrated that hiPSCs retain certain gene expressions of the parent cells, and this suggests that iPSCs of different origins may possess different capacities to differentiate. A complete study using various mouse iPSCs has demonstrated that the origin of the iPSCs has a profound influence on the tumor-forming propensities in a cell transplantation therapy model<sup>[92]</sup>. Mouse tail-tip fibroblast iPSCs (mesoderm origin) have shown the highest tumorigenic propensity, whereas gastric epithelial cells and hepatocyte iPSCs (both are endoderm origin) have shown lower propensities<sup>[93]</sup>. The recent evidence suggests that epigenetic memory of the somatic cell of origin is retained in the iPSCs, and



**Figure 3** Human induced pluripotent stem cells generated from human foreskin fibroblasts using single lentiviral stem cell cassette kit (Millipore, United States) method. A, B: Human induced pluripotent stem cell (hiPSC) colonies resembling embryonic stem cells in morphology were observed, and iPSC with a flat, packed, tight colony morphology and a high nucleus to cytoplasm ratio (A,  $\times 40$ ) were positive for Oct4 marker on immunocytochemistry (B,  $\times 200$ ); C, D: hiPSCs were differentiated into hepatocytes. At day 13, these differentiated cells exhibited polygonal morphology (C,  $\times 400$ ) and showed pink color (glycogen storage) on periodic acid schiff staining (D,  $\times 200$ ).



**Figure 4** Flow diagram of potential applications of induced pluripotent stem cell-derived hepatocytes. Induced pluripotent stem cells (iPSCs) are capable of self-renewal and are able to differentiate into hepatocytes *in vitro*. iPSC-derived hepatocytes can be applied to disease modeling, drug toxicity screening assays, and clinical applications.

that may influence their directed differentiation potential into blood cells<sup>[94,81]</sup> or hepatocytes<sup>[92]</sup>. In the mouse, iPSCs have been generated from derivatives of all three embryonic germ layers, including mesodermal fibroblasts, epithelial cells of endodermal origin and ectodermal keratinocytes, whereas human iPSCs have been produced from mesoderm (fibroblasts and blood cells) or ectoderm (keratinocytes and neural stem cells) and endoderm (hepatocytes)<sup>[81]</sup>. It is therefore extremely important to establish human iPSC lines of multiple origins and thoroughly examine the source impact on both the safety issues and

their differentiation potentials.

Recently, it has been demonstrated that iPSC-derived hepatocytes can restore liver function in an animal model of liver failure<sup>[95]</sup>. These results indicate the utility of hiPSC-derived hepatocytes as an alternative treatment for patients with end-stage liver disease. Researchers investigated and analyzed the potential of hiPSC-derived hepatocytes to model inborn liver diseases such as  $\alpha_1$ -antitrypsin deficiency, familial hypercholesterolemia, glycogen storage disease type 1a, hereditary tyrosinemia, and Crigler-Najjar syndrome<sup>[6]</sup>. Genetic diseases of the liver modeled in hiPSC-

**Table 5** Direct conversion approaches for specific cell types

| Ref.                                     | Key factors  | Direct converted cell type   |
|--|--|--|
| Vierbuchen <i>et al</i> <sup>[109]</sup> | Brn2, Ascl1, and Myt1l                                     | Transdifferentiated mouse fibroblasts into functional neuronal cells       |
| Ieda <i>et al</i> <sup>[110]</sup>       | Gata4, Mef2c, and Tbx5                                     | Transdifferentiated mouse dermal fibroblasts into cardiomyocyte-like cells |
| Szabo <i>et al</i> <sup>[111]</sup>      | Oct4   | Transdifferentiated human fibroblast cells into hematopoietic progenitors  |
| Huang <i>et al</i> <sup>[112]</sup>      | Gata4, Hnf1 $\alpha$ and Foxa3, and inactivation of p19Arf | Transdifferentiated mouse tail-tip fibroblasts into hepatocyte-like cell   |

Brn2: Brain-2; Ascl1: Achaete-scute homolog 1; Myt1l: Myelin transcription factor 1; Gata4: GATA binding protein 4; Mef2c: Myocyte enhancer factor 2; Tbx5: T-box protein 5; Hnf1 $\alpha$ : Hepatocyte nuclear factor 1 $\alpha$ ; Foxa3: Forkhead box protein A3; p19: protein p19; Oct: Octamer-binding transcription factor.

derived human hepatocytes create new opportunities to develop autologous cell transplantation therapy to correct genetic defects in liver diseases.

### Liver stem cells

The liver has a massive regenerative capacity. When liver regeneration is impaired, oval shaped cells emerge and are implicated in liver tissue repair<sup>[96]</sup>. These cells are derived from the canals of Hering, which are located in the periportal region of the liver and account for 0.3%-0.7% of the liver mass<sup>[97]</sup>. In rodents, these liver progenitor cells are called oval cells, while in humans they are known as hepatic progenitor cells<sup>[98]</sup>. These cells are phenotypically similar to fetal hepatoblasts and also have a bipotent differentiation potential. Oval cells or hepatic progenitors are difficult to isolate because of the lack of definitive markers. Various markers have been used to identify oval cells in adult liver, including liver stem cell and hematopoietic markers, such as OV6, Thy-1, CD34, c-kit, and Sca-1<sup>[99]</sup>. Hepatic progenitors have been isolated from fetal liver using the specific surface marker, epithelial cell adhesion molecule (EPCAM). These EPCAM<sup>+</sup> cells showed positive for hepatic progenitor markers such as CD29, CD49f and CD90<sup>[86]</sup>. Clinical studies have identified and confirmed the efficacy of fetal liver hepatic progenitors in end-stage liver diseases<sup>[100]</sup>. However, the clinical application of this cell source is limited due to the difficulty in obtaining large numbers of fetal liver cells, as well as ethical and immune rejection issues. Another stem cell population found in the fetal liver is side population cells which represents another potential source of liver progenitor cells<sup>[101]</sup>, but these cell numbers are very much fewer in fetal liver. There is increasing evidence in the literature suggesting that bone marrow is another source of hepatic progenitor cells<sup>[102,103]</sup>. Autologous bone marrow-derived stem cell transplantations have been performed in patients with liver diseases but it is difficult to assess overall clinical benefit from these therapies<sup>[104]</sup>.

In support of a role in liver regeneration, oval cell activation has been detected in chronic liver injury caused by inflammation, chronic hepatic necrosis, chronic alcoholism induced cirrhosis and hepatitis models<sup>[105,106]</sup>. Although the full complements of signals required for oval cell activation are still unknown, both continuous metabolic stress and chemical hepatotoxic substances have been implicated as potential oval cell activators when hepatocyte proliferation is inhibited<sup>[105,106]</sup>. A recent study reported the production of a chemokine known

as stromal derived factor-1 $\alpha$  in the liver following tissue damage<sup>[107]</sup>. The role of liver stem cells in physiology, pathophysiology and therapy is not yet exactly known; therefore, it needs to be further investigated<sup>[108]</sup>. Although a number of successful techniques have been developed, stem cell-derived hepatocytes from adult, fetal and embryonic sources are found to retain immature fetal liver characteristics, which are not similar in primary hepatocyte functionality. Therefore, the elucidation of other key developmental factors and tissue culture environments, together with iPSC technology, are essential in order to obtain functional hepatocytes for hepatic applications.

### DIRECT CONVERSION

Apart from the methods discussed above, overexpression of lineage-specific transcription factors in somatic cells is a new approach (direct conversion) to generate specific cell types including neurons, cardiomyocytes, blood progenitors and hepatocyte-like cells; as summarized in Table 5<sup>[109-112]</sup>. This method could be useful as an alternative approach for autologous cell-replacement therapies. Unlike iPSCs and ESCs, directly converted cells may not easily multiply in the lab since they do not have pluripotency properties. Therefore, this approach may have limitations. Choosing highly proliferative starting somatic cells is essential in the direct conversion approach.

### CONCLUSION

Thinking outside the liver explores the potential of iPSCs as an unlimited source for *in vitro* disease modeling and for drug toxicity studies and clinical applications. Patient-specific iPSCs or custom-made iPSCs may have future promising implications without immune rejection. However, iPSC technology has several technical issues to be addressed such as generation of iPSCs without viral integration, elimination of tumor formation and genetic mutations that need to be eliminated before the cells are put to clinical applications. Despite limitations, iPSC-derived hepatocytes are a very promising population for cell therapies in hepatology.

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**P- Reviewers** Gassler N, Tam PK **S- Editor** Cheng JX  
**L- Editor** Logan S **E- Editor** Xiong L



## Endoscopic ultrasound-guided ethanol ablation therapy for tumors

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Supported by A grant from the Shanghai Science and Technology Committee Foundation, No. 11D21921605

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Received: February 9, 2013 Revised: May 14, 2013

Accepted: May 18, 2013

Published online: June 14, 2013

### Abstract

Endoscopic ultrasonography (EUS) has evolved into a useful therapeutic tool for treating a broad range of tumors since being introduced into clinical practice as a diagnostic modality nearly three decades ago. In particular, EUS-guided fine-needle injection has proven a successful minimally invasive approach for treating benign lesions such as pancreatic cysts, relieving pancreatic pain through celiac plexus neurolysis, and controlling local tumor growth of unresectable malignancies by direct delivery of anti-tumor agents. One such ablative agent, ethanol, is capable of safely ablating solid or cystic lesions in hepatic tissues *via* percutaneous injection. Recent research and clinical interest has focused on the promise of EUS-guided ethanol ablation as a safe and effective method for treating pancreatic tumor patients with small lesions or who are poor operative candidates. Although it is not likely to replace radical resection of localized lesions or systemic treatment of metastatic tumors in all patients, EUS-guided ablation is an ideal method for patients who refuse or are not eligible for surgery. Moreover, this treatment modality

may play an active role in the development of future pancreatic tumor treatments. This article reviews the most recent clinical applications of EUS-guided ethanol ablation in humans for treating pancreatic cystic tumors, pancreatic neuroendocrine tumors, and metastatic lesions.

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**Key words:** Endoscopic ultrasonography; Ethanol; Tumor ablation; Pancreas cancer; Cystic tumor; Neuroendocrine tumors; Celiac plexus neurolysis

**Core tip:** Ethanol, a commonly used ablative agent, has been used to successfully and safely ablate solid and cystic hepatic lesions *via* percutaneous injection. Endoscopic ultrasonography (EUS)-guided ethanol ablation, a minimally invasive approach, was recently developed and has been successfully applied as treatment of pancreatic cysts, pancreatic neuroendocrine tumors, and abdominal metastatic lesions. Although it is not likely to replace radical resection for treating localized lesions or systemic therapy for managing metastatic tumors, EUS-guided ablation therapies represent an attractive alternative treatment modality for patients who refuse or are not eligible for surgery.

Zhang WY, Li ZS, Jin ZD. Endoscopic ultrasound-guided ethanol ablation therapy for tumors. *World J Gastroenterol* 2013; 19(22): 3397-3403 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3397.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3397>

### INTRODUCTION

Endoscopic ultrasonography (EUS) has been used in clinical practice for more than 30 years<sup>[1,2]</sup> since its development for the original purpose of diagnosing pancre-

atic disease and carrying out malignancy staging. In the early 1990s, the introduction of curvilinear array echoendoscope made it possible to perform fine-needle aspiration (FNA) biopsy under direct endosonographic visualization. The minimally invasive and safe nature of FNA led to a boon in use of therapeutic EUS techniques, including EUS-guided fine needle injection (EUS-FNI). More recently, EUS-FNI has been applied as a pancreatic cancer treatment aimed at controlling pain through nerve blockade, as a solid tumor therapy for introduction of brachytherapy seeds and viral vectors, and as a tool for ablation therapy<sup>[3-6]</sup>.

Ethanol, a commonly used ablative agent, gained popularity according to its advantageous features of cost effectiveness, ready availability, and rapidly ablation function. The mechanism of ethanol ablation mainly involves cell death by causing cell membrane lysis, protein denaturation, and vascular occlusion<sup>[7]</sup>. Percutaneous ethanol injection has been used in the ablation of renal cysts, hepatic cysts, and solid tumors, such as liver or adrenal tumors<sup>[8-12]</sup>. EUS-guided ethanol injection is superior to the percutaneous application because it offers real-time image monitoring of the lesions located deep in the pancreas. In addition, EUS can provide precise measurement of lesions and identification of surrounding structures, and can readily deliver therapeutic agents to a target site, thereby minimizing damage to non-tumor tissue. Some EUS-guided ethanol ablation procedures for pancreatic tissue have been shown to be safe and feasible in animal models<sup>[13,14]</sup>.

In this review, the recent applications, features, and outcomes of EUS-guided ethanol injection treatment for pancreatic cystic tumors, pancreatic neuroendocrine tumors (pNETs), celiac plexus, and metastatic lesions in humans are summarized.

## EUS-GUIDED ETHANOL ABLATION THERAPY FOR PANCREATIC CYSTIC TUMORS

Pancreatic cysts are common, with the incidence of asymptomatic cysts being reported at about 2.5% in the general population<sup>[15]</sup>. The advances of various imaging modalities, such as computed tomography (CT) scanning, magnetic resonance imaging, and EUS, have been accompanied by an increase in detection of pancreatic cystic neoplasms (PCNs). Pancreatic cystic tumors are mainly classified as either mucinous cystic neoplasms (MCNs), serous cystic neoplasms (SCNs), or intraductal papillary mucinous neoplasms (IPMNs), according to histopathological features<sup>[16]</sup>. Some cystic tumors of the pancreas, such as MCNs and IPMNs, have the potential to become malignant and are often treated by surgical resection when malignancy is suspected. Although surgical resection is generally recommended, pancreatic cyst ablation is an increasingly popular treatment option, especially *via* injection of ethanol or other ablative agents into the cyst cavity under EUS guidance.

Recent studies have shown that EUS-guided ethanol injection into pancreatic cysts is safe and feasible, slows or inhibits growth, and avoids the risks associated with surgical resection. Gan *et al*<sup>[17]</sup> published the first report of safety and feasibility for the ethanol lavage and ablation of pancreatic cystic lesions using EUS-FNI. In that study, 25 patients with pancreatic cysts (MCNs,  $n = 13$ ; IPMNs,  $n = 4$ ; SCNs,  $n = 3$ ; pseudocysts,  $n = 3$ ; uncertain etiology,  $n = 2$ ) were treated with escalating doses (5%-80%) of ethanol for 3-5 min under EUS guidance. The results demonstrated that this procedure effectively reduced tumors without inducing complications, such as pancreatitis, in the short- and long-term follow-up periods. Eight patients (35%) had complete resolution of the cysts, and five patients undergoing surgical resection showed histological evidence of epithelial ablation.

To further determine whether EUS-guided ethanol lavage can decrease pancreatic cyst size, a multicenter, randomized, prospective trial was performed in 2009<sup>[18]</sup>. The primary aim of that study was to compare the change of pancreatic cyst size after EUS-guided lavage with 80% ethanol or saline solution alone. Of the 42 patients enrolled, 25 patients were initially treated with 80% ethanol lavage and 17 patients were administered saline solution lavage. Three months after the first lavage, a second pancreatic cystic lavage was performed. Patients who initially received an ethanol lavage received a second ethanol lavage, and those who received an initial saline solution lavage were then given an 80% ethanol lavage. The results indicated that EUS-guided 80% ethanol injection resulted in a greater decrease in size of pancreatic cystic tumors compared with the saline solution injection, and that the overall resolution rate of pancreatic cystic tumors was 33.3%. Incidences of major complications, such as abdominal pain, intracystic bleeding during lavage, and acute pancreatitis, were similar in the two groups. Furthermore, nine of the study patients (75%) were followed-up for two years after initial cyst resolution, and no evidence of cyst recurrence was found in any patient<sup>[19]</sup>.

To improve the effect of ethanol ablation therapy, Oh *et al*<sup>[20-22]</sup> performed EUS-guided ethanol lavage with a paclitaxel injection to treat cystic tumors of the pancreas. An initial study found that complete resolution of pancreatic cystic tumors was achieved in 11 of 14 patients after treatment with ethanol and paclitaxel injection; in addition, minor complications, including hyperamylasemia and abdominal pain, were observed in one patient<sup>[20]</sup>, but no cases of acute pancreatitis occurred. The study's collective findings demonstrated that ethanol lavage with paclitaxel injection is a safe, feasible, and effective method to treat pancreatic cystic tumors. However, only a small number of patients and only short-term outcomes were assessed in this study. A subsequent study by this group was carried out involving a larger population ( $n = 52$ ) with a longer-term follow-up period ( $n = 47$  for  $> 12$  mo). All patients underwent 99% pure ethanol injection into the collapsed cyst for a 3-5 min lavage. After the injected ethanol was aspirated, paclitaxel solution was injected into the cyst cavity. Ultimately,

complete resolution was achieved in 29 of the patients (62%). No treatment-related complications, including bleeding, bowel perforation, or severe pancreatitis, were observed<sup>[22]</sup>.

DiMaio *et al.*<sup>[23]</sup> retrospectively analyzed the effectiveness of multiple EUS-guided ethanol lavages for pancreatic cystic tumor treatment. The results showed that multiple ethanol lavage treatments resulted in a greater decrease in the size and surface area of PCNs compared with only one ethanol lavage treatment. Complete cyst resolution was not seen in any patient after the first EUS-ethanol lavage, but was achieved in 5 of the 13 patients (38%) who underwent two sequential EUS-ethanol lavage treatments. Again, no treatment-related complications were observed, with the exception of one patient complaining of minor abdominal pain.

Collectively, these preliminary studies suggest that ethanol ablation is relatively safe and feasible for clinical use in humans. Therefore, EUS-guided ablation of pancreatic cystic tumors *via* ethanol injection, a minimally invasive technique, may be an attractive choice to treat patients who refuse or are not eligible for surgery. However, there are a number of limitations in these studies. First, some studies only included a treatment group, and no control group was included. Second, there was not a definitive diagnosis made prior to application of the ethanol ablation procedure. Inclusive criteria were based on the imaging findings and some benign lesions might have been treated unnecessarily. Third, the patients were followed-up for a relatively short period of time for documentation of cyst resolution. Thus, EUS-guided cyst ablation is an experimental therapy modality that should be used with caution.

EUS-guided pancreatic cyst ablation is mainly used currently for the following indications: (1) patients who refuse surgery or are high-risk surgical candidates; (2) cysts that appear to be benign and are morphologically indeterminate; and (3) asymptomatic cysts that increase in size during the follow-up period. Before ethanol ablation for pancreatic cysts can be widely accepted, a prospective, randomized, controlled trial with a long follow-up period is required to determine the clinical efficacy of ethanol ablation therapy compared with surgical resection.

## EUS-GUIDED ETHANOL ABLATION THERAPY FOR PANCREATIC NEUROENDOCRINE TUMORS

pNET account for a small percentage of all pancreatic tumors (1.3%), but their incidence is increasing<sup>[24]</sup>. Although surgical enucleation or resection is considered as the treatment of choice for pNET, a few patients are not suitable candidates for surgery because of old age or comorbidities. Recently, successful application of EUS-guided ablation therapy using ethanol has been reported for the treatment of pNET. Jürgensen *et al.*<sup>[25]</sup> reported successful resolution of a 13 mm insulinoma by EUS-

guided ethanol ablation therapy in a 78-year-old woman (diagnosed by laboratory findings and EUS-FNA). Because the patient was in poor general condition and refused surgical resection, EUS-guided ethanol injection was performed. Although mild procedure-associated pancreatitis was experienced after the ethanol ablation procedure, complete resolution was achieved in the patient. The patient had durable glycemic control throughout the recovery and follow-up and no indications of insulinoma were observed by follow-up EUS.

Muscatiello *et al.*<sup>[26,27]</sup> described another case of successful ethanol ablation involving a patient with a pancreatic endocrine tumor. A female patient with multiple endocrine neoplasia type 1 (11 and 7 mm, respectively) underwent 40% ethanol ablation therapy. Two months after the ethanol ablation, EUS with contrast enhancement revealed areas of fibrosis. However, subsequent resolution of the accompanying perturbed levels of vasoactive intestinal peptide and chromogranin A was observed. The main complication in this case was a small pancreatic necrotic lesion, which was presumably caused by minimal ethanol effusion and managed by laparoscopic necrosectomy.

Deprez *et al.*<sup>[28]</sup> reported treatment of a patient with insulinoma of the pancreas head involving a 98% ethanol injection under EUS guidance. The patient achieved complete resolution (evidenced by imaging) at 3 mo after the procedure and remained asymptomatic and normoglycemic for more than two years later. Vleggaar *et al.*<sup>[29]</sup> reported an ethanol ablation operation for an 82-year-old patient with a pNET, for which 96% ethanol was delivered to under EUS guidance. Two months after the ablation therapy, EUS examination indicated that a significant reduction in the diameter of the tumor had been achieved. Finally, Levy *et al.*<sup>[30]</sup> retrospectively reviewed eight patients with insulinomas who received US-guided ethanol ablation. Five of those patients underwent EUS-guided ethanol injection and the remaining three underwent intra-operative ultrasound (IOUS)-guided ethanol injection. No complications occurred during or after the EUS-guided procedure. The IOUS-guided ethanol injection, however, was associated with minor peritumoral bleeding ( $n = 1$ ), pseudocyst ( $n = 1$ ), and pancreatitis with peri-pancreatic fluid ( $n = 1$ ). Hypoglycemia-related symptoms completely disappeared in five patients who underwent EUS-guided ethanol injection, and significantly improved in the three patients who underwent IOUS-guided ethanol injection.

Based on these case reports, EUS-guided ethanol ablation seems to be an alternative treatment option for pancreatic solid tumors. However, some issues remain that require further study, such as the choice of target area and adequate ethanol dose to achieve successful ablation without causing serious complications. In addition, EUS-guided ethanol ablation therapy for pNETs appears to harbor the possibility of late relapse requiring re-intervention, as well as incomplete ablation and risk of metastasis. While currently the strongest indication for intratumoral therapy is patients who refuse surgery

or are poor surgical candidates, a clinical trial enrolling more patients with longer follow-up is required to more definitively determine the particular physiological indications.

## EUS-GUIDED ETHANOL ABLATION THERAPY FOR CELIAC PLEXUS ABLATION

Pain is one of the major complications of advanced pancreatic cancer. It is estimated that pain is present in 80%-85% of pancreatic cancer patients at the time of diagnosis, and it can be difficult to control, even with high doses of analgesics<sup>[31,32]</sup>. Celiac plexus neurolysis (CPN), a chemical splanchnicectomy of the celiac plexus, has been well established as an effective method for controlling pain and decreasing morphine consumption in patients with locally advanced or unresectable pancreatic cancer<sup>[32,33]</sup>. Before the advent of EUS, CPN was performed by radiological guidance or intraoperative method, both of which are associated with a risk of the serious complications of paraesthesia, paraplegia, and pneumothorax<sup>[34,35]</sup>. With the development of the EUS technique, EUS-guided CPN (EUS-CPN) has become a novel option to treat cancer-related pain in patients with inoperable pancreatic cancer.

EUS-CPN consists of an injection of absolute alcohol, a neurolytic agent, into the celiac ganglia to permanently destroy neural tissue of celiac ganglia. The first applications of EUS-CPN were reported by Wiersema *et al*<sup>[36]</sup>. Thirty patients with pain due to intra-abdominal malignancies were injected with 98% dehydrated absolute alcohol under EUS guidance into the celiac plexus *via* the transgastric route. The majority of patients (79%-88%) experienced a significant decrease in pain scores at the 10 wk post-treatment (median follow-up time).

Subsequent studies have demonstrated that EUS-CPN is a safe and effective method to relieve severe pain from advanced pancreatic cancer<sup>[3,37-39]</sup>. In a meta-analysis of these EUS-CPN studies, pain relief was observed in about 80% of 289 patients with pain due to pancreatic cancer<sup>[40]</sup>. While EUS-CPN can be delivered on either or both sides of the aorta, a recent study showed that bilateral injection was more effective than a central single injection<sup>[41]</sup>. The common complications of EUS-CPN, transient diarrhea and hypotension, were observed in 9% and 10%-15% of cases, respectively, and both complications were self-limiting in most cases<sup>[37]</sup>.

## EUS-GUIDED ETHANOL ABLATION THERAPY FOR OTHER TUMORS

EUS-guided injection of alcohol is also used for ablation of other abdominal tumors, such as gastrointestinal stromal tumor (GIST) and intra-abdominal metastatic lesions located in the liver, adrenal glands, and pelvic lymph nodes. Günter *et al*<sup>[42]</sup> reported a case of EUS-guided

ethanol ablation of a GIST involving a 59-year-old man with a 4 cm lesion in the muscularis propria of the stomach that was diagnosed by EUS and EUS-guided FNA. Severe comorbidity precluded surgery and the patient was treated with an injection of 1.5 mL of 95% ethanol under EUS guidance. Seven weeks after the injection, no endosonographic evidence of residual tumor was seen, but a 1.5-cm ulcer at the treatment site was present, which was resolved by acid suppression therapy. Two years after the ethanol ablation treatment, a follow-up examination showed complete remission of the tumor. No severe complications were observed, except for self-limiting pain in the upper abdomen.

Recently, two cases of EUS-guided ethanol ablation of a solid hepatic metastasis carcinoma have been reported<sup>[43,44]</sup>. In the first, a 65-year-old man with a 3.3-cm lesion in the left liver and a markedly elevated carcinoembryonic antigen level was considered as having metastatic cancer from either a pancreatic adenocarcinoma or prior colorectal cancer. Multiple EUS-guided injections of absolute alcohol were administered over several years. The lesion size decreased over time, as well as the levels of corresponding tumor markers, indicating that this approach was effective in controlling tumor growth. Furthermore, the multiple EUS-guided hepatic tumor injections did not result in major complications, except for a small subcapsular hematoma that resolved spontaneously<sup>[43]</sup>.

Our group also reported a case of a 47-year-old man with a hepatic metastatic carcinoma from a pancreatic adenocarcinoma treated by injection of anhydrous ethanol under EUS guidance (Figure 1). One month later, an abdominal CT follow-up scan revealed a decrease in size of the lesion (Figure 2). No significant procedure-related complications were observed, except for a transient low fever<sup>[44]</sup>.

Artifon *et al*<sup>[45]</sup> described a case of EUS-guided alcohol ablation of a 5 cm left adrenal metastatic carcinoma in a 52-year-old man. For this patient, the main presenting complaint was abdominal pain and the diagnosis of left adrenal metastasis from non-small-cell lung carcinoma was made by EUS-FNA. Three days after the alcohol ablation therapy treatment (15 mL of 98% absolute alcohol), the symptom of abdominal pain disappeared. At the one-month follow-up, EUS revealed a hyperechoic nodule between the pancreas and the left kidney, which was presumed to represent alcohol ablation-induced fibrosis.

More recently, DeWitt *et al*<sup>[46]</sup> reported the successful EUS-guided alcohol ablation of metastatic pelvic lymph nodes in a patient with rectal cancer. The two injected metastatic pelvic lymph nodes received 4 and 2 mL of ethanol, respectively, which achieved local complete resolution at 10 mo post-treatment. No procedure-related complications were reported.

The basic characteristics and outcomes of the studies described herein of EUS-guided ethanol ablation therapy for pancreatic cystic tumors, pNETs, and other tumors are summarized in Tables 1-3, respectively.

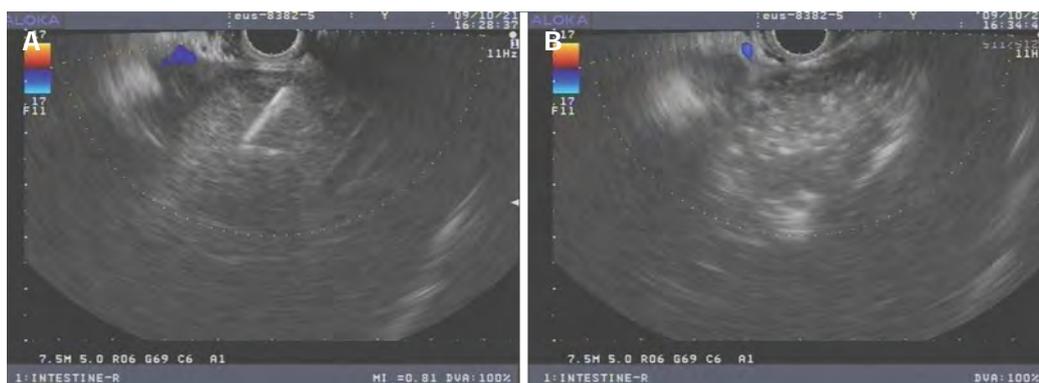


Figure 1 Endoscopic ultrasonography image of hepatic tumor. A: During ethanol injection; B: Hyperechoic appearance after ethanol injection.

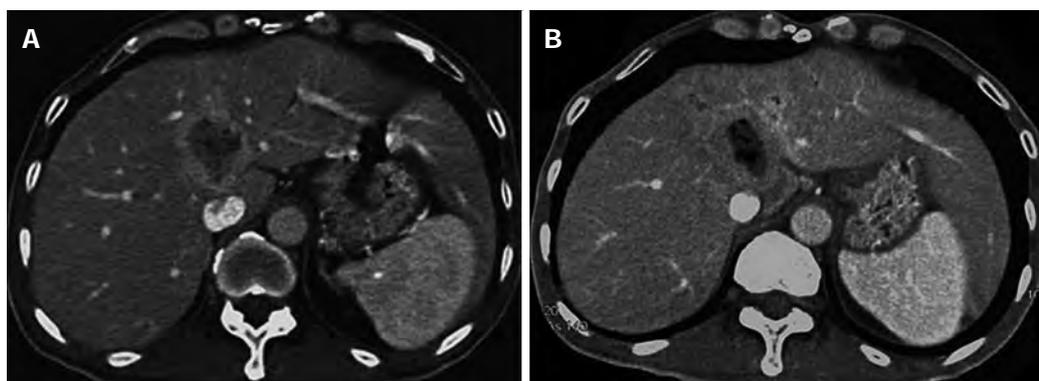


Figure 2 Computer tomography scans of hepatic metastatic carcinoma. A: Before ethanol injection; B: After ethanol injection.

Table 1 Summary of endoscopic ultrasonography-guided ethanol ablation for pancreatic cystic tumor

| Ref.                                | <i>n</i> | Median size, mm (range) | Ablative agents        | Complete resolution | Complications   |
|-------------------------------------|----------|-------------------------|------------------------|---------------------|---|
| Gan <i>et al</i> <sup>[17]</sup>    | 25       | 19.4 (6-30)             | Ethanol                | 35%                 | None  |
| Oh <i>et al</i> <sup>[20]</sup>     | 14       | 25.5 (17-52)            | Ethanol and paclitaxel | 79%                 | Acute pancreatitis ( <i>n</i> = 1)<br>Hyperamylasemia ( <i>n</i> = 6)<br>Vague abdominal pain ( <i>n</i> = 1)   |
| Oh <i>et al</i> <sup>[21]</sup>     | 10       | 29.5 (20-68)            | Ethanol and paclitaxel | 60%                 | Mild pancreatitis ( <i>n</i> = 1)   |
| DeWitt <i>et al</i> <sup>[18]</sup> | 42       | 22.4 (10-58)            | Saline vs ethanol      | 33%                 | Abdominal pain at 2 h ( <i>n</i> = 2)<br>Abdominal pain at 7 d ( <i>n</i> = 5)<br>Pancreatitis ( <i>n</i> = 1)<br>Acystic bleeding ( <i>n</i> = 1)    |
| Oh <i>et al</i> <sup>[22]</sup>     | 52       | 31.8 (17-68)            | Ethanol and paclitaxel | 62%                 | Fever ( <i>n</i> = 1)<br>Vague abdominal discomfort ( <i>n</i> = 1)<br>Mild pancreatitis ( <i>n</i> = 1)<br>Splenic vein obliteration ( <i>n</i> = 1) |

## CONCLUSION

With the advent of curvilinear EUS, therapeutic EUS has emerged as an important approach to manage malignancies. While prominent advances have been made in applications of EUS-FNI for specific antitumor therapy, the field of EUS-guided ethanol ablation therapy for malignancies continues to evolve. Currently, our understanding of the safety and efficacy of EUS-guided ethanol ablation as a tumor therapy is primarily limited by the small sample sizes and short-term follow-up periods of related case studies. Prospective, large trials should be

performed to better evaluate this technique: its indications and complications before it is recommended for widespread use in clinical practice. For example, particular patient populations may receive more benefit than others from the EUS-guided ethanol ablation with or without systemic therapies, and prospective clinical trials will help to define these patients.

## ACKNOWLEDGMENTS

We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

**Table 2 Summary of endoscopic ultrasound-guided ethanol ablation for pancreatic neuroendocrine tumor**

| Ref.                                     | n | Maximum diameter (mm) | Ethanol | Volume (mL) | Complications  |
|--|---|-----------------------|---------|-------------|--|
| Jurgensen <i>et al</i> <sup>[25]</sup>   | 1 | 13                    | 95%     | 8.0         | Pain in the upper abdomen<br>A mild increase of serum lipase activity              |
| Muscatiello <i>et al</i> <sup>[26]</sup> | 1 | 11 and 7              | 40%     | 2.0         | A small pancreatic necrotic lesion   |
| Deprez <i>et al</i> <sup>[28]</sup>      |   |                       | 98%     | 3.5         | A mild elevation of pancreatic enzymes<br>Hematoma ulceration of the duodenal wall |
| Vleggaar <i>et al</i> <sup>[29]</sup>    | 1 | 10                    | 96%     | 0.3         | None   |
| Levy <i>et al</i> <sup>[30]</sup>        | 5 | 18                    | 95%     | 0.1         | None   |
|  |   |                       |         | 0.4         |  |
|  |   | 20                    | 98%     | 0.1         |  |
|  |   |                       |         | 0.3         |  |
|  |   | 21                    | 98%     | 1.0         |  |
|  |   |                       |         | 0.3         |  |
|  |   | 8                     | 98%     | 3.0         |  |
|  |   |                       |         | 1.5         |  |
|  |   | 16                    | 99%     | 0.7         |  |
|  |   |                       |         | 1.0         |  |

**Table 3 Cases of endoscopic ultrasound-guided ethanol ablation for other tumor**

| Target tumor   | Maximum diameter (mm) | Ethanol    | Volume (mL) | Complications               |
|--|-----------------------|------------|-------------|-----------------------------|
| Gastrointestinal stromal tumor <sup>[42]</sup>                                       | 40                    | 95%        | 1.5         | None                        |
| Hepatic metastasis carcinoma from colorectal carcinoma <sup>[43]</sup>               | 33                    | 98%        | 6.0         | A tiny subcapsular hematoma |
| Hepatocellular carcinoma from pancreatic adenocarcinoma <sup>[44]</sup>              | 35                    | 100%       | 10.0        | Low-grade fever             |
| Left adrenal metastasis carcinoma from non-small-cell lung carcinoma <sup>[45]</sup> | 50                    | 98%        | 15.0        | None                        |
| Pelvic metastatic lymph nodes from rectal cancer <sup>[46]</sup>                     | 11 and 10             | Not stated | 4.0 and 2.0 | None                        |

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**P- Reviewers** Nakajima N, Oh HC, Reshetnyak VI, Tanno S  
**S- Editor** Zhai HH **L- Editor** A **E- Editor** Xiong L



## Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India

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Supported by Council of Scientific and Industrial Research, New Delhi; Government of India

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Received: November 24, 2012 Revised: January 1, 2013

Accepted: February 2, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To study the interplay between butyrate concentration and butyrate-producing bacteria in fecal samples of ulcerative colitis (UC) patients *vs* control individuals.

**METHODS:** Fecal samples were collected from 14 control individuals (hemorrhoid patients only) and 26 UC patients (severe:  $n = 12$ , moderate:  $n = 6$ , remission:  $n = 8$ ), recruited by the gastroenterologist at the Department of Gastroenterology, All India Institute of Medical Sciences, New Delhi, India. Disease activity in UC patients was determined by clinical colitis activity index. We employed fluorescent *in situ* hybridization in combination with flow cytometry to enumerate the clostridium cluster population targeted by 16S rRNA gene probe. Major butyrate-producing species within this cluster were quantified to see if any change existed in control *vs* UC patients with different disease activity. This observed change was further validated by quantitative polymerase chain reaction. In addition to this,

we carried out gas chromatography to evaluate the changes in concentration of major short chain fatty acids (SCFAs), namely acetate, *n*-butyrate, *iso*-butyrate, in the above samples. Student *t* test and Graph pad prism-6 were used to compare the data statistically.

**RESULTS:** There was a significant decrease of *Clostridium coccooides* (control,  $25.69\% \pm 1.62\%$  *vs* severe,  $9.8\% \pm 2.4\%$ ,  $P = 0.0001$ ) and *Clostridium leptum* clusters (control,  $13.74\% \pm 1.05\%$  *vs* severe,  $6.2\% \pm 1.8\%$ ,  $P = 0.0001$ ) in fecal samples of UC patients. Furthermore, we demonstrated that some butyrate-producing members of the clostridial cluster, like *Fecalibacterium prausnitzii* (control,  $11.66\% \pm 1.55\%$  *vs* severe,  $6.01\% \pm 1.6\%$ ,  $P = 0.0001$ ) and *Roseburia intestinalis* (control,  $14.48\% \pm 1.52\%$  *vs* severe,  $9\% \pm 1.83\%$ ,  $P = 0.02$ ) were differentially present in patients with different disease activity. In addition, we also demonstrated decreased concentrations of fecal SCFAs, especially of *n*-butyrate (control,  $24.32 \pm 1.86$  mmol/ $\mu$ L *vs* severe,  $12.74 \pm 2.75$  mmol/ $\mu$ L,  $P = 0.003$ ), *iso*-butyrate (control,  $1.70 \pm 0.41$  mmol/ $\mu$ L *vs* severe,  $0.68 \pm 0.24$  mmol/ $\mu$ L,  $P = 0.0441$ ) and acetate (control,  $39.51 \pm 1.76$  mmol/ $\mu$ L *vs* severe,  $32.12 \pm 2.95$  mmol/ $\mu$ L,  $P = 0.047$ ), in the fecal samples of UC patients. The observed decrease of predominant butyrate producers of clostridial clusters correlated with the reduced SCFA levels in active UC patients. This was further confirmed by the restoration in the population of some butyrate producers with simultaneous increase in the level of SCFA in remission samples.

**CONCLUSION:** Our observations indicate that decreases in members of the clostridial cluster resulting in reduced butyrate levels contribute to the etiology of UC.

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**Key words:** Fecal microbiota; Ulcerative colitis; Short chain fatty acids; Clostridial cluster; Fluorescent *in situ* hybridization-flow cytometry; Quantitative polymerase chain reaction

Kumari R, Ahuja V, Paul J. Fluctuations in butyrate-producing bacteria in ulcerative colitis patients of North India. *World J Gastroenterol* 2013; 19(22): 3404-3414 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3404.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3404>

## INTRODUCTION

Inflammatory bowel disease (IBD), comprising of Crohn's disease and ulcerative colitis (UC), is a class of chronic inflammatory disorders of the intestine. An increasing trend in the incidence and prevalence of IBD in the Asian population has been recognized for the past two decades<sup>[1]</sup>. The dynamic balance between commensal microbiota and host defensive responses at the mucosal frontier has a pivotal role in the initiation and pathogenesis of chronic IBD<sup>[2]</sup>. Whether the exaggerated immune response is exerted to all commensal bacteria, or to a subset or a single strain of bacteria, is not known<sup>[3]</sup>. Differences in fecal microbiota of healthy subjects and IBD patients have been enumerated using different techniques<sup>[4-6]</sup>. Impaired cellular metabolism, such as butyrate oxidation and short chain fatty acid (SCFA) fermentation, has shown strong association with altered gut microbiota in UC patients<sup>[7,8]</sup>.

SCFAs, such as acetate, propionate and butyrate, are produced by intestinal microbial fermentation of mainly undigested dietary carbohydrates, specifically resistant starches and dietary fiber, but also in a minor part generated by dietary and endogenous proteins in the intestine<sup>[9]</sup>. SCFAs are important for normal intestinal biology<sup>[10]</sup>. They also stimulate colonic sodium and fluid absorption and exert proliferative effects on the colonocytes<sup>[11]</sup>. Therefore, monitoring the fluctuations in SCFA concentration may help in understanding the relation of dysbiosis with UC.

Members of *Clostridium leptum* (*C. leptum*) and *Clostridium coccoides* (*C. coccoides*) groups together constitute the majority of Firmicutes (low G + C content bacteria)<sup>[12]</sup>, producing large amounts of butyrate that function as an energy source for colonic epithelial cells and inhibit mRNA expression of proinflammatory cytokines in the mucosa by inhibiting nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation<sup>[13]</sup>. Butyrate has been reported to help in prevention of colorectal cancer<sup>[14]</sup>. Evidence indicates that bacteria related to *Eubacterium hallii* (*E. hallii*), *Roseburia* species and *Eubacterium rectale* (*E. rectale*) within cluster XIVa and *Faecalibacterium prausnitzii* (*F. prausnitzii*)-related bacteria within cluster IV are normally the two most abundant groups of human fecal bacteria that produce butyrate<sup>[15]</sup>. These species-level probes account for a mean of 7.7% of the population of the total human fecal microbiota<sup>[16]</sup>. However, both the clostridial clusters harbor a diverse collection of several species of butyrate producers and non-butyrate producers as well<sup>[17]</sup>.

Dynamics of predominant butyrate producers at the species level during disease activity and their correlation with the fluctuation in SCFA have not been established

clearly. Here we explored the alteration in population of dominant butyrate-producing bacterial species present in fecal samples of UC patients at different disease stages. Abundance of the butyrate-producing clostridial cluster group was estimated by fluorescent *in situ* hybridization (FISH) in combination with flow cytometry and real time polymerase chain reaction (PCR). Further enumeration was carried out for known predominant members of butyrate-producing bacteria such as (1) *F. prausnitzii* as a member of the *C. leptum* group; and (2) *E. hallii* and *Roseburia intestinalis* (*R. intestinalis*) as members of the *C. coccoides* group. We also checked the level of butyrate, *iso*-butyrate and acetate in fecal samples to see if any correlation exists with butyrate-producing bacteria during different disease conditions.

## MATERIALS AND METHODS

### Fecal sample collection and processing

Disease activity in UC patients was determined by simple clinical colitis activity index<sup>[18]</sup> and the patients with total index score of 7-10, > 10 and 0-2 were assigned moderate, severe and remission category, respectively<sup>[19]</sup>. The patients were recruited by the gastroenterologist at the Department of Gastroenterology of the All India Institute of Medical Sciences, New Delhi, India. Clinical and demographic features of UC patients and controls are shown in Table 1. Patients who had hemorrhoids only and showed no evidence of small and large intestinal disease were enrolled as control individuals in this study. No study patient had received any antibiotic treatment in the past three months before sample collection. Patients under any antibiotic or probiotic treatments were excluded from this study. The fecal samples were collected in sterile stool specimen containers and stored at -80 °C within 3 h of sample collection until further processing.

### Probes and oligonucleotides

All probes were designed from the *16S rRNA* gene. EUB 338 conserved within the bacterial domain was used as a positive control probe<sup>[20]</sup>; conversely NONEUB338 (reverse of EUB)<sup>[21]</sup> was used as a negative control probe. The positive control probe was double labeled with fluorescein isothiocyanate (FITC) at both the 5' and 3' end<sup>[22]</sup>, whereas the negative control probe was labeled with FITC at the 5' end and Cy5 at the 3' end. We used two group-specific probes labeled with Cy5 at their 5' end (Sigma, India) and three species-specific probes labeled with FITC at their 5' end (Sigma, India). Two competitors (unlabeled) and 5 helper oligonucleotides (unlabeled) were used to increase the accessibility of the Clep 1156 probe<sup>[23]</sup> (Table 2).

### Analysis of fecal samples by FISH-flow cytometry

About 1 g of fecal sample was suspended in 9 mL of phosphate buffered saline (PBS) and vortexed with 10-15 glass beads for 5 min to homogenize the sample. This suspension was centrifuged at 1500 rpm for 1 min

**Table 1 Clinical and demographic features of ulcerative colitis patients and controls *n* (%)**

| Feature               | UC ( <i>n</i> = 26)   | Control ( <i>n</i> = 14) |
|-----------------------|-----------------------|--------------------------|
| Sex (F/M)             | 11 (42.30)/15 (57.69) | 7 (50)/7(50)             |
| Age at diagnosis (yr) |                       |                          |
| mean ± SD             | 38.35 ± 11.49         | 35.00 ± 14.14            |
| 15-40                 | 18 (69.23)            | 8 (57.14)                |
| > 40                  | 8 (30.76)             | 6 (42.85)                |
| Disease behavior      |                       |                          |
| Severe                | 12 (46.15)            | -                        |
| Moderate              | 6 (23.07)             | -                        |
| Remission             | 8 (30.76)             | -                        |
| Disease extent        |                       |                          |
| Proctitis             | 4 (15.38)             | -                        |
| Left sided colitis    | 6 (23.00)             | -                        |
| Pancolitis            | 6 (23.00)             | -                        |
| None of the above     | 10 (38.46)            | -                        |
| Smoking history       |                       |                          |
| Yes                   | 3 (11.53)             | 2 (14.28)                |
| No                    | 23 (88.46)            | 12 (85.70)               |
| Treatment history     |                       |                          |
| Immunosuppressant     | 16 (61.53)            | -                        |
| Steroids              | 14 (53.84)            | -                        |
| Appendectomy Y/N      | 4 (15.38)/22 (84.61)  | 0/14                     |
| Family history Y/N    | 2 (7.69)/24 (92.30)   | 0/14                     |

UC: Ulcerative colitis; F: Female; M: Male; Y: Yes; N: No.

to pellet down the debris and the supernatant was collected. To fix the cells, 1 mL of this supernatant was incubated with 4% paraformaldehyde (1:3 ratio) at 4 °C, overnight. The fixed cells were washed twice with PBS and incubated in ethanol-PBS solution (1:1 ratio) at -20 °C for 2 h. For each hybridization reaction, 60 µL of fixed cells were used. The fixed cells were washed twice with PBS and resuspended in 50 µL hybridization buffer (900 mmol/L NaCl, 20 mmol/L Tris-HCl of pH 8, and 0.01% sodium dodecyl sulfate at pH 7.2). All hybridizations were performed in the dark at 50 °C for 16 h in the hybridization solution containing 4 ng/µL of the appropriate labeled probe. One hundred and fifty microliter of hybridization solution (without probe) was added to stop the reaction and cells were pelleted at 1610 *g* for 10 min. Hybridized cells were further resuspended in prewarmed washing buffer [65 mmol/L NaCl, 20 mmol/L Tris-HCl, 5 mmol/L diaminoethanetetraacetic acid (EDTA), 0.01% sodium dodecyl sulfate, pH 7.2] and incubated at 50 °C for 20 min to remove non-specific binding of the probe. Finally, cells were pelleted down at 6000 rpm for 10 min and suspended in 200 µL PBS<sup>[5,24,25]</sup>. An aliquot of 100 µL was added to 0.5 mL of flow sheath solution (Becton Dickinson) for flow cytometry analysis.

#### Data acquisition by flow cytometry

Data acquisition was performed with a FACS calibur flow cytometer (Becton Dickinson) which is equipped with an air-cooled argon-ion laser providing 15 mW at 488 nm light combined with a 635 nm red-diode laser. The 488 nm laser was used to measure the forward angle light scatter (FSC, in the 488/10 nm band pass filter), the side angle light scatter (SSC, in the 488/10 nm band

pass) and the green fluorescence intensity conferred by FITC labeled probes (filter 1 in the 530/30 nm band pass filter). The red-diode laser was used to detect the red fluorescence conferred by Cy5 labeled probes (filter 4 in a 661/16 nm band pass filter). The acquisition threshold was set in the side scatter channel. All the parameters were collected as logarithmic signals. The rate of events in the flow was set at low (12 µL/s). A total of 25000 events were collected and subsequent analyses were conducted using the Cell Quest Software (Becton Dickinson).

#### Enumeration of bacterial groups in fecal samples

Enumeration of bacterial groups was performed by a double staining method in the same reaction tube where the hybridization of the EUB338 probe labeled with FITC and the genus-specific probe labeled with Cy5 were combined. This led us to estimate the abundance of bacterial groups targeted by the respective Cy5-labeled probe as a proportion of total bacteria labeled with the EUB338 FITC probe. Next, the abundance of known butyrate producers was enumerated as cells hybridized with the FITC-labeled species-specific probe as a proportion of total cells hybridized with the respective genus-specific Cy5-labeled probe. Each time, the proportion of hybridized bacteria was corrected by subtracting the background fluorescence obtained with hybridization of the negative control probe NONEUB338.

#### Quantitative polymerase chain reaction

Genomic DNA from human fecal samples (220 mg) was extracted using the Qiagen stool DNA kit and eluted in 50 µL of Tris-EDTA buffer. About 20 ng of DNA from each sample was used to analyze the bacterial population. All primer sets used in the study were designed from the *16S rRNA* gene as shown in Table 2. Genus-specific primers were used to amplify respective genus and species from genomic DNA of the fecal samples of healthy individuals. The amplified product was cloned and sequenced and sequences were deposited in the EMBL database to obtain the accession numbers (Table 3). These *16S rRNA* gene fragments containing plasmids were used as reference strains. The standard curves were constructed by serial dilutions of each reference clone prepared from 0.05 to 500000 pg/tube, corresponding to 1 × 10 to 1 × 10<sup>7</sup> copy numbers. The standard curve of the reference clones was used to extrapolate the numbers of bacteria present in the fecal samples. With the molecular mass of the plasmid and insert known, the copy number was calculated as follows: mass in Daltons (g/molecule) = [size of double-stranded (ds) product in base pairs (bp)] (330 Da × 2 nucleotides (nt)/bp)/Avogadro's number.

Thus, the precise number of molecules (molecules/µL) = Conc./mass in Daltons<sup>[26]</sup>.

#### Gas chromatography analysis of fecal SCFA

Fecal SCFAs were analyzed using gas chromatography/flame ionization detection (GC-FID). An aliquot of fecal

**Table 2 Probes and oligonucleotides employed in the study**

| Primer or probe | Target (phylogenetic group)                     | Sequence (5'-3') from 16S rRNA gene | Used in FISH-flow cytometry or qPCR | Ref.       |
|-----------------|---|-------------------------------------|-------------------------------------|------------|
| NON338          | No bacteria                                     | ACATCCTACGGGAGGC                    | Probe <sup>1</sup>                  | [21]       |
| EUB338          | Most bacteria                                   | GCTGCCTCCCGTAGGAGT                  | Probe <sup>1</sup>                  | [20]       |
| Erec 482        | <i>C. coccoides</i> / <i>E. rectale</i> cluster | GCTTCTTAGTCARGTACCG                 | Probe <sup>1</sup>                  | [34]       |
| Clep 1156       | <i>Clostridium leptum</i> subgroup              | GTTTTRTCAACGGCAGTC                  | Probe <sup>1</sup>                  | [35]       |
| Fpra-655        | <i>F. prausnitzii</i>                           | CGCTACCTCTGCACTAC                   | Probe <sup>1</sup>                  | [16]       |
| Rint-623        | <i>R. intestinalis</i> subcluster               | TTCCAATGCAGTACCGGG                  | Probe <sup>1</sup>                  | [16]       |
| Ehal-057        | <i>E. halli</i> L2-7/ <i>E. hallii</i>          | TTCGACTGCCACCTACGC                  | Probe <sup>1</sup>                  | [16]       |
| Cp1             | Competitor 1                                    | GRTTTRTCAAYCGGCAGTC                 | Competitor <sup>1</sup>             | [23]       |
| Cp2             | Competitor 1                                    | GTVTTRTCBACGGCAGTC                  | Competitor <sup>1</sup>             | [23]       |
| H1174           | Helper oligonucleotide                          | TTGACGTCRTCCCCACCTTCCTCC            | Helper <sup>1</sup>                 | [23]       |
| H1129           | Helper oligonucleotide                          | TAGAGTGMTCTGTGCGTA                  | Helper <sup>1</sup>                 | [23]       |
| H1090           | Helper oligonucleotide                          | GGTIGCGCTCGTTGCGGGACTTAA            | Helper <sup>1</sup>                 | [23]       |
| H750            | Helper oligonucleotide                          | TCGHGCTCAGCGTCAG                    | Helper <sup>1</sup>                 | [23]       |
| H122            | Helper oligonucleotide                          | GAAGGCAGGTTACTCACGC                 | Helper <sup>1</sup>                 | [23]       |
| Clep FP         | <i>C. leptum</i> subgroup                       | CGTCAGCTCGTGTGTCGAGAT               | Primer set <sup>2</sup>             | [36]       |
| Clep RP         |   | CGTCATCCCACTTCCTCC                  |                                     |            |
| C.cocci FP      | <i>C. coccoides</i> subgroup                    | GCCACATTGGGACTGAGA                  | Primer set <sup>2</sup>             | [36]       |
| C.cocci RP      |   | GCTTCTTAGTCAGGTACCG                 |                                     |            |
| Fpraus FP       | <i>F. prausnitzii</i>                           | GATGGCCTCGCGTCCGATTAG               | Primer set <sup>2</sup>             | [37]       |
| Fpraus RP       |   | CCGAAGACCTTCTTCCTC                  |                                     |            |
| Rint FP         | <i>Roseburia</i> / <i>E. rectale</i> cluster    | CKGCAAGTCTGATGTGAAAG                | Primer set <sup>2</sup>             | This study |
| Rint RP         |   | GCGGGTCCCGTCAATTCC                  |                                     |            |
| Ehal FP         | <i>E. halli</i> L2-7/ <i>E. hallii</i> members  | GCGTAGGTGGCAGTGCAA                  | Primer set <sup>2</sup>             | [38]       |
| Ehal RP         |   | GCACCGRAGCCTATACGG                  |                                     |            |

<sup>1</sup>Respective probe or oligonucleotide was used in fluorescent *in situ* hybridization (FISH)-flow cytometry; <sup>2</sup>Respective primer was used in quantitative polymerase chain reaction (qPCR). FP: Forward primer; RP: Reverse primer; *R. intestinalis*: *Roseburia intestinalis*; *E. hallii*: *Eubacterium hallii*; *F. prausnitzii*: *Fecalibacterium prausnitzii*; *C. coccoides*: *Clostridium coccoides*; *C. leptum*: *Clostridium leptum*; *E. rectale*: *Eubacterium rectale*.

**Table 3 Accession number of reference strain used in the study**

| Bacteria               | Source                     | Accession No. |
|------------------------|----------------------------|---------------|
| <i>C. leptum</i>       | Healthy human fecal sample | AM042697      |
| <i>F. prausnitzii</i>  | Healthy human fecal sample | JX556686      |
| <i>R. intestinalis</i> | Healthy human fecal sample | JX556688      |
| <i>E. hallii</i>       | Healthy human fecal sample | JX556687      |

*C. leptum*: *Clostridium leptum*; *F. prausnitzii*: *Fecalibacterium prausnitzii*; *R. intestinalis*: *Roseburia intestinalis*; *E. hallii*: *Eubacterium hallii*.

content (250 mg) was extracted with 1 mL of extraction buffer [0.1% (w/v) HgCl<sub>2</sub> and 1% (v/v) H<sub>3</sub>PO<sub>4</sub>] supplemented with 0.045 mg/mL 2,2-dimethylbutyrate (as internal standard). The resulting slurry was centrifuged for 30 min at 5000 g at 4 °C, and the supernatant was filtered through a 0.2-µm filter. SCFAs in the supernatant collected were analyzed using a GC (Shimadzu-2010) equipped with FID and a stabilwax column (Restek, United States) of 30 m length, 530 µm diameter and 1 µm film thickness. The system was run with nitrogen as carrier gas at an inlet constant pressure of 18.1 kPa. Samples were run at an initial temperature of 120 °C for 0.5 min, and then with 8 °C/min change in temperature till it reached 220 °C and was held at 220 °C for 8 min for a total program time of 20.5 min<sup>[27]</sup>. SCFAs were identified using external standards consisting of acetate, *iso*-butyrate, *n*-butyrate (Sigma, India) and the concentration was calculated using the area percentage method.

### Ethics statement

Ethical clearance for the study was obtained from the Institute Ethics Committee, All India Institute of Medical Sciences, New Delhi. Written informed consent was obtained from all the participants.

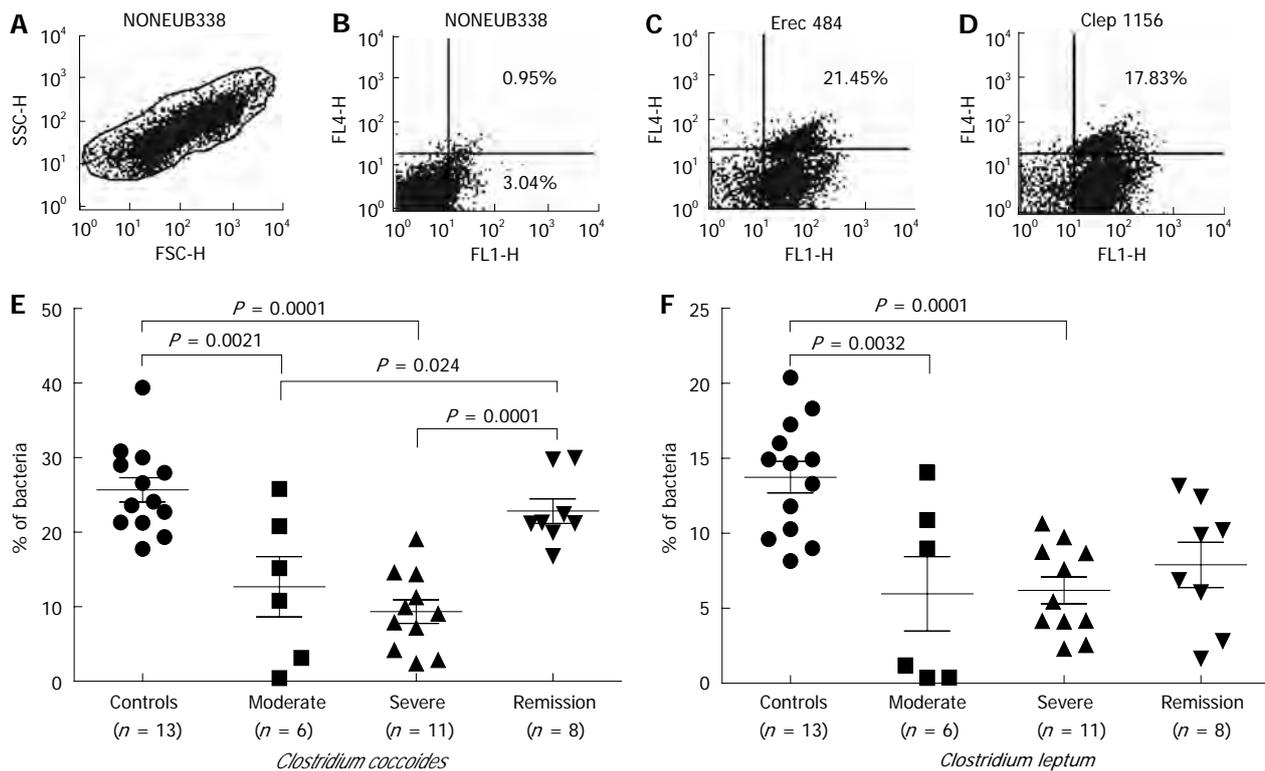
### Statistical analysis

The mean cell proportion and number of bacteria in fecal samples were estimated by FISH and qPCR in triplicate, and the results were expressed as a percentage of bacteria and number of bacteria, respectively. SCFA level was determined by GC, and the results were expressed in mmol/µL. Student's *t* test was employed to check any significant changes in the SCFA concentrations with the changes in the disease activity. Graphpad prism-6 was used to analyze FISH and qPCR data.

## RESULTS

### Analysis of *C. leptum* and *C. coccoides* groups

**FISH-flow cytometry:** In Figure 1A, the region R1 corresponds to relative size (FSC) with granularity (SSC) of the bacteria during flow cytometry with NONEUB338 hybridized cells. This region R1 was gated for further dot plots. Flow cytometric analysis of the hybridized samples gave a shift in signal of 1 log unit compared to the nonhybridized cells, enabling the specific detection and enumeration of the different bacterial groups (Figure 1B-D). Scoring of bacteria could not be achieved uni-



**Figure 1** Flow cytometric analysis of fecal microflora using 16S rRNA targeted probes. A: The region R1 corresponding to relative size (the forward angle light scatter)/granularity (the side angle light scatter) of the bacteria was delineated. This region R1 was gated for further dot plots; B: Bacteria from fecal samples were hybridized with NONEUB338 probe; C: EUB338 and Erec 482; D: EUB338 and Clep 1156. A shift in fluorescence to higher intensities was obtained upon hybridization with positive control or group-specific probe: right lower and upper quadrant compared to the lower left quadrant. The signal in lower left quadrant represents debris. The events in upper right quadrant represent the proportion of bacterial cells hybridized with the group-specific probe within the total bacterial cells hybridized with the universal bacterial probe EUB338-fluorescein isothiocyanate (FITC). The enumeration of targeted cells was corrected by subtracting the background fluorescence, which was measured using the negative control NONEUB338 probe. Fluorescent *in situ* hybridization (FISH)-flow cytometry data were expressed as the mean  $\pm$  SE as enumerated by FISH-flow cytometry in control, moderate, severe and remission samples of ulcerative colitis; E: Erec 482; F: Clep 1156.

formly; therefore, sample size differed in each category.

In UC patients belonging to the moderate and severe disease categories, we observed significant decreases in members of the *C. coccooides* and *C. leptum* groups compared to control individuals (Figure 1E and F). Among the *C. coccooides* cluster, decreases in moderate disease samples attained the *P* value of 0.0021, while there was a *P* value of 0.0001 in the case of severe patient samples in comparison to controls. However, in the case of *C. leptum*, the *P* values were 0.0032 in moderate and 0.0001 in severe categories of samples, respectively, as compared to controls. Samples in the remission stage showed significant restoration in the population of the *C. coccooides* group (*P* = 0.0001); although an increasing trend was observed in the members of the *C. leptum* group, this did not attain a significant value.

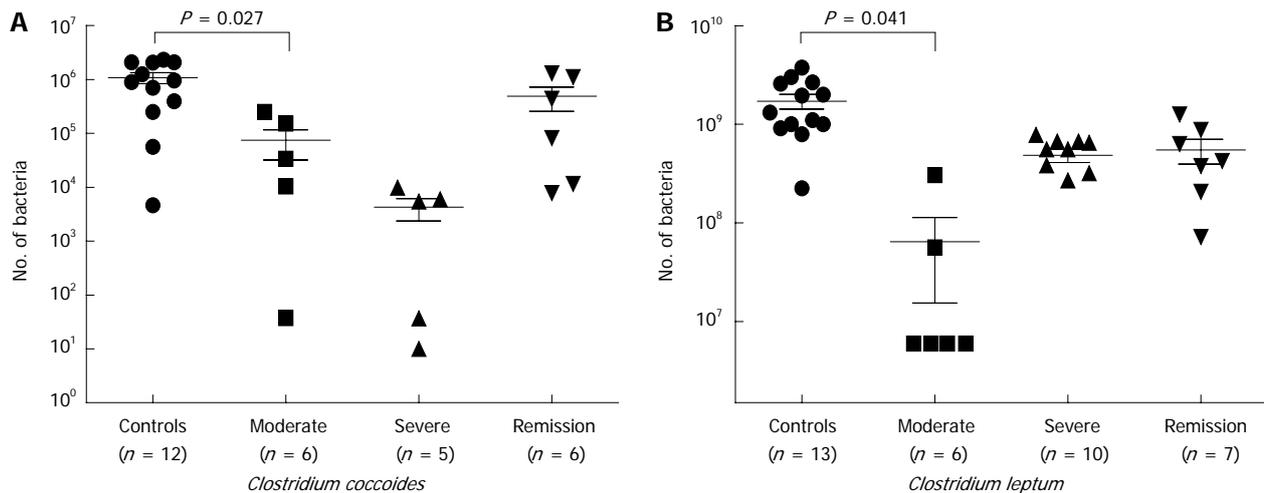
**Quantitative polymerase chain reaction:** In order to validate our FISH-flow cytometry data, we carried out a qPCR study (Figure 2A and B), where we observed significant decreases in both the members of *C. coccooides* group (*P* = 0.027) and *C. leptum* group (*P* = 0.041) in samples of moderate and severe disease stages. Members of both the clusters showed restoration of bacteria in

samples of remission category; however, this did not attain a significant *P* value.

**SCFA quantification by GC:** We further quantified the change in concentration of fecal SCFAs, namely acetate, *n*-butyrate and *iso*-butyrate, in control *vs* UC patient samples by GC. The concentrations of butyrate (*P* = 0.003), *iso*-butyrate (*P* = 0.044) and acetate (*P* = 0.047), were significantly reduced in severe UC samples when compared with control samples (Figure 3). As expected, during the remission stage *n*-butyrate level significantly restored back to normal level (*P* = 0.05) as seen in control individuals, confirming that the decrease observed during disease conditions reflects the loss of butyrate-producing bacteria (Figure 3C).

**Evaluation of predominant butyrate producers**

**FISH-flow cytometry:** Next, we evaluated the concentration of predominant members of both clostridial clusters (XIVa and IV). The population of *F. prausnitzii*, a member of the *C. leptum* group, was significantly low in UC samples of severe (*P* = 0.0001) category of disease in comparison to control samples (Figure 4A). Samples from the remission stage did not show significant restora-



**Figure 2** Quantitative polymerase chain reaction data showing the numbers of bacteria ( $\pm$  SE) in fecal samples of control vs ulcerative colitis patients. A: *Clostridium coccooides*; B: *Clostridium leptum*. The Y axis represents number of bacteria and X axis represents the sample category.

tion of *F. prausnitzii* in our FISH-flow cytometry experiment, as was observed for the *C. leptum* cluster during remission (Figures 1F and 2B).

*R. intestinalis*, a member of the *C. coccooides* group, was also significantly low ( $P = 0.02$ ) in patient samples of severe category disease (Figure 4B); however, this did not show significant restoration in the samples of remission stage. The population of *E. hallii*, another member of the *C. coccooides* group, decreased in disease conditions, but not significantly (Figure 4C). We failed to detect *E. hallii* in the majority of samples either by flow cytometry or qPCR, indicating low abundance of these bacteria in our study population. An increase in the population of *R. intestinalis* was recorded during remission stage in comparison to severe stage of the disease (Figure 4B and C). This was in agreement with our FISH-flow cytometry data of *C. coccooides* group. Abundance of *R. intestinalis*, *E. hallii* and *F. prausnitzii* was calculated out of total microbiota, as shown in Table 4. We detected a higher representation of *R. intestinalis* compared to *E. hallii* and *F. prausnitzii*. Therefore, we can infer that at the species level, abundance of the members of the *C. coccooides* cluster was higher in comparison to the members of the *C. leptum* cluster.

#### Quantitative polymerase chain reaction

As expected, the population of *F. prausnitzii* was significantly reduced in the samples of severe category of UC disease ( $P = 0.045$ ) (Figure 4D) as compared to control. In addition, abundance of the same species was restored at remission stage when compared to severe stage ( $P = 0.041$ ) (Figure 4D). Similarly, the *R. intestinalis* population showed significant reduction in samples of moderate ( $P = 0.015$ ) and severe ( $P = 0.001$ ) stage of the disease in comparison to control (Figure 4E). The recovery in the population of *R. intestinalis* was also seen in the remission category of samples when compared to severe category ( $P = 0.018$ ) (Figure 4E). However, qPCR analysis in the case of *E. hallii* did not show any significant reduction; in ad-

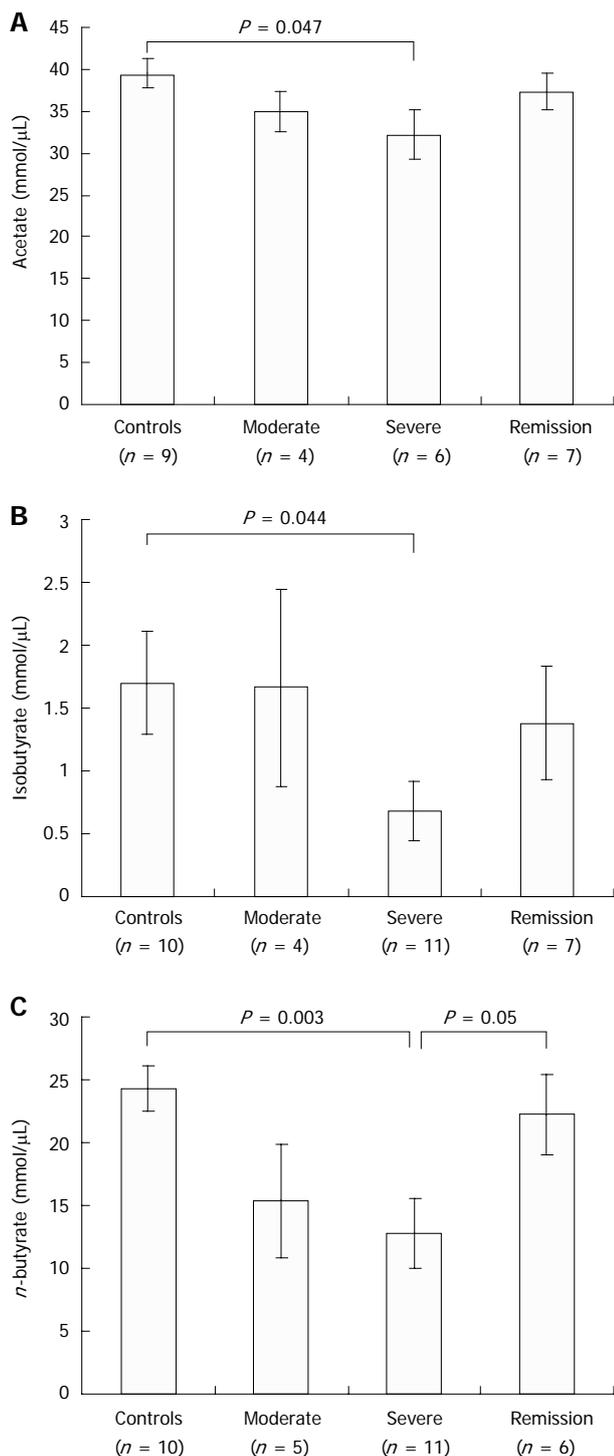
dition, *E. hallii* was undetected in the majority of samples observed by FISH-flow cytometry (Figure 4F).

## DISCUSSION

Our study revealed significant reduction in the members of both *C. coccooides* and *C. leptum* groups in fecal samples obtained from the severe disease category of UC patients in comparison to controls. FISH-flow cytometry and qPCR analysis of fecal samples belonging to the above groups supported the observations made by Takaishi *et al.*<sup>[4]</sup> and Sokol *et al.*<sup>[5]</sup>.

We quantified the abundance of predominant butyrate-producing species of clostridial clusters to see their association with acetate and butyrate. Since in most cases butyryl-CoA:acetate CoA-transferase rather than butyrate kinase appears to perform the final step in butyrate synthesis, we targeted bacteria possessing butyryl CoA:acetate CoA-transferase for butyrate synthesis, *e.g.*, *F. prausnitzii* and *Roseburia spp/E. rectale* which apparently lack butyrate kinase activity<sup>[15,28]</sup>. It is known that *Roseburia spp.* and *F. prausnitzii* strains contribute in butyrate production and many strains are associated with the net consumption of both acetate and carbohydrate<sup>[15]</sup>. Our study revealed that both the above species exhibited low abundance in the samples of severe category of UC and tended to restore their population during remission.

*F. prausnitzii* has been shown to exhibit anti-inflammatory effects on cellular and trinitrobenzenesulphonic acid-induced colitis models, partly due to secreted metabolites that are able to block NF- $\kappa$ B activation and interleukin 8 production<sup>[29]</sup>. In our observations (Table 4), *F. prausnitzii* accounted for 11.66%  $\pm$  1.55% of the *C. leptum* group, and *R. intestinalis* and *E. hallii* accounted for 14.48%  $\pm$  1.52% and 5.93%  $\pm$  0.54% of the *C. coccooides* group, respectively, in control individuals. In total these three species accounted for 6.83% of the total fecal flora. Previous studies have already reported low counts of *F. prausnitzii* in UC patients<sup>[30]</sup>.



**Figure 3** Short chain fatty acids in fecal samples of ulcerative colitis patients vs control samples, analyzed by gas chromatography. A: Acetate; B: Iso-butyrate; C: Butyrate. The Y axis represents concentration of respective short chain fatty acid and X axis represents the sample category.

We observed a significant decrease in concentrations of butyrate, iso-butyrate and acetate in UC fecal samples compared to controls through SCFA quantification by GC analysis, supporting earlier observations<sup>[4,31]</sup>.

An intriguing link between the level of SCFA and an intracellular energy sensor for the maintenance of intestinal barrier function has been suggested<sup>[10]</sup>. Our study

further substantiates a link between the fluctuations of butyrate production and the changes in the numbers of butyrate producers during UC. Lack of butyrate availability may lead to compromised intestinal barrier function resulting in increased exposure of luminal content to the host immune system, thus exaggerating the immune response<sup>[10]</sup>.

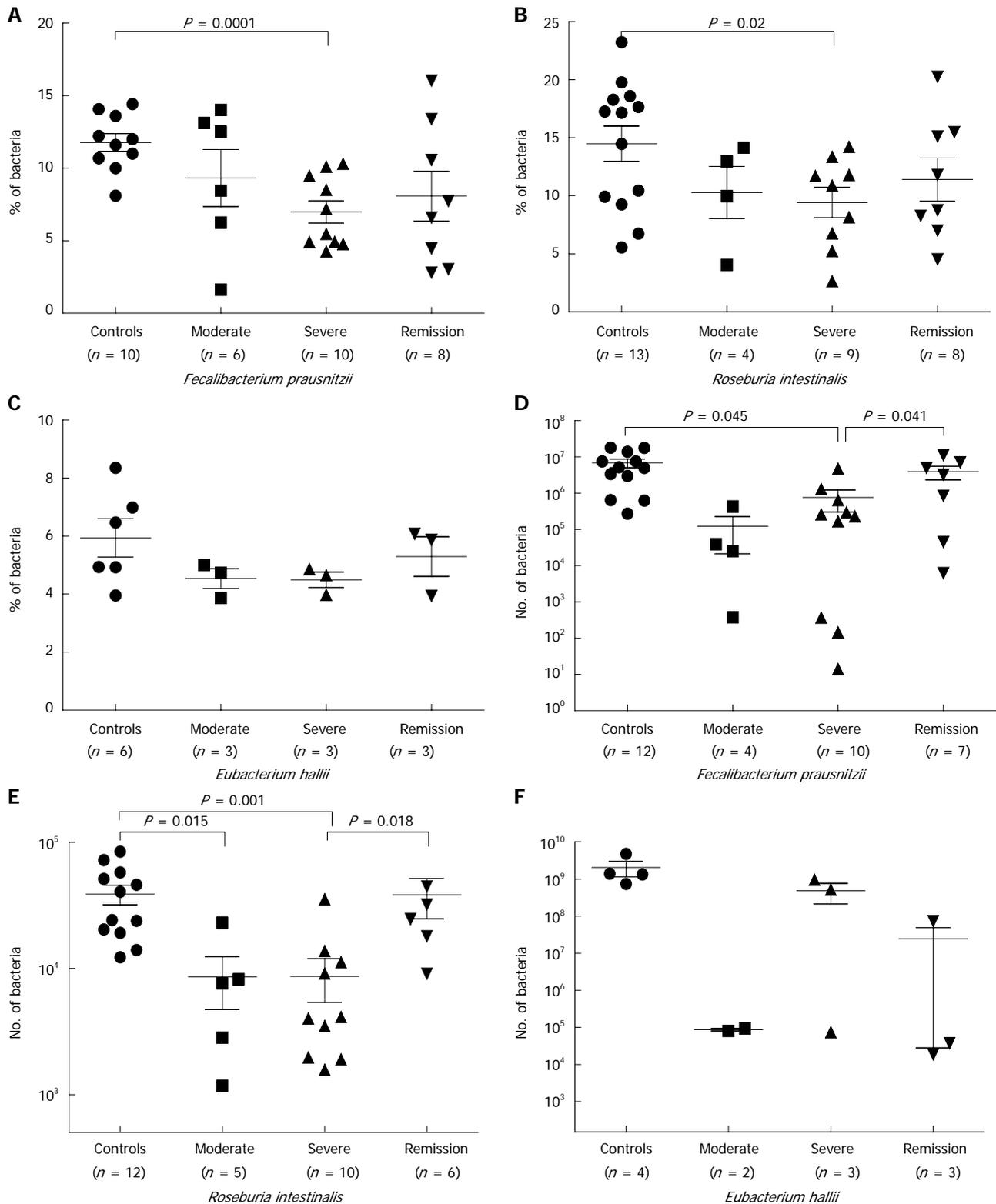
The reduced population of dominant butyrate producers and decreases in concentrations of butyrate and acetate in diseased samples indicate impaired butyrate supply in the colon, which may lead to energy deficiency for colonocytes. The resurgence of butyrate-producing bacteria at the remission stage and simultaneous increases in butyrate concentration, as observed here, show an association with UC and support the energy deficiency hypothesis of IBD<sup>[32]</sup>. Earlier studies have shown a requirement of acetate by *F. prausnitzii* and *R. intestinalis* for survival and other activities like production of butyrate<sup>[15,33]</sup>. Thus, major butyrogenic species depend on other bacteria, including net producers of acetate (*e.g.*, amyolytic bifidobacteria) and other bacterial species capable of degrading a variety of complex carbohydrates<sup>[15]</sup>. These bacteria play an important role in net butyrate production and may be associated with dysbiosis during UC.

Acetate and butyrate are major SCFAs and decreases in their concentration may affect the overall SCFA concentration. SCFAs in the colon maintain mild acidic conditions which boost the formation of butyrate by favoring growth of butyrate-producing bacteria and allowing them to compete against gram-negative bacteria such as *Bacteroides spp* to maintain the homeostasis<sup>[9]</sup>. Thus, a reduced butyrate level may lead to an increased Gram-negative bacteria population due to reduced competition. This was validated by our observation of increased representation of *Bacteroides* in UC samples (data not shown).

Our data showing reduced abundance of three predominant butyrate-producing species indicate that during UC, butyrate deficiency may not be solely due to reduced uptake of butyrate by the inflamed mucosa as reported by Canani *et al.*<sup>[9]</sup>, but also due to reduced abundance of predominant butyrate producers and conversely lower production of butyrate.

The limitation of our findings is that most of the microbial data are not presented in absolute values but in percentages; therefore, it is difficult to critically assess the real changes in the microbial composition. The study is performed in a very restricted number of subjects due to the exclusion criteria followed during sample collection.

In conclusion, our study shows a decreased abundance of predominant butyrate producers like *R. intestinalis* and *F. prausnitzii* belonging to clostridial clusters in the UC disease condition. This decrease was found to correlate with reduced SCFA concentration in UC patients. We thus provide evidence that reduced butyrate levels during the diseased state are due to less abundance of these species in UC. It is evident from our data that decreased levels of these species resulting in reduced butyrate levels may be associated with the etiology of UC.



**Figure 4** Analysis of predominant butyrate-producing bacteria. A-C: *Fecalibacterium prausnitzii* (A), *Roseburia intestinalis* (B), and *Eubacterium rectale* (C) by fluorescent *in situ* hybridization-flow cytometry; D-F: *Fecalibacterium prausnitzii* (D), *Roseburia intestinalis* (E), and *Eubacterium hallii* (F) by quantitative polymerase chain reaction. The Y axis represents number of bacteria and X axis represents the status of patients. Horizontal bars with asterisks represent comparison between ulcerative colitis and control conditions.

This was further demonstrated in our analysis of the samples from remission patients, where the targeted organisms tended to revert back to normal level. It would

be of interest in the future to extend this approach to studying the diversity of each member of the clostridial cluster, including changes in non-butyrate producers.

**Table 4** Enumeration of butyrate producers in fecal samples through fluorescent *in situ* hybridization-flow cytometry (mean  $\pm$  SE)

| Cluster or species                  | Out of total microbiota | Species                | Out of respective cluster |
|-------------------------------------|-------------------------|------------------------|---------------------------|
| Control fecal samples               |                         |                        |                           |
| <i>C. coccoides</i> cluster         | 25.69% $\pm$ 1.62%      | <i>R. intestinalis</i> | 14.48% $\pm$ 1.52%        |
|                                     |                         | <i>E. hallii</i>       | 5.93% $\pm$ 0.54%         |
| <i>C. leptum</i> cluster            | 13.74% $\pm$ 1.05%      | <i>F. prausnitzii</i>  | 11.66% $\pm$ 1.55%        |
| <i>R. intestinalis</i> <sup>1</sup> | 3.71% $\pm$ 0.39%       |                        |                           |
| <i>E. hallii</i> <sup>1</sup>       | 1.52% $\pm$ 0.13%       |                        |                           |
| <i>F. prausnitzii</i> <sup>1</sup>  | 1.60% $\pm$ 0.21%       |                        |                           |
| UC fecal samples (moderate)         |                         |                        |                           |
| <i>C. coccoides</i> cluster         | 12.70% $\pm$ 4.60%      | <i>R. intestinalis</i> | 10.00% $\pm$ 2.25%        |
|                                     |                         | <i>E. hallii</i>       | 4.03% $\pm$ 0.3%          |
| <i>C. leptum</i> cluster            | 6.40% $\pm$ 3.40%       | <i>F. prausnitzii</i>  | 7.5% $\pm$ 2.3%           |
| <i>R. intestinalis</i> <sup>1</sup> | 1.27% $\pm$ 0.10%       |                        |                           |
| <i>E. hallii</i> <sup>1</sup>       | 0.51% $\pm$ 0.01%       |                        |                           |
| <i>F. prausnitzii</i> <sup>1</sup>  | 0.48% $\pm$ 0.14%       |                        |                           |
| UC fecal samples (severe)           |                         |                        |                           |
| <i>C. coccoides</i> cluster         | 9.80% $\pm$ 2.40%       | <i>R. intestinalis</i> | 9% $\pm$ 1.83%            |
|                                     |                         | <i>E. hallii</i>       | 4.2% $\pm$ 0.2%           |
| <i>C. leptum</i> cluster            | 6.20% $\pm$ 1.80%       | <i>F. prausnitzii</i>  | 6.01% $\pm$ 1.6%          |
| <i>R. intestinalis</i> <sup>1</sup> | 0.82% $\pm$ 0.04%       |                        |                           |
| <i>E. hallii</i> <sup>1</sup>       | 0.41% $\pm$ 0.01%       |                        |                           |
| <i>F. prausnitzii</i> <sup>1</sup>  | 0.37% $\pm$ 0.02%       |                        |                           |
| UC fecal samples (remission)        |                         |                        |                           |
| <i>C. coccoides</i> cluster         | 12.99% $\pm$ 2.65%      | <i>R. intestinalis</i> | 11.40% $\pm$ 1.83%        |
|                                     |                         | <i>E. hallii</i>       | 5.07% $\pm$ 0.6%          |
| <i>C. leptum</i> cluster            | 7.89% $\pm$ 1.50%       | <i>F. prausnitzii</i>  | 7.40% $\pm$ 2.32%         |
| <i>R. intestinalis</i> <sup>1</sup> | 1.48% $\pm$ 0.04%       |                        |                           |
| <i>E. hallii</i> <sup>1</sup>       | 0.65% $\pm$ 0.01%       |                        |                           |
| <i>F. prausnitzii</i> <sup>1</sup>  | 0.58% $\pm$ 0.03%       |                        |                           |

<sup>1</sup>Abundance of *Roseburia intestinalis* (*R. intestinalis*), *Eubacterium hallii* (*E. hallii*) and *Fecalibacterium prausnitzii* (*F. prausnitzii*) were calculated out of total microbiota. *Clostridium coccoides* (*C. coccoides*) and *Clostridium leptum* (*C. leptum*) were enumerated as proportion of total bacterial cell hybridized with group-specific probe. *R. intestinalis*, *E. hallii* and *F. prausnitzii* were enumerated as proportion of cell hybridized with species-specific probe. UC: Ulcerative colitis.

## COMMENTS

### Background

Inflammatory bowel disease (IBD), comprising of Crohn's disease and ulcerative colitis (UC), is a class of chronic inflammatory disorders of the intestine and involves impaired barrier function. A rising trend in the incidence and prevalence of inflammatory bowel IBD in the Asian population has been recognized for the past two decades. IBD is considered as an exaggerated immune response, exerted either to all commensal bacteria or to a subset or to a single strain of bacteria.

### Research frontiers

Differences in fecal microbiota of healthy subjects and IBD patients have been identified. Commensal gut microbiota have been reported to exert various multiple beneficial effects on host gut epithelium. Short chain fatty acids (SCFAs), like butyrate, produced by microbial fermentation of undigested carbohydrates have been depicted to regulate transepithelial transport, colonocyte proliferation and differentiation, mucosal inflammation, intestinal motility and barrier function. The homeostasis between commensal microbiota and host defensive responses at the mucosal level has a pivotal role in the initiation and pathophysiology of chronic IBD. Therefore, monitoring the fluctuations in butyrogenic bacteria and SCFA level may help in understanding the relation of dysbiosis with UC.

### Innovations and breakthroughs

This work was carried out to study the fluctuations in butyrate-producing bacteria and simultaneous measurement of butyrate concentration in fecal samples of UC vs control individuals belonging to the northern part of India. The data were also correlated with patients differing in disease activity. Decreases in the level of butyrate producers in UC patients coincided with the changes in butyrate concentration during active stage disease. However, the levels reverted back to normal during remission stage showing the role of these bacteria in the disease etiology. Such comparisons have not been made so far to show the

above relationship with disease activity.

### Applications

It is speculated that by measuring the level of these species and concentration of SCFA in stool samples (a non-invasive method), authors can indicate the status of UC patients.

### Terminology

Dysbiosis (also called dysbacteriosis) refers to a condition with microbial imbalances on or within the body. Dysbiosis is most prominent in the digestive tract or on the skin, but can also occur on any exposed surface or mucous membrane such as the vagina, lungs, mouth, nose, sinuses, ears, nails or eyes. It has been associated with different illnesses, such as IBD, as imbalances in the intestinal microbiome may be associated with bowel inflammation and chronic fatigue syndrome.

### Peer review

This is a methodologically well performed, interesting report, addressing the potential role of gut commensal microbiota in the pathophysiology of UC.

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L- Editor Logan S E- Editor Zhang DN



## Disruption of interstitial cells of Cajal networks after massive small bowel resection

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Supported by Grants from the Program for Innovative Research Team of Shanghai Municipal Education Commission and Special Foundation of Shanghai Municipal Public Health Bureau, LJ06021; the National Natural Science Foundation of China, No. 30772270, 30972427; and the Scientific Foundation of Nantong University, No. 10Z046

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Received: December 15, 2012 Revised: February 22, 2013

Accepted: April 13, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To investigate the disruptions of interstitial cells of Cajal (ICC) in the remaining bowel in rats after massive small bowel resection (mSBR).

**METHODS:** Thirty male Sprague-Dawley rats fitting entry criteria were divided randomly into three experimental groups ( $n = 10$  each): Group A rats underwent bowel transection and re-anastomosis (sham) and tissue samples were harvested at day 7 post-surgery. Group B and C rats underwent 80% small bowel resection with tissue harvested from Group B rats at day 7 post-surgery, and from Group C rats at day 14 post-surgery. The distribution of ICC at the site of the resid-

ual small bowel was evaluated by immunohistochemical analysis of small intestine samples. The ultrastructural changes of ICC in the remnant ileum of model rats 7 and 14 d after mSBR were analyzed by transmission electron microscopy. Intracellular recordings of slow wave oscillations were used to evaluate electrical pace-making. The protein expression of c-kit, ICC phenotypic markers, and membrane-bound stem cell factor (mSCF) in intestinal smooth muscle of each group were detected by Western blotting.

**RESULTS:** After mSBR, immunohistochemical analysis indicated that the number of c-kit-positive cells was dramatically decreased in Group B rats compared with sham tissues. Significant ultrastructural changes in ICC with associated smooth muscle hypertrophy were also observed. Disordered spontaneous rhythmic contractions with reduced amplitude ( $8.5 \pm 1.4$  mV vs  $24.8 \pm 1.3$  mV,  $P = 0.037$ ) and increased slow wave frequency ( $39.5 \pm 2.1$  cycles/min vs  $33.0 \pm 1.3$  cycles/min,  $P = 0.044$ ) were found in the residual intestinal smooth muscle 7 d post mSBR. The contractile function and electrical activity of intestinal circular smooth muscle returned to normal levels at 14 d post mSBR (amplitude,  $14.9 \pm 1.6$  mV vs  $24.8 \pm 1.3$  mV; frequency,  $30.7 \pm 1.7$  cycles/min vs  $33.0 \pm 1.3$  cycles/min). The expression of Mscf and c-kit protein was decreased at 7 d ( $P = 0.026$ ), but gradually returned to normal levels at 14 d. The ICC and associated neural networks were disrupted, which was associated with the phenotype alterations of ICC.

**CONCLUSION:** Massive small bowel resection in rats triggered damage to ICC networks and decreased the number of ICC leading to disordered intestinal rhythmicity. The mSCF/c-kit signaling pathway plays a role in the regulation and maintenance of ICC phenotypes.

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**Key words:** Interstitial cells of Cajal; c-kit; Slow wave; Massive small bowel resection; Intestinal dysfunction

**Core tip:** Several gastrointestinal motility diseases are associated with altered numbers of interstitial cells of Cajal (ICC). Short bowel syndrome is also characterized by disordered intestinal motility immediately after surgery. We have investigated the alterations in numbers and functional changes of ICC that occur as a result of short bowel syndrome. In summary, our study showed modifications of the ultrastructure morphology of ICC, altered numbers of ICC and subsequent altered electrophysiological functional activity in the ileum after massive small bowel resection. However, the association between motility disorders and the changes of ICC should be further evaluated.

Chen J, Du L, Xiao YT, Cai W. Disruption of interstitial cells of Cajal networks after massive small bowel resection. *World J Gastroenterol* 2013; 19(22): 3415-3422 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3415.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3415>

## INTRODUCTION

Short bowel syndrome (SBS) is characterized by disordered intestinal motility immediately after surgery<sup>[1]</sup>. After massive small bowel resection (mSBR), the remaining bowel undergoes a compensatory process termed adaptation. During the process of adaptation, the small intestine smooth muscle cells undergo dramatic changes in gross morphology and ultrastructure. Adaptation is associated with the hypertrophy of smooth muscle, which is a physiological response to the increased functional requirement placed on the residual small bowel<sup>[2]</sup>. In addition, hypertrophy of smooth muscle tissue produces distinct motility disorders in the intestinal remnant, resulting in malabsorption and loss of nutrients because of diarrhea. Therefore, there is a critical need for further investigation of the mechanisms regulating resection-induced adaptation and potential targets that could be developed as therapeutic strategies. In a previous study, we found that resection-induced intestinal adaptation in a rat SBR model involved both the mucosal and intestinal smooth muscle layers. However, the mechanisms regulating adaptation remain unclear<sup>[3]</sup>.

Interstitial cells of Cajal (ICC) reside in the tunica muscularis of the gastrointestinal tract<sup>[4]</sup>. ICC play a crucial role in gastrointestinal motility in concert with the enteric nervous system, which is composed of both the myenteric (inter-muscular) plexus and the submucosal plexus<sup>[5]</sup>. ICC are present in organs containing smooth muscle tissue and are pacemaker cells that provide the basal electrical rhythm, which controls peristalsis in the gastrointestinal tract<sup>[6]</sup>. After receiving inputs from motor neurons, ICC generate and propagate electrical activity<sup>[7,8]</sup>. ICC are specialized cells in the gastrointestinal tract smooth muscle organs that express the c-kit receptor tyrosine kinase<sup>[9]</sup>. ICC can be identified by the expression

of CD117 (c-kit), which is a membrane receptor with tyrosine kinase activity<sup>[5,10,11]</sup>.

Intracellular signaling *via* c-kit plays a key role in the development and maintenance of the ICC phenotype and functional activity of ICC in the gastrointestinal tract<sup>[7,12]</sup>. Maintenance of the ICC phenotype requires membrane-bound stem cell factor (mSCF) produced locally within the tunica muscularis<sup>[13-16]</sup>. To date, remarkably few studies have investigated the functional changes of ICC that occur as a result of SBS. We hypothesize that the disruption to ICC activity involves the downregulation of the mSCF/c-kit signaling pathway following massive mSBR. In the current study, alterations in ICC phenotype and pacemaker activity were evaluated in a model of mSBR.

## MATERIALS AND METHODS

### *Experimental design and animal model*

Thirty male Sprague-Dawley rats weighing 250-300 g were obtained from the Experimental Animal Center of Shanghai Jiaotong University School of Medicine. All animals were housed in metabolic cages with free access to food and water and acclimated to their environment for 5 d before experimentation. Animals were maintained under standardized temperature, humidity and 12 h light-dark cycles. The rats were divided randomly into three experimental groups ( $n = 10$  each): Group A rats underwent bowel transection and re-anastomosis (sham); Groups B and C rats underwent 80% small bowel resection; Group A and Group B (SBS1W) bowel tissues were harvested at day 7 post-surgery; Group C (SBS2W) bowel tissues were harvested at day 14 post-surgery.

Animals were fasted for 16 h prior to laparotomy, and intestinal surgery was performed the following morning, as previously described<sup>[17]</sup>. All operative procedures were performed under anesthesia by intraperitoneal (*ip*) injection of pentobarbital sodium (30 mg/mL), which was administered at doses of 33-40 mg/kg body weight. Briefly, during surgery, the abdomen was opened by a midline incision and the ligament of Treitz and the ileal-cecal junction was identified and marked. For SBR rats, enterectomy was performed by removing approximately 80% of the small intestine, leaving approximately 10 cm of the terminal ileum and 5 cm of the proximal jejunum, which were anastomosed. For sham-operated control rats, the laparotomy and all surgical manipulations were the same as above, but the resection procedure was not carried out. All animals received fluid resuscitation by *ip* injection of saline (10 mL 0.9% NaCl) before the abdominal wall was closed and surgery concluded. After recovery, rats were transferred back to individual cages and given water *ad libitum* overnight, after which their regular diet was reinstated.

All experimental protocols were approved by the local Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the Science and Technology Commission of the People's Republic of China (STCC Publication No. 2, revised 1988).

### Immunohistochemistry

Immunohistochemical analysis was performed on tissue samples of smooth muscle at the same site of the ileum from both control and mSBR rats. The bowel was opened along the mesenteric border and the lumen contents were washed away with Krebs Ringer buffer. Segments of the bowel were pinned to the base of a Sylgard silicone elastomer dish and the mucosa was removed by sharp dissection. After dissection, the tunica muscularis were embedded in Tissue-Tek<sup>®</sup> OCT compound medium (Sakura Finetek United States, Inc., Torrance, CA, United States). The embedded tunica muscularis was cut at a thickness of 30  $\mu\text{m}$  with a freezing microtome (Leica Microsystems GmbH, Wetzlar, Germany) and stored at  $-20\text{ }^{\circ}\text{C}$  until use. The sections were fixed with iced acetone for 10 min. After endogenous peroxidase activity was quenched with 3% hydrogen peroxide, the sections were preincubated in 10% goat serum/0.2% Triton X-100/0.1 mol/L phosphate buffered saline (PBS) for 1 h at room temperature. The sections were incubated with antibodies against c-kit protein (1:50 dilution, rabbit polyclonal c-kit antibody, Santa Cruz Technologies, Santa Cruz, CA, United States) in 0.1 mol/L PBS containing 1% goat serum for 2 h. The sections were then incubated for 1 h at room temperature with biotinylated anti-rabbit immunoglobulin G (1:200; Vector Labs, Burlingame, CA, United States). Positive staining was visualized using an avidin-biotin-peroxidase complex system (Vectastain ABC Elite Kit, Vector Labs). The degree of expression of c-kit antibody was analyzed using image analysis software (Zeiss).

### Transmission electron microscopy

Tissue samples from intestinal smooth muscle at the same site of the ileum from both control and mSBR rats were fixed with 3% glutaraldehyde at room temperature. After fixation, the tissues were washed overnight in 0.1 mol/L sodium cacodylate buffer [6% sucrose and 1.25 mmol/L  $\text{CaCl}_2$  (pH 7.4)] at  $4\text{ }^{\circ}\text{C}$  and postfixed with 1% osmium tetroxide in 0.05 mol/L sodium cacodylate buffer (pH 7.4) at  $4\text{ }^{\circ}\text{C}$  for 2 h. The tissues were stained with saturated uranyl acetate for 3.5 h at room temperature, dehydrated in graded alcohol and embedded in Eponate 12 resin (Ted Pella, Inc., United States). The tissue was sectioned parallel and transverse to the long axis of the circular muscle layer. At suitable sites, 3  $\mu\text{m}$  sections were cut and stained with 2% toluidine blue. After examination of the toluidine blue stained sections, ultrathin sections of selected areas were obtained with the ultramicrotome using a diamond knife, mounted on 200-mesh grids, and stained with uranyl acetate and lead citrate. The grids were observed with a JEM1200EX electron microscope.

### Electrophysiological experiments

Following euthanasia, a 50 mm mid-segment of the remaining small bowel was removed and placed in Krebs solution (mmol/L; NaCl 117, KCl 4.7,  $\text{NaHCO}_3$  25,  $\text{KH}_2\text{PO}_4$  1.2,  $\text{MgSO}_4$  1.2, *D*-glucose 11,  $\text{CaCl}_2$  2.6). The bowel was opened along the mesenteric border, and the luminal contents were washed away with Krebs solution.

Segments of the bowel were pinned to the base of a Sylgard silicone elastomeric dish (Dow Corning, Midland, MI, United States), and the mucosa was removed by sharp dissection<sup>[18]</sup>. Strips of smooth muscle tissue (8 mm  $\times$  4 mm) were cut parallel to the longitudinal muscle oral to the site of the occlusion clips. The muscle was placed in a recording chamber with the submucosal aspect of the muscle facing upwards at  $37\text{ }^{\circ}\text{C}$  in an atmosphere of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . Cells were impaled with KCl-filled glass microelectrodes with resistances of 50-90 M $\Omega$ . Electrical responses were recorded and amplified through a high input impedance amplifier (SYS-773 Duo 773 Electrometer, WPI, United States). Experiments were performed in the presence of nifedipine (1  $\mu\text{mol/L}$ ; Sigma, St Louis, MO, United States) in order to reduce contractions and facilitate the extended period of cell impalement. Slow waves in mouse intestine have been previously shown to be unaffected by nifedipine<sup>[19]</sup>.

### Western blotting

Smooth muscle tissues were lysed in radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] for the detection of protein expression levels. The Bradford method (Pierce, Rockford, IL, United States) was used to determine protein concentrations. Tissue lysates (20  $\mu\text{g}$  of total protein per lane) were subjected to electrophoretic separation by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond, GE Healthcare Biosciences, Pittsburgh, PA, United States). Nonspecific binding was reduced by incubation of the membrane in 5% milk. Western blots were performed using antibodies directed against c-kit (1:200 dilution, rabbit polyclonal c-kit antibody, Santa Cruz Technologies, Santa Cruz, CA, United States), murine stem cell factor (mSCF) (1:200 dilution, mouse monoclonal SCF antibody, Santa Cruz Technologies), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:600 dilution, rabbit monoclonal antibody, CWbiotech company, Beijing, China). Alkaline phosphatase conjugated secondary antibodies (CWbiotech Company Beijing, China) were used to detect protein bands. TIFF images were captured by Adobe Photoshop and analyzed by Quantity one image software (NIH, MA, United States).

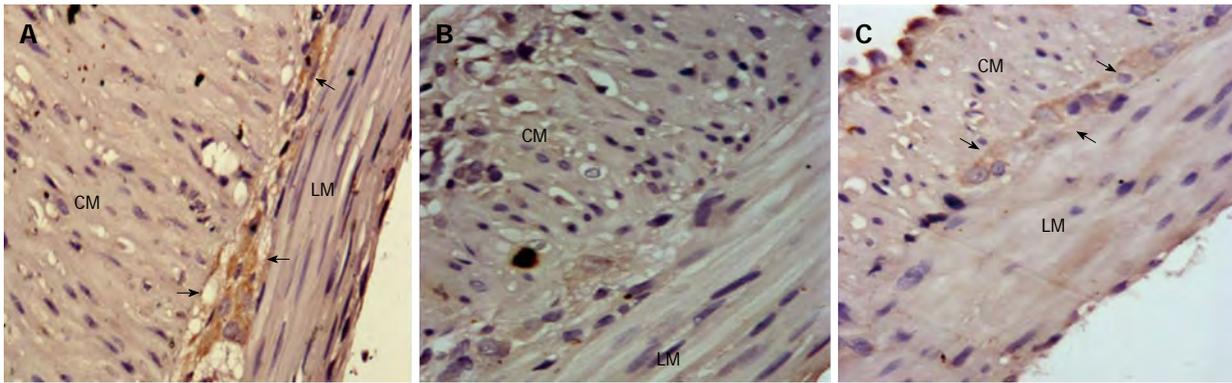
### Statistical analysis

The results are presented as mean  $\pm$  SE. Statistical differences between the groups were determined using a one-way analysis of variance with the SigmaStat program (SPSS, United States).  $P < 0.05$  was considered statistically significant.

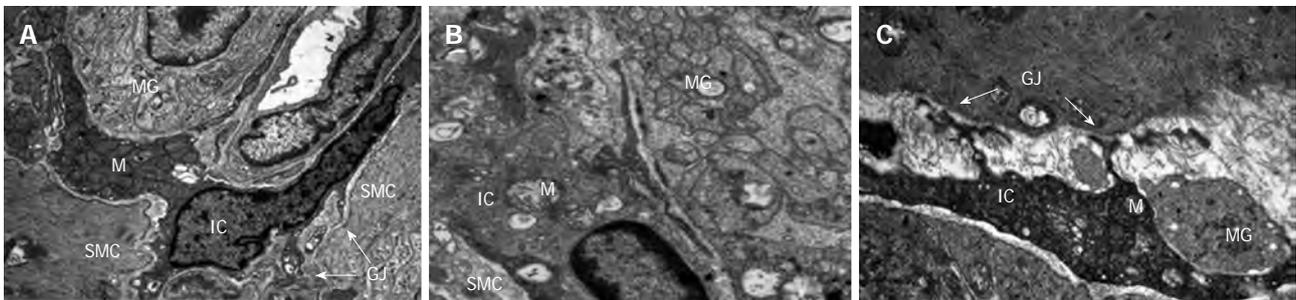
## RESULTS

### Distribution of c-kit(+) cells

Representative pictures of c-kit immunopositivity at the myenteric plexus are shown in Figure 1. In sham-operated rats, c-kit-positive cells were predominantly present



**Figure 1** c-kit immunopositivity at the level of the myenteric plexus in ileal whole-mount preparations. A: In the sham-operated group, c-kit-positive cells were predominantly present at the myenteric plexus level; B: Compared with sham-operated tissues, the number and density of c-kit immunopositive cells were significantly decreased in the short bowel syndrome (SBS) 1W group; C: In the SBS2W group, the number of interstitial cells of Cajal had returned to normal levels. The arrows showed c-kit immunopositivity is positive (original magnification  $\times 400$ ). LM: Longitudinal muscle; CM: Circular muscle.



**Figure 2** Ultrastructural morphological changes in interstitial cells of Cajal. A: At the ultrastructural morphological level, interstitial cells of Cajal (ICC) showed typical myofilaments and organelles of ICC, such as mitochondria and smooth sarcoplasmic reticulum in the sham-operated group; B: Ultrastructural changes of ICC were observed in the short bowel syndrome (SBS) 1W group, such as clear cytoplasm, sparse mitochondria, scarce smooth endoplasmic reticulum and a reduced number of contacts between nerves and ICC; C: In the SBS2W group, ultrastructural changes of ICC were ameliorated (original magnification  $\times 3750$ , 2.5 K). SMC: Smooth muscle cell; GJ: Gap junction; M: Mitochondria; MG: Myenteric ganglion.

at the myenteric plexus level. However, the number and density of c-kit immunopositive cells were significantly decreased in SBS1W rats ( $P < 0.05$ ) compared with sham tissues. However, after 2 wk, the number and density of ICC were clearly increased and approached sham levels, as indicated by c-kit positive cells (Figure 1).

### Ultrastructural morphological changes

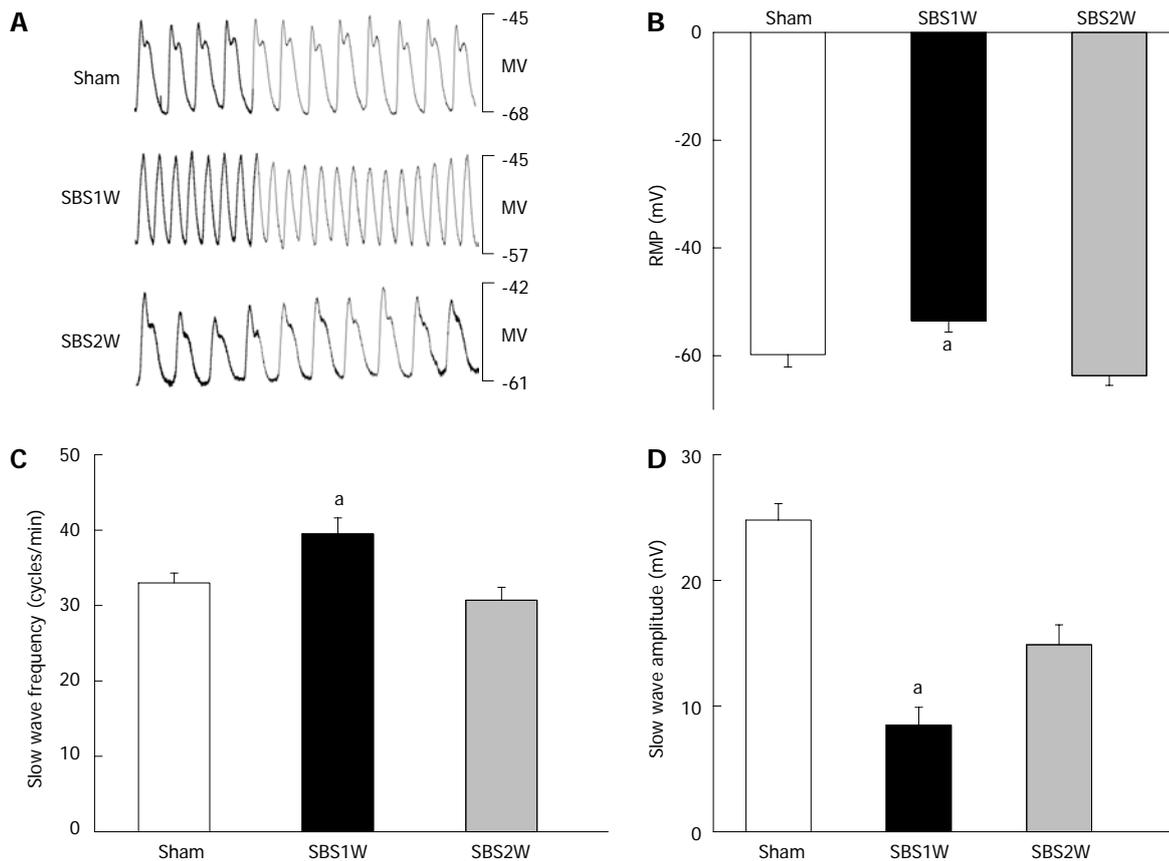
In sham rats, ultrastructural features of ICC in the small intestine were characterized by a less electron-dense cytoplasm and abundant mitochondria in sham rats (Figure 2A). However, the basal lamina or caveolae were not present. Intermediate filaments and thin filaments were apparent as thin processes. Along the length of overlapping processes, ICC predominantly formed large gap junctions between adjoining cells (Figure 2A). In certain areas, slender cytoplasmic processes of ICC were in close contact with a varicosity of the myenteric ganglion. Another part of the same cellular process was connected to a muscle cell *via* a gap junction, suggesting a functional relationship between these cell types.

Altered ICC ultrastructure was observed in the small intestine of SBS1W rats including clear cytoplasm, sparse mitochondria, scarce smooth endoplasmic reticulum,

and a reduced number of contacts between nerves and ICC (Figure 2B). The ultrastructural abnormalities of ICC in the small intestine of SBS2W rats exhibited some improvement compared to SBS1W rats, but the ultrastructural morphology had not returned to normal as observed in the sham-operated rats (Figure 2C).

### Electrophysiological studies

Electrophysiological studies were performed on circular muscles of the ileum in the remaining small bowel at day 7 or 14 following mSBR. The second component of slow waves was absent in SBS1W rats (Figure 3A). Circular muscle cells from the ileum of sham-operated rats ( $n = 7$ ) exhibited resting membrane potentials (RMP) averaging  $-63.7 \pm 1.8$  mV and slow waves of  $24.8 \pm 1.3$  mV in amplitude and a frequency of  $33 \pm 1.3$  cycles/min. The electrical activities of ileum circular muscle cells were markedly different in animals 7 d after mSBR. The slow waves had considerably reduced RMP and amplitude and increased in frequency (RMP,  $-53.5 \pm 2.1$  mV; amplitude,  $8.5 \pm 1.4$  mV; frequency,  $39.5 \pm 2.1$  cycles/min; Figure 3B-D, respectively;  $P < 0.05$ ) compared with sham-operated tissues. However, 14 d after mSBR, the electrical activity of the circular layer showed no significant chang-



**Figure 3** Electrical activity recorded from the small intestines (ileum) from sham-operated and short bowel syndrome rats. A: Slow waves in sham-operated group were biphasic, consisting of an upstroke and plateau component. Slow waves short bowel syndrome (SBS) 1W rats lacked an obvious secondary component; B: Resting membrane potentials (RMP) in each group were recorded, and a depolarized membrane potential appears to be a common feature of intestine smooth muscle in SBS rats; C: Slow wave frequency; D: Slow wave amplitude. <sup>a</sup> $P < 0.05$  vs sham group ( $n = 7$ ).

es ( $P > 0.05$ ) in RMP, amplitude and frequency (RMP,  $-59.8 \pm 2.3$  mV; slow wave amplitude,  $14.87 \pm 1.6$  mV; frequency,  $30.7 \pm 1.7$  cycles/min) compared with sham-operated tissues.

#### Expression levels of mSCF and c-kit are involved in the maintenance of the ICC phenotype and function after mSBR

In order to investigate the roles of mSCF and c-kit in the recovery process of the electrophysiological function of smooth muscle, we determined the expression of these two proteins in mSBR and sham rat intestinal smooth muscle tissues by Western blot (Figure 4A and C). The protein expression levels of mSCF and c-kit were normalized to the internal control GAPDH (Figure 4B and D). The protein expression levels of mSCF and c-kit were significantly decreased with mSBR in the SBS1W group ( $n = 4$ ;  $P < 0.05$ ). The protein expression levels returned to levels found in sham-operated rats by day 14 after mSBR, which coincided with the recovery of the electrical activities of ileal circular muscle cells.

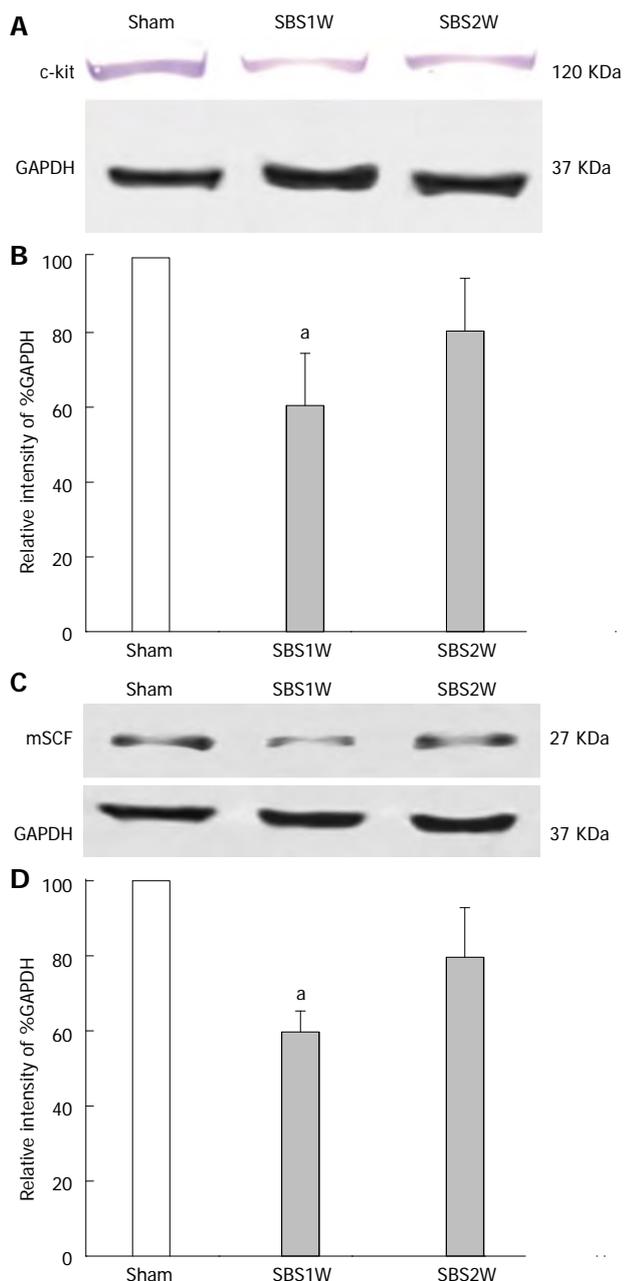
## DISCUSSION

After mSBR, adaptive alterations in the function of the remaining bowel are often accompanied by disorders of intestinal motility. However, the mechanisms regulating

the adaptation and motility disorders are not clear. A number of factors have been implicated in the pathogenesis of intestinal dysfunction, including changes in the number, density and ultrastructural morphology of ICC<sup>[11,20-22]</sup>. Alterations in the normal function of ICC have been reported in many intestinal disorders. In our previous studies, the unexpected finding of contractile dysfunction after mSBR prompted the investigation of whether intestinal dysfunction after mSBR was also mediated by ICC depletion<sup>[3]</sup>.

ICC are found between and within smooth muscle layers of the gastrointestinal tract from the esophagus to the internal anal sphincter<sup>[23,24]</sup>. ICC specifically express the proto-oncogene c-kit that encodes a receptor tyrosine kinase. *c-kit* expression can be clearly detected with the immunohistochemistry method and is a valuable tool for determining ICC structure, localization and distribution of cell networks.

Two separate functional groups of ICC exist in the lumen of the gastrointestinal tract: myenteric ICC (ICC-MY) and intramuscular ICC (ICC-IM). Networks of ICC-MY are located within the intermuscular space at the level of the myenteric plexus between the circular and longitudinal muscle layers. The plexus of ICC-MY, like the sino-atrial node, is the dominant pacemaker center that triggers the generation of slow waves, which are



**Figure 4** Changes in the expression profile of c-kit and membrane-bound stem cell factor in response to massive small bowel resection. A, C: The protein expression levels of c-kit and membrane-bound stem cell factor (mSCF) were downregulated in the first week after massive small bowel resection in the short bowel syndrome (SBS) 1W group and increased in the second week of the SBS2W group; B, D: Relative expression was determined by normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). <sup>a</sup> $P < 0.05$  vs sham group ( $n = 4$ ).

essential for orderly segmenting and peristaltic contractions in the tunica muscularis<sup>[25,26]</sup>. A second population of cells, ICC-IM, is localized within the muscle layers of the gastrointestinal tract and is innervated preferentially by enteric motor nerves. ICC-IM are closely associated with not only enteric motor nerves but also vagal afferent nerves. Elongated ICC-IM mediate efferent inputs to smooth muscle cells and the pacemaker apparatus, as well as relay afferent mechanical signals<sup>[27,28]</sup>.

We hypothesize that ICC depletion is central to the pathogenesis of many intestinal disorders. ICC are reduced or otherwise dysfunctional in several gastrointestinal tract dysmotilities, including achalasia, diabetic and idiopathic gastroparesis, mechanical ileus, intestinal pseudo-obstructions, slow-transit constipation, inflammations and malformations<sup>[29-31]</sup>. Rolfe *et al.*<sup>[30]</sup> reported that intestinal neuronal dysplasia was associated with loss or deficiency of ICC networks in the neonatal period. In addition, Chang *et al.*<sup>[32]</sup> reported that electrical slow waves were significantly disrupted and accompanied by disruption of ICC function and network in the obstructed ileum of mice.

In the current study, an absence or reduction in the number of ICCs was observed in the remaining bowel of an animal model of SBS. Utilizing intracellular recording of smooth muscle cells in isolated segments of the remnant ileal tissues, a reduction of rhythmic contractions and disruption of electrical slow waves were associated with the disruption of ICC and their network in the remnant ileum at 7 d in SBS rats. Slow waves are composed of two components: one produced by electrogenic propagation of driving potentials from the ICC-MY and the other formed by slow potentials from the ICC-IM<sup>[32,33]</sup>. In the current study, the frequency of slow waves changed in response to RMP changes as depolarization increased the frequency. Slow waves detected in SBS1W rats were lacking the secondary wave component compared with those in sham-operated and SBS2W rats. These differences suggested that alterations in the function of ICC account for the disruption of slow waves. This finding supports the concept that when ICC and their network were disrupted from the SBS ileum, the amplitude of slow waves decreased and the shape of slow waves and neural responses were lost due to the loss of ICC<sup>[12,32]</sup>.

Furthermore, c-kit signaling is essential for normal development of ICC and is required for long-term ICC survival and function<sup>[7,8]</sup>. This was first demonstrated by the blockade of c-kit signaling postnatally with an antagonistic anti-kit antibody. Inhibition of c-kit signaling resulted in a severe anomaly of gut motility with depletion of c-kit-positive ICC-MY cells in the myenteric regions of the small intestine<sup>[33]</sup>. Albertí *et al.*<sup>[34]</sup> also reported a deficient population of ICC in Ws/Ws rats harboring mutations in c-kit. In the gastrointestinal tract, c-kit expression on the cell surface of ICC is activated by mSCF produced by surrounding smooth muscle cells. Maintenance of ICC requires mSCF produced locally within the tunica muscularis. Another study reported a reduction in the content of mSCF in the stomach of non-obese diabetic mice<sup>[35]</sup>. These mice also exhibited marked depletion of ICC and a reduction in expression level of mSCF to one-third of that in normal mice. These studies support the hypothesis that reduced mSCF/c-kit signaling may underlie the decrease in ICC numbers and its network in obstructed mice<sup>[36]</sup>.

In our study, there was a reduction in mSCF/c-kit in the remnant ileum of SBS1W rats. The correlation

between the decrease in ICC numbers and reduction in mSCF/c-kit protein expression levels suggest that reduced mSCF/c-kit in the gastrointestinal tract may be involved in the disruption of ICC function in the remnant ileum of rats with SBS. Although previous work has shown that inflammatory diseases can induce the disruption of ICC and their network, the underlying mechanism by which the expression of mSCF in intestinal smooth muscle is decreased during SBS remains unclear. Additional studies are warranted to determine the role of mSCF and c-kit in the disruption of ICC.

In conclusion, the current study showed modifications of the ultrastructure morphology of ICC, phenotypic changes in ICC and subsequent altered electrophysiological functional activity in the ileum after mSBR, which may be associated with the mechanical alterations. In addition, the association between motility disorders after mSBR and the changes in ICC should be further evaluated.

## COMMENTS

### Background

Motility disorders are a prevalent condition observed in the residual small bowel after massive small bowel resection (mSBR). A number of factors have been implicated in the pathogenesis of intestinal dysfunction. A potential role for interstitial cells of Cajal (ICC) networks in intestinal disorders after mSBR has not previously been reported.

### Research frontiers

ICC are organized in distinct networks and serve several different functions. ICC generate the electrical slow wave and also set the smooth muscle membrane potential, are mechanosensors and modulate neuronal input to smooth muscle. Moreover, ICC play a protective role in gastrointestinal motility, although disruption of ICC networks are certainly not the only reason for intestinal dysfunction in some cases.

### Innovations and breakthroughs

The authors show that disruption of ICC networks is associated with intestinal dysfunction in rats after mSBR. This role of the ICC has not previously been reported and identifies a potential new therapeutic target intestinal disorders after mSBR.

### Applications

A greater understanding of mechanism of intestinal dysfunction after mSBR will help in utilizing its diverse effects more efficiently. Pharmacotherapy (such as glucagon-like peptide-2, which is supposed to improve the function of intestinal ileum in rats with short bowel syndrome) holds promise as an adjuvant treatment modality for short bowel syndrome.

### Terminology

Phenotypic changes in ICC refers to changes in the structure and function of ICC.

### Peer review

The authors investigate an attractive subject of Cajal network involvement in intestinal dysfunction after small bowel resection, using an experimental rat model. Experimental setup and methodology are appropriate and the study hypothesis and overall manuscript are well presented.

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## Human liver tissue metabolic profiling research on hepatitis B virus-related hepatocellular carcinoma

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Received: December 13, 2012 Revised: March 21, 2013

Accepted: April 3, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To select characteristic endogenous metabolites in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) patients and to identify their molecular mechanism and potential clinical value.

**METHODS:** An ultra performance liquid chromatography and linear trap quadrupole-Orbitrap XL-mass spectrometry platform was used to analyze endogenous metabolites in the homogenate of central tumor tissue, adjacent tissue and distant tissue obtained from 10 HBV-related HCC patients. After pretreatment with Mzmine software, including peak detection, alignment and normalization, the acquired data were treated with Simca-P+software to establish multivariate statistical analysis based on a pattern recognition technique and characteristic metabolites highly correlated with changing trends in metabolic profiling were selected and further identified.

**RESULTS:** Based on data acquired using Mzmine software, a principal component analysis model ( $R2X = 66.9\%$ ,  $Q2 = 21.7\%$ ) with 6 principal components and an orthogonal partial least squares discriminant analy-

sis model ( $R2X = 76.5\%$ ,  $R2Y = 93.7\%$ ,  $Q2 = 68.7\%$ ) with 2 predicted principal components and 5 orthogonal principal components were established in the three tissue groups. Forty-nine ions were selected, 33 ions passed the 2 related samples nonparametric test ( $P < 0.05$ ) and 14 of these were further identified as characteristic metabolites that showed significant differences in levels between the central tumor tissue group and distant tumor tissue group, including 9 metabolites (*L*-phenylalanine, glycerophosphocholine, lysophosphatidylcholines, lysophosphatidylethanolamines and chenodeoxycholic acid glycine conjugate) which had been reported as serum metabolite biomarkers for HCC diagnosis in previous research, and 5 metabolites (betasitosterol, quinaldic acid, arachidyl carnitine, tetradecanal, and oleamide) which had not been reported before.

**CONCLUSION:** Characteristic metabolites and metabolic pathways highly related to HCC pathogenesis and progression are identified through metabolic profiling analysis of HCC tissue homogenates.

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**Key words:** Hepatocellular carcinoma; Metabolomics; Characteristic metabolites; Potential biomarker; Ultra performance liquid chromatography-mass spectrometry

**Core tip:** An ultra performance liquid chromatography-mass spectrometry platform was used in the present study to identify characteristic metabolites in hepatitis B virus-related hepatocellular carcinoma tumor tissues. From an orthogonal partial least squares discriminant analysis model established to determine metabolic profiling in the central tumor tissue group, adjacent tissue group and distant tissue group, 49 ions were selected and 14 of these were identified as characteristic metabolites. The detection of these metabolites in tumor tissue not only confirmed the targeted traceability of previously reported serum biomarkers related to cancer diagnosis, but also provided novel targets for anticancer research.

Liu SY, Zhang RL, Kang H, Fan ZJ, Du Z. Human liver tissue metabolic profiling research on hepatitis B virus-related hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(22): 3423-3432 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3423.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3423>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is currently an important research topic in basic and clinical investigations due to its high malignancy, fast development, high mortality, complicated pathogenesis and significant individual differences. There have been a number of previous investigations on the molecular biology and proteomics of HCC<sup>[1-3]</sup>. However, the occurrence and development of this disease are not simply determined by innate genetic differences. The introduction of metabolomics has provided more information in HCC investigations. Metabolomics is the study of biological systems where changes in metabolites after specific stimulation or interference are determined<sup>[4-7]</sup>. This type of study focuses on the end products of biological systems, which are a reflection of both the physiological and biochemical status, and are affected by both genotype and environment and are close to biological characteristics. Thus it has the advantage of investigating the objective basis and generative mechanism of individual differences in metabolites.

Current metabolomic investigations in HCC are mainly focused on identifying characteristic metabolites in serum or urine, revealing changes in the metabolic network, and finally selecting potential biomarkers with clinical application and exploring pathologic mechanisms<sup>[8,9]</sup>. For example, Wu *et al.*<sup>[10]</sup> used gas chromatography/mass spectrometry (MS) to analyze metabolic profiling in 20 male HCC patients and 20 healthy male volunteers. One hundred and three ions were detected and 66 ions were identified. Following *t* test analysis, 18 metabolites were significantly different between the central tumor tissue group and the distant tumor tissue group ( $P < 0.05$ ). The ability to distinguish these 18 ions combined with alpha-fetoprotein was analyzed by the receiver operating characteristic curve (0.9275). This non-invasive method was considered quite promising. Tan *et al.*<sup>[11]</sup> also used metabolic analytical methods to select serum biomarkers for small HCC diagnosis. Metabolic profiling was performed in a diethylnitrosamine-induced rat HCC model, which is similar to human HCC in the histopathology of liver disease development. The alterations in three metabolites, taurocholic acid, lysophosphoethanolamine 16:0 and lysophosphatidylcholine 22:5, were reported to be correlated with disease progression and considered as potential biomarkers. In addition, serum metabolic profiling was performed in 262 HCC patients, 76 liver cirrhosis patients and 74 HBV patients. The results showed that the sensitivity and specificity of these ions all reached 80%, which was better than alpha-fetoprotein in distinguishing HCC-

liver cirrhosis. This research laid the foundation for the clinical application of metabolic biomarkers. At the same time, Soga *et al.*<sup>[12]</sup> research group reported that gamma-glutamyl dipeptides were used as biomarkers for liver disease diagnosis. The metabolic profiling of 248 serum samples, including patients with 9 types of liver disease, was performed using a capillary electrophoresis-MS platform. Gamma-glutamyl dipeptides were selected and found to be predictive of a decrease in glutathione. Multiple regression analysis showed that the selected biomarkers had the ability to distinguish different liver diseases.

In animal models, serum and urine analysis and sample collection are easy to perform, and HCC serum and urine metabolic profiling can reflect specific signs and reveal pathological mechanisms, however, the targeting ability of these analyses is still limited. The present study used a ultra performance liquid chromatography and linear trap quadrupole (UPLC-LTQ)-Orbitrap XL MS analytical platform to perform metabolic profiling analyses on homogenates of hepatitis B virus (HBV)-related HCC tumors removed by surgery. A metabolic profiling model was established and metabolic pathways highly related to HCC were characterized for further investigations on the genesis and progression of HCC and the potential clinical value of characteristic metabolites.

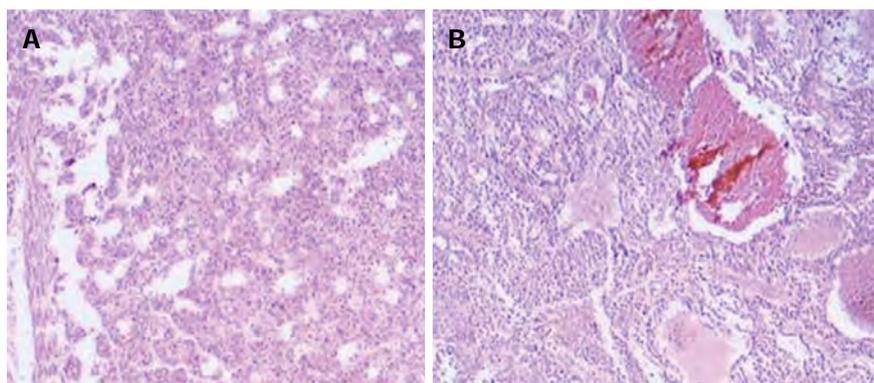
## MATERIALS AND METHODS

### Chemicals and instruments

All solvents were high performance liquid chromatography (HPLC) grade and used without modification. Formic acid and acetonitrile (ACN) were obtained from Merck (KGaA Merck, Germany). Distilled water was produced using a Milli-Q Reagent Water System (Millipore, Billerica, MA, United States). All standard [*L*-phenylalanine, glycerophosphocholine, chenodeoxycholic acid glycine conjugate and lysophosphatidylcholine (lysoPC) (18:0)] preparations were purchased from Sigma-Aldrich (St. Louis, MO, United States). Ultra performance liquid chromatography was performed on a Thermo Fisher Accela system (Thermo Fisher Scientific, Franklin, MA, United States). MS was performed on a Thermo Fisher (Thermo Fisher Scientific, Franklin, MA, United States) LTQ Orbitrap XL hybrid mass spectrometer. Other equipment included a Multifuge X1R high-speed centrifuge (Thermo Fisher Scientific, United States).

### Sample collection

The 10 HBV-related HCC patients included in this study were all from the Department of Hepatobiliary Surgery, Tianjin Third Central Hospital, and included 5 females and 5 males with average age of  $54.2 \pm 9.2$  years. All patients voluntarily joined this study and gave informed consent. Tissue from the central area of the tumor, adjacent tissue (1-2 cm from the tumor) and distant tissue (5 cm from the tumor) were collected. Thirty samples (10 samples in each group) were washed in hypothermic normal saline, dried using neutral filter paper and then stored at  $-80^{\circ}\text{C}$ .



**Figure 1** Pathological image of hepatocellular carcinoma tissues (hematoxylin-eosin staining,  $\times 100$ ). A: Moderately differentiated hepatocellular carcinoma (HCC), cancer emboli were observable in vessels; B: Moderately differentiated HCC, focal carcinoma tissue was observed in adjacent liver tissue.

Collection of samples was performed according to the following instructions: (1) all 10 patients had chronic persistent HBV (no other hepatotropic virus infection, no long-term drinking behavior, no autoimmune hepatitis, no schistosomiasis infection and no genetic metabolic liver disease); (2) all 10 patients had solitary tumors  $< 5$  cm in diameter, and Barcelona Clinic Liver Cancer stage A1; (3) tissue pathological immunohistochemistry examination diagnosed HCC using hepatitis B surface antigen (+); the pathological section is shown in Figure 1. Samples were all HCC grade II-III differentiation. Invasive growth was detected using the appropriate specific stain. No cancer cells were detected at the incisional edge; (4) no radiotherapy or chemotherapy was performed before surgery; (5) no evidence of endocrine or metabolic disease; (6) renal function, liver function, blood routine and hydropower solution pH were all in the normal range; (7) no severe infection was detected and parenteral nutrition was used; and (8) patients' dietary requirements were managed by the Nutrition Department of Tianjin Third Central Hospital to a relatively uniform standard, as a result, exogenous dietary influence on metabolic profiling was limited to the lowest level.

#### Sample pretreatment

Samples were thawed at room temperature and then weighed, 1:3 ultrapure water was added to each sample according to their weight before being homogenized. Tissue homogenate was subjected to ultrasonication at  $130\text{ W} \times 60\%$  for 3 cycles. One cycle included 10 s of ultrasonication and a 10 s interval (ultrasonication was performed in an ice-bath). Homogenate (400  $\mu\text{L}$ ) was centrifuged (4  $^{\circ}\text{C}$ , 15000  $g$ , 30 min). Supernatant (100  $\mu\text{L}$ ) was mixed with 400  $\mu\text{L}$  methanol to precipitate proteins. The mixture was centrifuged at 4  $^{\circ}\text{C}$ , 15000  $g$  for 30 min. The supernatant was filtered using a 0.22  $\mu\text{m}$  membrane. Quality control (QC) was the equivalent volume mixture in each sample.

#### Sample analysis

Chromatography conditions were as follows: Chromatography was performed on a Thermo Fisher Accela system

(Thermo Fisher Scientific, Franklin, MA, United States) which was equipped with a binary solvent delivery manager, and a sample manager. The analytical column was a Thermo Hypersil GOLD (2.1 mm *id*  $\times$  50 mm 1.9  $\mu\text{m}$ ) C18 reversed phase column.

**Mobile phase:** Phase A: 0.1% formic acid (volume ratio), 1 mL of formic acid was added to a 1 L bottle of HPLC-grade water; Phase B: 95% ACN and 0.1% formic acid. And 950 mL ACN, 50 mL HPLC-grade water, and 1 mL formic acid were combined.

**Chromatographic separation:** Chromatographic separation was performed isocratically within 15 min and the injection volume was 10  $\mu\text{L}$ . The flow rate was set at 200  $\mu\text{L}/\text{min}$ . The sample manager and column oven temperature were set at 4  $^{\circ}\text{C}$  and 20  $^{\circ}\text{C}$ , respectively. The chromatographic elution gradient was initialized at 5% Phase B and held for 3 min. In consecutive 10 min periods, Phase B was gradually escalated to 50%, and then a rapid increase in Phase B to 95% was completed within 3 min. After 4 min of maintaining the high volume of organic phase gradient, Phase B was immediately reduced to 5% and this elution gradient was used to balance the analytical column for the final 4 min.

MS was performed on a Thermo Fisher (Thermo Fisher Scientific, Franklin, MA, United States) LTQ Orbitrap XL hybrid mass spectrometer<sup>[13]</sup>, operating in the positive ion mode with an ion source voltage of 4.5 kV, a capillary voltage of 30 V, cone voltage of 150 V, desolvation temperature of 275  $^{\circ}\text{C}$ , sheath gas flow of 30 arb and assistant gas flow of 5 arb (99.999% nitrogen). Data were collected over 15 min in centroid mode over the mass range 50-1000  $m/z$ . The MS resolution was at 100000 full width half maximum (FMHW) and the calibration standards were provided by Thermo Fisher Scientific (caffeine, Ultramark 1621 and *L*-methionyl-arginyl-phenylalanylalanine acetate  $\text{H}_2\text{O}$ ). MS/MS analysis was carried out with collision-induced dissociation (CID) collision energy 35 (normalization collision energy) and the collision gas was 99.999% helium.

There were 14 QC samples throughout the test (equal

volume mixture of each analyzed sample). Before the samples were tested, 8 QC samples were analyzed continuously and the remaining QC samples were inserted into the sequence after every 5 samples were analyzed.

The sequence of samples was randomly generated by the excel function before and after sample analysis (including QC), and cross-contamination was avoided by inserting a blank between adjacent samples. The whole experiment lasted 780 min.

### **Ethics statement**

All samples were collected in accordance with the ethical guidelines and written consent protocols mandated by the Tianjin Third Central Hospital. The Institutional Review Board approved the collection of serum for comprehensive metabolite characterization. All patients and all control individuals were approached using approved ethical guidelines and those who agreed to participate in this study, were required to sign consent forms. Patients could refuse entry, discontinue participation, or withdraw from the study at any time without prejudice for further treatment or management. All participants provided written consent.

### **Statistical analysis**

MZmine 2.0 was used for peak detection, alignment and normalization. The filter conditions were: chromatography peak intensity signal/noise > 30, retention time tolerance =  $\pm 0.1$  min, and m/z tolerance =  $\pm 0.01$ .

One of the aims of this study was to establish a model to predict the tissue metabolic profile in HCC. With the help of Simca-P+12.0.1.0 (Umetrics, Sweden), software based on chemometrics methods, the OPLS-DA supervised model was established. The detailed process was as follows: The variables were firstly traded by Pareto scaling. The principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) model of all the samples were established and checked by cross validation<sup>[14,15]</sup>. The preliminary selection of characteristic metabolites was accomplished using the corresponding variable importance (VIP) value, confidence interval and coefficient plot generated by the OPLS-DA model. The selected metabolites were then preliminarily confirmed by the S and SUS diagram. Finally, the variances were evaluated by SPSS 16.0 software (SPSS, United States), using the 2 related samples non-parametric test.

## **RESULTS**

### **Data pretreatment and QC analysis**

The total ion chromatogram of the central tumor tissue group, adjacent tissue group and distant tissue group acquired by the UPLC-MS platform are shown in Figure 2. After pretreatment and standardization using MZmine 2.0, there were 211 integral peaks following extraction ion chromatography detected in QC samples and 215 peaks in test samples.

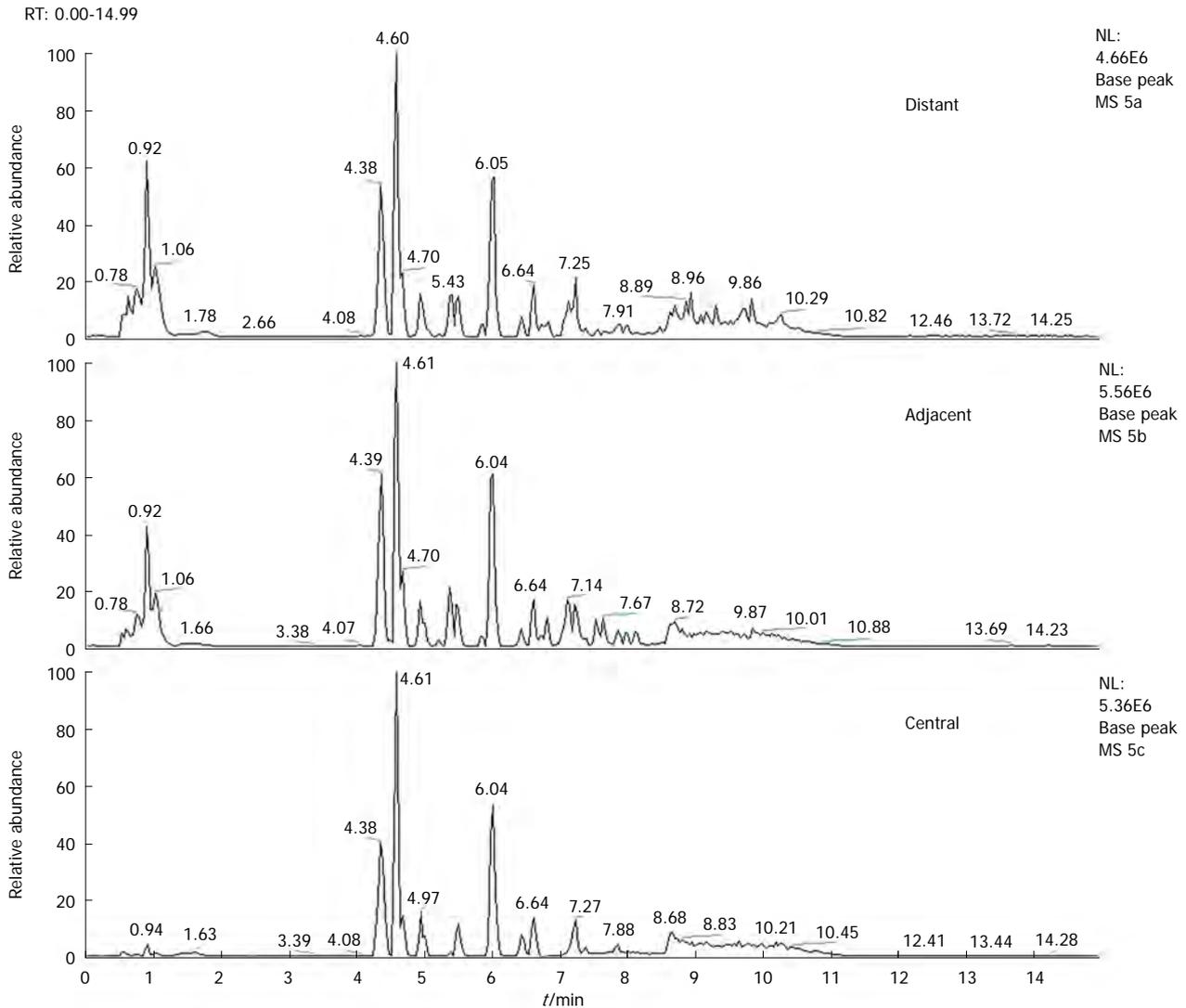
The stability of the UPLC-MS system was adequately assessed by the analysis of QC samples during the entire experimental period<sup>[16]</sup>. Through PCA of 14 data sets of QC samples, a PCA model with 2 principal components was established (Figure 3). Figure 3 shows the score plot of QC sample sequence versus first principal component (the most influential factor which varied with time). From the QC principal component score plot we found that the UPLS-MS system was stable after the first 8 continuous QC injections. In the test sample sequence, a QC sample was inserted after every 5 test samples to evaluate the stability of the system during the entire analytical process. The results showed that the detection system was stable throughout the experiment after the first 8 QC samples were injected (no outliers exceeding  $\pm 2$  SD were detected in the QC samples). According to a related article<sup>[17]</sup>, the QC standard was set as follows: (1) ion peaks were defined as reliable peaks when their intensity was in the range of  $\pm 30\%$  average ion intensity; (2) a QC sample was qualified if its 70% ion peaks were reliable; and (3) experimental data were accepted only when 60% QC was qualified. In the present experiment, 11 of 14 QC samples (reliable ion peaks distributed among 70.5%-86.3%) inserted into the test sample sequence qualified and the qualified ratio was 78.5%, which meant that the analytical results were trusted.

### **Ability of the metabolic profile to distinguish disease states**

A PCA model with 6 principal components was established (R2X = 66.9%, Q2 = 21.7%) and the score plot of its first two principal components is shown in Figure 4A. It can be seen that the central tumor tissue group and distant tissue group showed a clustering tendency in the direction of first predictive principal component (X axis). The adjacent tissue group was located between the central tumor tissue and distant tissue groups. Although the clustering tendency was not significant, its distribution could assess the development of HCC. An OPLS-DA model was established using all 30 samples. The model had 2 predictive principal components and 5 orthogonal principal components (R2X = 76.5%, R2Y = 93.7%, Q2 = 68.7%). As shown in Figure 4B, the score plot of the first predictive principal component and first orthogonal principal component showed significant clustering tendency. In the X axis direction, the changing tendency of metabolic profiling in the three groups reflected the development of HCC, at the same time differences between the three groups in metabolic profiling were significant. As a result, it was concluded that the first principal component could reflect the development of HCC.

### **Selection of characteristic metabolites**

Through metabolic profiling analysis of the homogenates of HCC tumor tissue, endogenous metabolites highly related to HCC were detected. The main focus of the present study was the metabolites which had an obvious impact on clustering tendency of the central tumor tissue group and distant tissue group. First, the OPLS-DA



**Figure 2 Total ion chromatogram of tissue metabolic profiling.** This was the total ion chromatogram of one single sample chosen randomly. Distant: Distant tissue group; Adjacent: Adjacent tissue group; Central: Central tumor tissue group; RT: Retention time.

model was established for the central tumor tissue group and distant tissue group (Figure 5A), which had 1 predictive principal component and 3 orthogonal principal components ( $R^2Y = 97.6\%$ ,  $Q^2 = 83.8\%$ ). The characteristic metabolites with the ability to distinguish disease were selected in the established OPLS-DA model after the following two processes: (1) Among ions with  $VIP > 1$ , excluded ions which included zero in the confidence interval in the VIP diagram (Figure 5B) and excluded ions which included zero in the confidence interval in the coefficient plot (Figure 5C, excluded ions were marked by black arrows)<sup>[18]</sup>, and (2) From the S-plot (Figure 5B), ions with a high degree of variation (high horizontal ordinate value) and reliability (high vertical ordinate value) were selected. After these two steps, 14 ions were selected. To limit the cover-up effect, a “blank” OPLS-DA model was established which excluded the 14 selected ions. The OPLS-DA model had 1 predictive principal component and 3 orthogonal principal components ( $R^2Y = 97.1\%$ ,  $Q^2 = 74.2\%$ ). Because the ability of the “blank”

model to distinguish disease was still high, other characteristic metabolites were selected following the two steps previously mentioned, and 35 ions were selected. Among the total 49 ions selected, 33 ions passed the 2 related samples nonparametric test ( $P < 0.05$ ).

#### Identification of characteristic metabolites

Some characteristic metabolites were identified by MS graphs when compared with the chromatographic peaks and mass spectrographic peaks of the standard (including MS1 and MS2). Identification of other selected ions was performed as follows: Firstly, because of the high resolution of the Orbitrap XL mass spectrometer (resolution set as 100000 FMHW), characteristic metabolites were preliminarily identified by checking accurate  $m/z$  on the Human Metabolome DataBase (<http://hmdb.ca/>). The matching metabolites were retained for further identification according to the rules that  $m/z$  deviation was below 0.01, with equal charge number and with suitable ionization mode. Secondly, after MS/MS scanning, MS2 graphs

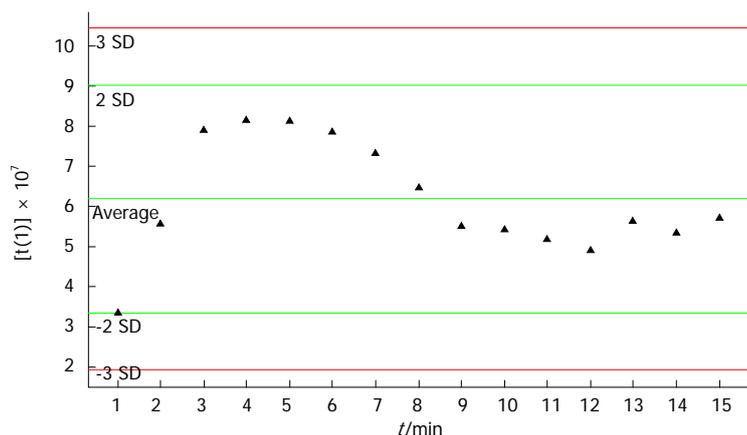


Figure 3 Score plot of principal component [t(1)] for quality control principal component analysis model. Each point represents a quality control sample.

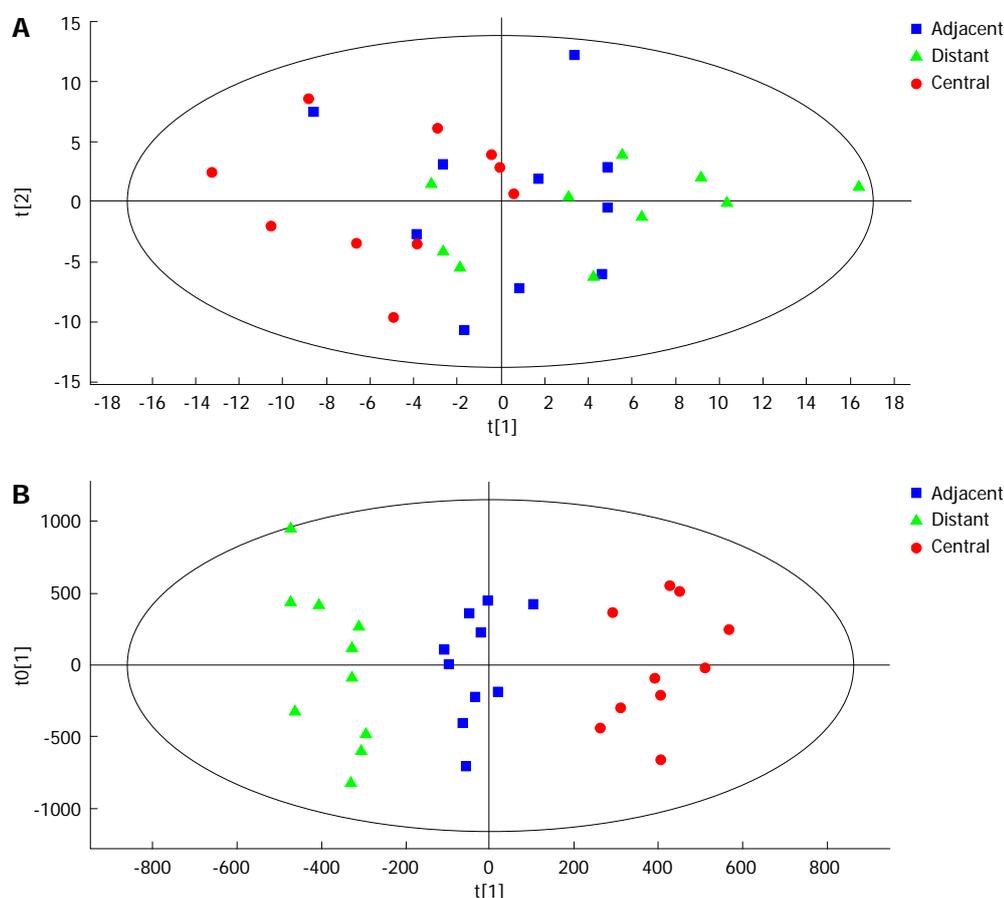


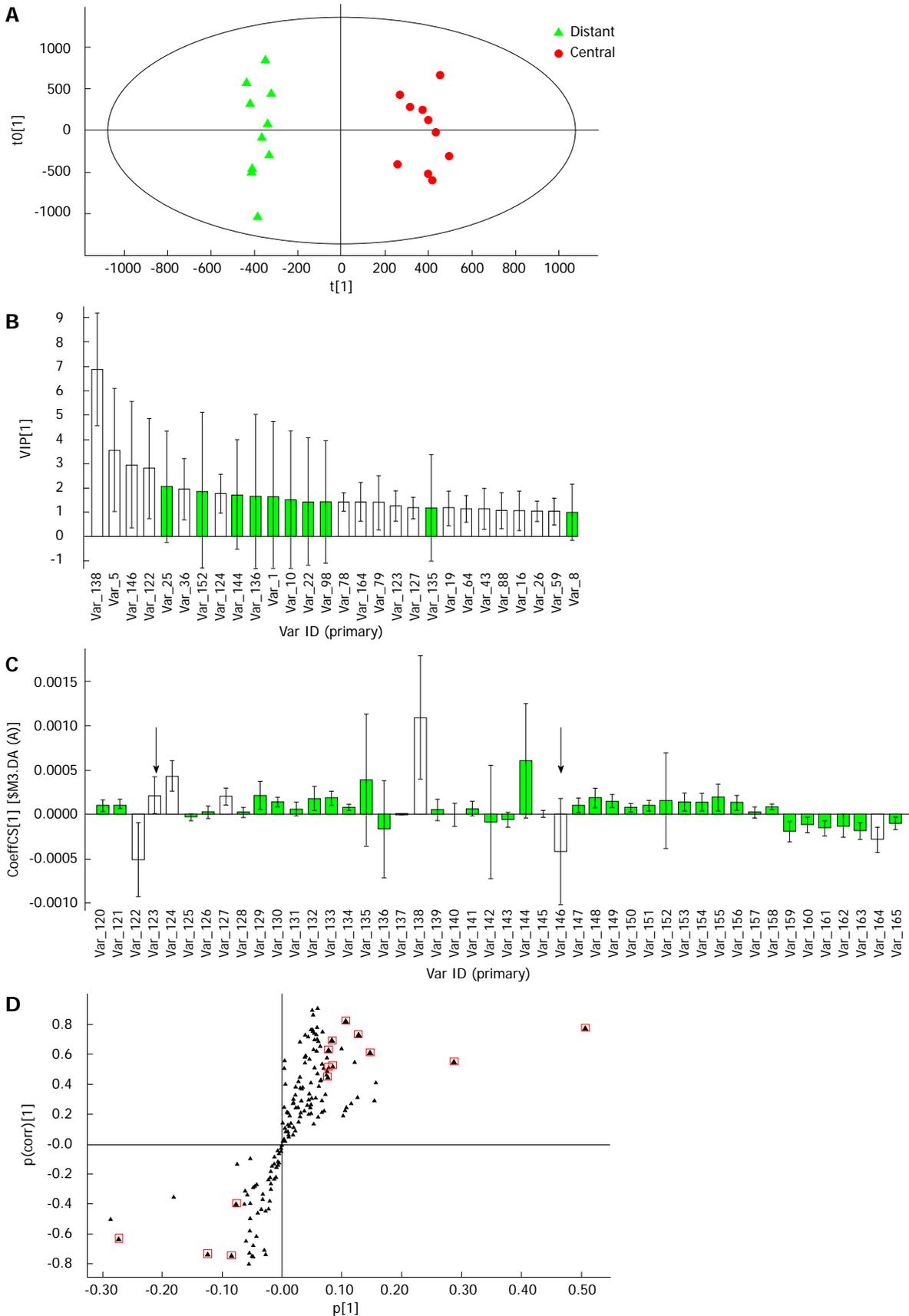
Figure 4 Ability of metabolic profiling to distinguish disease in tissue samples. A: Score plot of the first two components [t(1)]/[t(2)] of the tissue metabolic profiling principal component analysis model; B: Score plot of metabolic profiling orthogonal partial least squares discriminant analysis model. Each point in the figure represents a sample. Distant: Distant tissue group; Adjacent: Adjacent tissue group; Central: Central tumor tissue group.

of the characteristic ions were obtained and then compared with theoretical fragments of previous preliminary results according to the rules that MS2 *m/z* deviation was below 0.2, matching the top three peaks and at least 80% of secondary MS graphs (secondary MS fragments were generated by the ion trap CID model). The theoretical fragments were derived using Mass Frontier 6.0. The final identified results and statistical differences among

the three groups are shown in Table 1 (only 14 identified metabolites are listed).

## DISCUSSION

HCC, the fifth commonest cancer and the third most common cause of cancer-related death, accounts for 6% of all cancers worldwide<sup>[19,20]</sup>. Fifty percent of diagnosed



**Figure 5** Selecting process of characteristic metabolites. A: Orthogonal partial least squares discriminant analysis model of the central tumor tissue group and distant tissue group, each point in the figure represents a sample; B: Variable importance (VIP) diagram with confidence interval (green bars were excluded metabolites, the others were target metabolites); C: Coefficient plot of included variants in the VIP diagram (variants marked by arrows were excluded metabolites); D: S-plot of selected ions passed last two steps of filtering (points marked by red frame represent characteristic metabolites). Distant: Distant tissue group; Central: Central tumor tissue group.

**Table 1** Metabolite identification results

| m/z     | Retention time (min) | Metabolite   | Adduct <sup>2</sup>      | Content <sup>3</sup> |                   |                   |
|---------|----------------------|--|--------------------------|----------------------|-------------------|-------------------|
|         |                      |  |                          | Normal/adjacent      | Adjacent/tumor    | Normal/tumor      |
| 453.345 | 4.39632              | Sitosterol-β   | M + K [1+]               | Up <sup>a</sup>      |                   | Up <sup>a</sup>   |
| 166.086 | 1.05638              | <i>L</i> -phenylalanine <sup>1</sup>                 | M + H [1+]               |                      | Up <sup>a</sup>   | Up <sup>b</sup>   |
| 520.341 | 6.84401              | LysoPC [18:2 (9Z, 12Z)]                              | M + H [1+]               |                      | Up <sup>a</sup>   | Up <sup>b</sup>   |
| 258.111 | 0.77819              | Glycerophosphocholine <sup>1</sup>                   | M + H [1+]               |                      | Up <sup>b</sup>   | Up <sup>b</sup>   |
| 476.276 | 7.10121              | LysoPE [18:3 (9Z, 12Z, 15Z)/0:0]                     | M + H [1+]               |                      | Up <sup>a</sup>   | Up <sup>b</sup>   |
| 450.322 | 5.88844              | Chenodeoxycholic acid glycine conjugate <sup>1</sup> | M + H [1+]               |                      | Up <sup>b</sup>   | Up <sup>b</sup>   |
| 568.342 | 6.78248              | LysoPC [22:6 (4Z, 7Z, 10Z, 13Z, 16Z, 19Z)]           | M + H [1+]               |                      | Up <sup>a</sup>   | Up <sup>b</sup>   |
| 212.02  | 5.54796              | Quinaldic acid                                       | M + K [1+]               | Up <sup>b</sup>      |                   | Up <sup>a</sup>   |
| 456.406 | 8.78787              | Arachidyl carnitine                                  | M + H [1+]               |                      | Down <sup>a</sup> | Down <sup>b</sup> |
| 504.308 | 7.89672              | LysoPE (18:0/0:0)                                    | M + Na [1+]              |                      | Up <sup>a</sup>   | Up <sup>a</sup>   |
| 546.357 | 7.08546              | LysoPC (18:0) <sup>1</sup>                           | M + Na [1+]              |                      | Up <sup>a</sup>   | Up <sup>b</sup>   |
| 566.323 | 6.82403              | LysoPC [20:4 (5Z, 8Z, 11Z, 14Z)]                     | M + Na [1+]              |                      | Up <sup>b</sup>   | Up <sup>b</sup>   |
| 230.249 | 6.09076              | Tetradecanal   | M + NH <sub>4</sub> [1+] |                      | Down <sup>b</sup> | Down <sup>b</sup> |
| 282.28  | 9.02044              | Oleamide   | M + H [1+]               | Down <sup>b</sup>    |                   | Down <sup>b</sup> |

<sup>1</sup>Metabolites identified by standard comparison; <sup>2</sup>Ionospheric models of mass spectrometry cationic scanning; <sup>3</sup>Comparison of characteristic metabolites' integral peak area in the three groups. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 *vs* samples related non-parametric test. lysoPC: Lysophosphatidylcholine; lysoPE: Lysophosphatidylethanolamines.

HCC cases occur in China<sup>[21-23]</sup>. Because it takes a long time for HCC to occur in liver cirrhosis patients, early diagnosis of HCC is of great importance. Lots of effort has gone in discovering early biomarkers in human fluid, especially in serum, to monitor the occurrence and development of HCC<sup>[11,24,25]</sup>. Unfortunately, although the biomarkers discovered in serum have great potential value in clinical and subclinical areas, their targeting ability and specificity are still limited. The present study determined serum metabolic profiling of HCC and provided new research targets for molecular biology.

Tissue metabolic profiling, especially human tissue metabolic profiling has several advantages in metabolomics investigations<sup>[26,27]</sup>. For example, compared with serum metabolic profiling, tissue metabolic profiling reflects the metabolic changes in certain target lesions in tissues or organs instead of changes in the whole metabolic system. Thus, it has higher targeting ability, specificity and is affected by fewer exogenous influencing factors, such as psychological changes in patients. Compared with animal tissue metabolic profiling, where the model is generally induced by a single factor, human tissue metabolic profiling reflects the natural physiological changes of lesions and is trusted in investigations on the occurrence and development of certain diseases.

Following tissue metabolite profiling of HBV-related HCC, 215 ions were detected and after multivariate statistical analysis of their relative levels (integral peak area of extraction ion), 14 ions were selected as characteristic metabolites and then identified. Of these characteristic metabolites, 9 had been suggested as serum metabolite biomarkers for HCC diagnosis in previous reports, including *L*-phenylalanine, glycerophosphocholine, lysoPCs, lysophosphatidylethanolamines (lysoPEs) and chenodeoxycholic acid glycine conjugate<sup>[11,28-30]</sup>. The present results enhanced the targeting ability of these characteristic metabolites, as alterations in these metabolites occurred in HCC cells.

The remaining 5 identified characteristic metabolites have seldom been reported, including Beta-Sitosterol, Quinaldic acid, Arachidyl carnitine, Tetradecanal, and Oleamide. This was possibly due to alterations in these metabolites which were too small to be observed in serum or were masked by alterations in other metabolites. Although these metabolites did not show great value in clinical application as potential biomarkers, they still provided new targets for anticancer research.

Previous research has shown that the levels of 11 metabolites including beta-sitosterol, *L*-phenylalanine, lysoPCs, glycerophosphocholine, lysoPEs, chenodeoxycholic acid glycine conjugate and quinaldic acid were significantly high in the distant tissue group compared with the central tumor tissue group. In contrast, the levels of Arachidyl carnitine, tetradecanal and oleamide were significantly lower in the distant tissue group compared with the central tumor tissue group. A comparison of the levels of these metabolites in the adjacent tissue group showed that beta-sitosterol, quinaldic acid and oleamide were significantly lower in the central tumor tissue group, but relatively close to those in the central tumor tissue group. From these changes in metabolite levels, we can speculate that these endogenous metabolites correlate with the progression of adjacent tissue to central tumor tissue and contain important information on the occurrence and development of HCC. Further attention should be focused on these metabolites which will hopefully lead to the discovery of novel potential biomarkers for cancer diagnosis and disease course monitoring and provide new targets for tumor therapy.

In summary, the establishment of a metabolic profiling model and the analysis of the characteristic metabolites provided new perspectives for further research into the pathophysiology of HCC. Following investigations on homogenates of tumor tissue obtained during radical surgery of HCC, detection and identification of these previously reported potential biomarkers in human tissue

enhanced their targeted traceability and a comparison of the changes in these metabolites in the central tumor tissue group, adjacent tissue group and distant tissue group revealed metabolic information highly related to HCC development. More attention should be focused on these characteristic metabolites which will provide more potential biomarkers for HCC diagnosis and development. In addition, further research into the pathways related to these metabolites would provide new clinical targets for HCC therapy.

## ACKNOWLEDGMENTS

We would like to express my gratitude to Jie Gao and Yi-Jun Wang who helped with the sample collection and to Li Zhang who helped with the data collection. We also acknowledge the Liver Surgery Department of Tianjin Third Central Hospital for sample submission.

## COMMENTS

### Background

Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is the most common type of liver cancer in Asian populations with an extremely high incidence and poor survival rate. Unfortunately, there are no satisfactory definitive positive and negative markers for HCC diagnosis in clinical application at the present time.

### Research frontiers

Recently, metabolomics research provided a series of novel biomarkers in serum or urine with clinical value in the diagnosis and prognosis of HCC. However, the application of these biomarkers is still limited due to the lack of target traceability in the diseased organ. The detection of these previously reported characteristic metabolites in tumor tissue in the present research provided supporting proof for these novel biomarkers.

### Innovations and breakthroughs

The targeting ability of animal models, serum and urine analysis are limited although experiments and sample collection are easier to perform. The present research used homogenates of central, adjacent and distant HBV-related HCC tumor tissue removed by surgery to identify these metabolites. Thus, this method had higher targeting ability, specificity and was less affected by exogenous influencing factors. In addition, the analysis detected alterations in these metabolites that were barely observed in serum or urine.

### Applications

By detecting characteristic metabolites in tumor tissue, this research confirmed the traceability of 9 previously reported biomarkers detected in serum or urine provided supporting evidence for their potential clinical applications. The introduction of homogenates also suggested new avenues of research in metabolic analysis.

### Terminology

Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) analysis are data analysis methods based on projection technology. PCA can visually represent sample correlations (results distribution) such as clustering and migration. However, this method is easily affected by noise signals such as instrumental bias and operation error. In this situation, OPLS-DA analysis is often introduced in data analysis to focus experimental data in a particular direction according to a priori knowledge.

### Peer review

The manuscript selects 14 characteristic metabolites in liver tumor tissue that not only supports the clinical application of previous reported serum or urine biomarkers but also provides novel research targets for anticancer investigations.

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P- Reviewer Grizzi F S- Editor Zhai HH  
L- Editor A E- Editor Zhang DN



## Polysomnographic sleep aspects in liver cirrhosis: A case control study

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Supported by Grants from the Associação Fundo de Incentivo a Pesquisa and FAPESP-CEPID-Proc. 95/14303-3

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Received: September 19, 2012 Revised: January 22, 2013

Accepted: February 5, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To study sleep aspects and parameters in cirrhotic patients and assess the role of liver dysfunction severity in polysomnographic results.

**METHODS:** This was a case-control study. Patients with a diagnosis of liver cirrhosis were consecutively enrolled in the study. Clinical examinations and laboratory liver tests were performed in all patients, and disease severity was assessed using the Child-Pugh score. The control group consisted of age- and gender-matched healthy volunteers. All individuals answered a questionnaire about habits, behaviors, and complaints related to sleep and were submitted to polysomnography. Sleep parameters were compared between the two groups, and separate analyses were performed among classes

of Child-Pugh classification in the cirrhotic group.

**RESULTS:** Forty-two cirrhotic patients and forty-two controls were enrolled. Compared to the control group, the cirrhotic group exhibited lower sleep efficiency (mean  $\pm$  SD: 73.89%  $\pm$  14.99% vs 84.43%  $\pm$  8.55%,  $P < 0.01$ ), increased latency (151.27  $\pm$  93.24 min vs 90.62  $\pm$  54.74 min,  $P < 0.01$ ) and a lower percentage of rapid eye movement (REM) sleep (14.04%  $\pm$  5.64% vs 20.71%  $\pm$  6.77%,  $P < 0.05$ ) as well as a higher frequency of periodic limb movements (10.56  $\pm$  2.85/h vs 2.79  $\pm$  0.61/h,  $P < 0.01$ ). The comparison of sleep parameters among Child A, B and C cirrhotic patients revealed a significant reduction of REM sleep stage occurrence in individuals with severe liver disease (Child C patients) compared to Child A/B patients (polysomnography percentage of REM sleep stage of patients Child A: 16.1%  $\pm$  1.2%; Child B: 14.9%  $\pm$  1.2%; Child C: 8.6%  $\pm$  1.6%,  $P < 0.05$ ).

**CONCLUSION:** Cirrhosis was associated with shorter sleep time, reduced sleep efficiency, increased sleep latency, increased REM latency and reduced REM sleep. Additionally, disease severity influences sleep parameters.

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**Key words:** Liver cirrhosis; Sleep; Child-Pugh classification; Polysomnography; Rapid eye movement sleep; Periodic limb movements in sleep; Apnea-hypopnea index; Obstructive sleep apnea syndrome

Teodoro VV, Júnior MAB, Lucchesi LM, Cavignolli D, de Mello MT, Kondo M, Tufik S. Polysomnographic sleep aspects in liver cirrhosis: A case control study. *World J Gastroenterol* 2013; 19(22): 3433-3438 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3433.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3433>

## INTRODUCTION

Although modern sleep research has a short forty-year history, interest in the impact of sleep quality on bio-regulatory functions and human health dates back to ancient times<sup>[1-3]</sup>. The development of polysomnography (PSG) provided the ability to assess sleep structure and led to thorough characterizations of different sleep stages and sleep disorders<sup>[4]</sup>. The rapid eye movement (REM) sleep stage has acquired increasing relevance; it is considered fundamental to the maintenance of important intellectual functions such as memory, attention and mood<sup>[5]</sup>. The REM stage is characterized by cortical activation; this is evidenced by a rapid transition to higher frequency rhythms with rapid, low-voltage, irregular activity on electroencephalogram (EEG). It is also characterized by irregular breathing, heart rate and muscle atonia<sup>[2]</sup>. Time spent in REM sleep is markedly reduced in several organic dysfunctions and is associated with cardiovascular adverse events, such as systemic arterial hypertension<sup>[6-8]</sup>. Similarly, spontaneous or induced REM sleep deprivation was previously correlated with higher death rates<sup>[9]</sup>.

Sleep disturbances are commonly reported in liver cirrhosis (LC), particularly in patients with hepatic encephalopathy (HE)<sup>[10,11]</sup>. There are few clinical<sup>[12,13]</sup> and experimental<sup>[14]</sup> studies assessing the sleep patterns of LC patients without overt HE; however, to our knowledge, an approach based on full-night PSG (level 1) has never been performed<sup>[15-17]</sup>.

A study that used sleep questionnaires, neuropsychological tests and actigraph monitoring suggested that sleep complaints are an early sign of HE<sup>[17]</sup> and that insomnia and excessive daytime sleepiness (EDS) are often described in patients with liver disease<sup>[10,11,13,17]</sup>. Conversely, Vignatelli *et al.*<sup>[18]</sup> found only a small percentage of cirrhotic patients with EDS. Although clinical reports vary, one possible explanation for sleep dysfunction in LC patients is a disruption in melatonin circadian rhythms<sup>[13,14]</sup>; one study reported a delayed onset and peak of melatonin secretion, which caused a sleep phase delay and possibly EDS<sup>[19,20]</sup>.

Detailed information regarding PSG sleep aspects in LC patients is lacking. Moreover, no study to date has attempted to determine the influence of disease severity on sleep structure. This study aimed to characterize sleep patterns of LC patients using a full-night PSG-based approach focusing on the following: (1) sleep structure in LC; (2) sleep pattern variations associated with liver disease severity; and (3) detection of possible sleep disorders linked to LC. We then conducted a case-control study to compare PSG sleep aspects between LC patients and healthy volunteers.

## MATERIALS AND METHODS

### *Clinical and laboratory assessment*

Patients with a diagnosis of LC by either liver biopsy or analysis of clinical and laboratory data were enrolled in the cirrhotic group. They were invited to participate in

the study upon reporting to the Gastroenterology Out-patient Clinic of the Universidade Federal de São Paulo (UNIFESP). The exclusion criteria were the following: younger than 18 years, alcohol consumption or gastrointestinal bleeding in the last 6 mo, serum creatinine levels higher than 2.0 mg/dL, psychoactive drug intake in the last 2 wk and overt clinical HE in the initial assessment. Those who fulfilled the selection criteria had venous blood drawn, and a clinical evaluation was performed to determine liver disease severity according to the Child-Pugh score. At the same time, arterial blood was collected to determine arterial ammonia levels. LC etiology was determined in all patients. Selected patients were submitted to a full-night PSG examination in the same week as the initial assessment.

The control group was composed of age- and gender-matched volunteers from the UNIFESP-Instituto do Sono laboratories, who met the criteria listed above and were considered healthy according to clinical and laboratory evaluations. They also completed a PSG examination.

The research protocol was reviewed and approved by the institutional research ethics committee (protocol number 1503/04), and all participants provided informed consent before enrollment in the study.

### PSG

All PSG recordings were performed in the Instituto do Sono. Before PSG, all study participants completed a questionnaire about habits, behaviors, and complaints related to sleep. The questionnaire was developed by the internal staff of the Instituto do Sono<sup>[21]</sup> and validated for the local population.

Full-night PSGs were performed in all participants using the sleep laboratory digital system EMBLA S7000® (Embla Systems Inc., Broomfield, CO, United States). The subjects were instructed to go to sleep at their usual bedtime. The following physiological variables were simultaneously and continuously recorded during PSG: (1) four channels to EEG; (2) two channels to electrooculogram; (3) channels placed in the submentonian region, the anterior tibial muscle, the masseter region and the seventh intercostal space to the electromyogram; (4) electrocardiogram; (5) two channels for airflow monitoring (one for the thermocouple and the other for nasal pressure measurement); (6) the detection of respiratory efforts of the thorax (one channel) and the abdomen (one channel) for inductance plethysmography; (7) snore monitoring; (8) one channel for monitoring body position; and (9) oxy-hemoglobin saturation measurement.

All PSG sessions were monitored by trained technicians and visually scored according to standardized criteria<sup>[22]</sup>. EEG arousals and leg movement episodes were scored according to the "Manual for Scoring Sleep and Associated Events"<sup>[23,24]</sup>; apnea and hypopnea episodes as well as other detected sleep events were also scored and classified using recognized rules<sup>[25]</sup>.

The following PSG sleep parameters were recorded and systematically evaluated: (1) total recording time

**Table 1** General characteristics of the two groups evaluated (*n* (%) or mean  $\pm$  SD)

| Variables                | Control group<br>( <i>n</i> = 42) | Cirrhotic group<br>( <i>n</i> = 42) | <i>P</i><br>value |
|--------------------------|-----------------------------------|-------------------------------------|-------------------|
| Age (yr)                 | 48.4 $\pm$ 8.3                    | 50.0 $\pm$ 8.5                      | 0.32              |
| Males                    | 29 (69.0)                         | 33 (78.5)                           | 0.70              |
| BMI (kg/m <sup>2</sup> ) | 25.3 $\pm$ 3.4                    | 26.3 $\pm$ 4.4                      | 0.40              |
| LC etiology              |                                   |                                     |                   |
| Alcohol                  | -                                 | 15 (38)                             | -                 |
| HCV                      | -                                 | 12 (30)                             | -                 |
| Alcohol + HCV            | -                                 | 8 (20)                              | -                 |
| HBV                      | -                                 | 2 (4)                               | -                 |
| Alcohol + HBV            | -                                 | 1 (2)                               | -                 |
| Cryptogenic              | -                                 | 4 (6)                               | -                 |
| Child-Pugh               |                                   |                                     |                   |
| Child A                  | -                                 | 16 (38)                             | -                 |
| Child B                  | -                                 | 17 (40)                             | -                 |
| Child C                  | -                                 | 9 (22)                              | -                 |

BMI: Body mass index; LC: Liver cirrhosis; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

(TRT): the entire period under PSG monitoring; (2) total sleep time (TST): the entire PSG recorded while sleeping; (3) wake: the entire PSG recorded while the patient was awake; (4) sleep efficiency: the TST/TRT ratio, expressed as percentage; (5) sleep latency: the length of time to sleep onset; (6) latency to REM sleep: the latency of REM sleep stage onset; (7) sleep stages 1, 2, 3 + 4 and REM sleep stage (S1, S2, S3 + 4 and REM): the percentage (%) of time patients spent in sleep stages 1, 2, 3 + 4 or REM sleep stage, respectively; (8) apnea-hypopnea index (AHI): an index to express the mean number of apneas or hypopneas in a 1-h period; (9) periodic leg movements of sleep per hour (PLMS/h): the average number of PLM events in a one hour period; (10) arousals/h: the average number of arousals in a one hour period; (11) mean SpO<sub>2</sub>: the mean oxy-hemoglobin saturation; and (12) nadir SpO<sub>2</sub>: minimal oxy-hemoglobin saturation recorded during PSG. These parameters were analyzed and scored by a blinded specialist before between-groups comparisons were made.

### Statistical analysis

Statistical analyses were performed using STATISTICA software, version 5.1. The Student's *t*-test was used to compare the quantitative PSG parameters between groups. Analysis of variance and Tukey's *post-hoc* test were used to compare the sleep parameters among the three classes of liver disease according to the Child-Pugh score. Results were considered statistically significant if *P* < 0.05.

## RESULTS

Forty-two cirrhotic patients and forty-two controls who satisfied the selection criteria were evaluated between May 2003 and August 2005. The cirrhotic group consisted of 29 males (69%) with a mean age of 50.0  $\pm$  8.5 years, and the general demographic characteristics of cirrhotic patients did not differ significantly from those of

**Table 2** Polysomnographic parameters of the two groups evaluated (mean  $\pm$  SD)

| Variables              | Control group<br>( <i>n</i> = 42) | Cirrhotic group<br>( <i>n</i> = 42) | <i>P</i><br>value |
|------------------------|-----------------------------------|-------------------------------------|-------------------|
| TRT (min)              | 421.98 $\pm$ 38.20                | 445.65 $\pm$ 50.64                  | 0.07              |
| TST (min)              | 357.18 $\pm$ 53.42                | 329.67 $\pm$ 76.62                  | < 0.05            |
| Wake (min)             | 51.86 $\pm$ 29.91                 | 115.05 $\pm$ 67.92                  | < 0.01            |
| SE                     | 84.43% $\pm$ 8.55%                | 73.89% $\pm$ 14.99%                 | < 0.01            |
| Sleep Lat (min)        | 13.39 $\pm$ 14.40                 | 28.41 $\pm$ 29.28                   | < 0.01            |
| REM Lat (min)          | 90.62 $\pm$ 54.74                 | 151.27 $\pm$ 93.24                  | < 0.01            |
| S1                     | 5.14% $\pm$ 3.26%                 | 5.90% $\pm$ 3.12%                   | 0.77              |
| S2                     | 57.00% $\pm$ 8.73%                | 61.72% $\pm$ 7.38%                  | 0.29              |
| S3 + 4                 | 17.31% $\pm$ 7.00%                | 18.32% $\pm$ 6.64%                  | 0.73              |
| REM                    | 20.71% $\pm$ 6.77%                | 14.04% $\pm$ 5.64%                  | < 0.05            |
| AHI                    | 7.33 $\pm$ 1.01                   | 5.16 $\pm$ 0.80                     | 0.09              |
| PLMS/h                 | 2.79 $\pm$ 0.61                   | 10.56 $\pm$ 2.85                    | < 0.01            |
| Arousals/h             | 15.88 $\pm$ 5.46                  | 11.78 $\pm$ 7.06                    | < 0.01            |
| Mean SpO <sub>2</sub>  | 94.80% $\pm$ 1.62%                | 94.38% $\pm$ 2.05%                  | 0.06              |
| Nadir SpO <sub>2</sub> | 87.45% $\pm$ 5.24%                | 85.87% $\pm$ 8.98%                  | < 0.05            |

TRT: Total recording time; TST: Total sleep time; Wake: Minutes awake after sleep onset; SE: Sleep efficiency; Sleep Lat: Latency to sleep onset; REM: Rapid eye movement; REM Lat: REM sleep latency; S1: Stage 1; S2: Stage 2; S3: Stage 3; S3 + 4: Stages 3 and 4; AHI: Apnea-hypopnea index; PLMS/h: Number of periodic limbs movements of sleep per hour; Arousals/h: Number of arousals per hour of sleep; Mean SpO<sub>2</sub>: Mean oxy-hemoglobin saturation; Nadir SpO<sub>2</sub>: Minimal oxy-hemoglobin saturation.

the control group (Table 1). LC etiology and Child-Pugh scores are also shown in Table 1. The arterial ammonia measurements in cirrhotic patients were (mean  $\pm$  SD) 166.38  $\pm$  26.10 mol/L (normal value: 9-33 mol/L).

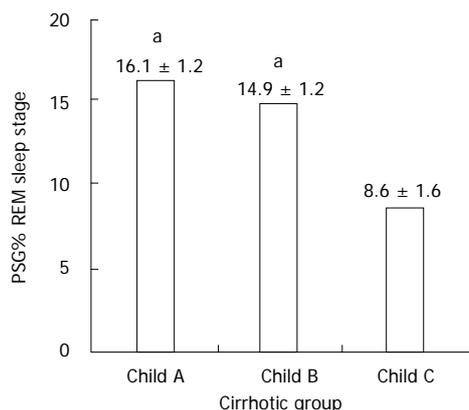
LC patients reported more sleep difficulties on the questionnaire, including trouble initiating sleep, non-restorative sleep and more episodes of napping during the day. Although the TRT and AHI were similar in both groups, a significant reduction in the TST, sleep efficiency and wake time were observed in the cirrhotic group. Increased sleep latency, REM latency, PLMS index and a decreased REM sleep percentage were also observed in the cirrhotic group (Table 2).

Comparison of sleep parameters among Child A, B and C cirrhotic patients revealed a significant reduction of REM sleep stage occurrence in individuals with severe liver disease (Child C patients) (Figure 1).

To assess the influence of alcoholism on sleep parameters, we divided the patients into two groups: those who had alcoholic etiology (alone or associated with viral hepatitis; *n* = 24) and those who had no history of alcoholism (*n* = 18). No significant differences were detected between the two groups in our study for any sleep parameter except sleep latency, which was longer in the group without alcoholic etiology (21.68  $\pm$  15.38 min *vs* 40.53  $\pm$  42.74 min for the alcoholic etiology group and without alcoholic etiology group respectively, *P* = 0.04).

## DISCUSSION

The sleep parameters evaluated by PSG in this study indicated worsening sleep in the cirrhotic group compared to the control group. This was due to decreased sleep



**Figure 1** Rapid eye movement sleep stage percentage comparison among different Child-Pugh score system classes. <sup>a</sup> $P < 0.05$  vs Child C group. Child A, B and C: Liver disease severity classes; PSG: Polysomnography; REM: Rapid eye movement.

efficiency, increased time to initiate sleep, increased latency of REM sleep, reduced REM sleep percentage and higher PLMS indices. As our data suggest, disease severity influenced sleep parameters, especially when data were classified by the patients' Child scale rating.

The sleep questionnaire results showed that the cirrhotic patients had more complaints about their sleep, such as difficulty initiating sleep and non-restorative sleep. These findings are consistent with the PSG results indicating lower sleep efficiency and higher sleep latency. Other studies have also described a higher number of sleep complaints in LC patients<sup>[12,26]</sup>. Moreover, LC patients report more episodes of napping during the day<sup>[10,19,27]</sup>. EDS appears to be attributable to a dysfunction of the neural circuit responsible for the maintenance of wakefulness and sleep states. The monoaminergic system, the systems of the locus coeruleus (noradrenergic) and the raphe nuclei (serotonergic) are important for directing attention and recruiting the cortex for processing external sensory stimuli<sup>[28]</sup>. High levels of ammonia can reduce serotonin and noradrenaline levels in the central nervous system, resulting in low alertness and attention<sup>[29,30]</sup>.

The higher incidence of PLMS in our sample of LC patients was not related to any clinical disorder or laboratory finding commonly associated with this phenomenon. This would include conditions such as anemia, renal failure, or low levels of iron or plasma transferritin<sup>[31]</sup>. PLMS is also associated sleep complaints, including difficulty falling asleep, multiple arousals and EDS<sup>[32,33]</sup>. These complaints were also reported by our group of patients and objectively confirmed by the PSG findings of higher sleep latency and higher arousal index.

This study did not find statistically significant differences between groups regarding AHI. This is in contrast with two previous studies<sup>[15,26]</sup>; however, it is consistent with the findings of Nikaina *et al.*<sup>[16]</sup>. Differing results may be explained by the presence or absence of ascites<sup>[34]</sup>. In fact, Nikaina *et al.*<sup>[16]</sup> found no significant differences in the index of respiratory events between controls and patients with compensated cirrhosis without ascites.

It is widely recognized that chronic alcohol abuse influences sleep parameters<sup>[35]</sup> and many patients in this study experienced cirrhosis of alcoholic etiology. However, in our study, we did not confirm this influence, perhaps due to the fact that patients had been in withdrawal for at least six months. The absence of differences suggests that LC may be seen as a determining factor of the sleep parameters observed for study in this group. The only difference that was found, in sleep latency, which was longer in the group without alcoholic etiology, cannot be easily explained; considering the effects of alcohol, the expected result would be the opposite of what was actually observed<sup>[35]</sup>.

The intense electrical and metabolic activity observed in REM sleep is the best argument supporting sleep as an active phenomenon<sup>[2,3]</sup>. The system responsible for the generation of REM sleep encompasses several specific nerve structures and follows a model of reciprocal interaction between the co-organizers and suppressor structures, which are located mainly in the brainstem and midbrain<sup>[2,3]</sup>. In the current study, both the latency and percentage of REM sleep of LC patients differed from those of controls, suggesting the hyperfunction of REM sleep suppressor mechanisms. Evidence from sleep deprivation studies suggests a role for dopamine during REM sleep<sup>[36]</sup>, and these studies have described the relationship of REM sleep in terms of dopamine D2 receptors<sup>[37]</sup>. Specific changes in the dopamine receptors of the brain of LC patients have been previously described<sup>[38,39]</sup>. Dopamine receptor dysfunction is associated with low concentrations of serotonin, which may suppress REM sleep and constitute a possible explanation for the PSG findings in our LC group.

It is possible that our findings of sleep impairment in subjects with cirrhosis are common to all forms of metabolic encephalopathy. However, our study's strength resides in the fact that we found differences in both subjective and objective parameters. In our observational study, LC patients had longer sleep latencies, shorter sleep time, worse sleep efficiency, increased REM sleep latencies and lower REM sleep percentages. The latter finding was negatively related to the severity of the disease. Therefore, we need to draw clinician's attention to the importance of sleep complaints and parameters regarding prognosis in cirrhotic patients.

## ACKNOWLEDGMENTS

The authors would like to thank Altay Lino de Souza and Ana Amelia Benedito Silva for their valuable suggestions and statistical analyses. All the efforts of the Associação Fundo de Incentivo a Pesquisa staff, particularly those of the sleep technicians, are deeply appreciated.

## COMMENTS

### Background

Liver cirrhosis (LC) is currently a serious clinical problem, and is considered a very common disease with a major impact on public health worldwide. It is a

chronic and irreversible process characterized by progressive replacement of the normal structure of the liver by fibrosis as a response to a continuous injury to this organ, leading to various clinical consequences, as alterations in sleep. In fact, sleep disturbances are commonly reported in LC.

### Research frontiers

Polysomnography provided the ability to assess sleep structure and led to a thorough characterization of sleep stages and sleep disorders. Sleep is divided into rapid eye movement (REM) and non-REM sleep stages. REM sleep has acquired increasing relevance because it is considered fundamental to the maintenance of important intellectual functions, such as memory, attention and mood. Time spent in REM is markedly reduced in several organic dysfunctions and is associated with cardiovascular adverse events, such as systemic arterial hypertension.

### Innovations and breakthroughs

There is a lack of detailed information regarding polysomnographic sleep aspects in patients with LC. Moreover, no study has attempted to determine the influence of disease severity on sleep structure. This study aimed to characterize the sleep patterns of patients with LC with a full-night polysomnographic based approach focusing on the following: (1) sleep structure in LC; (2) sleep pattern variations associated with liver disease severity; and (3) detection of possible sleep disorders linked to LC. Authors then compared polysomnographic sleep aspects between patients with LC and healthy volunteers.

### Applications

The study results suggest that hepatic cirrhosis was associated with shorter sleep time, reduced sleep efficiency, increased sleep latency, increased REM latency and reduced REM sleep. Additionally, disease severity influenced sleep parameters. Therefore, authors need to draw clinician's attention to the importance of sleep complaints and parameters regarding prognosis in cirrhotic patients.

### Peer review

In this paper, the authors study sleep aspects and parameters in cirrhotic patients and assess the role of liver dysfunction severity on polysomnographic results. This study is interesting and suggests that cirrhosis is associated with sleep disturbances and that disease severity influences sleep parameters.

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P- Reviewer Yang YF S- Editor Gou SX  
L- Editor A E- Editor Xiong L



## Deep sedation during gastrointestinal endoscopy: Propofol-fentanyl and midazolam-fentanyl regimens

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Received: November 3, 2012 Revised: December 22, 2012

Accepted: January 11, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To compare deep sedation with propofol-fentanyl and midazolam-fentanyl regimens during upper gastrointestinal endoscopy.

**METHODS:** After obtaining approval of the research ethics committee and informed consent, 200 patients were evaluated and referred for upper gastrointestinal endoscopy. Patients were randomized to receive propofol-fentanyl or midazolam-fentanyl ( $n = 100/\text{group}$ ).

We assessed the level of sedation using the observer's assessment of alertness/sedation (OAA/S) score and bispectral index (BIS). We evaluated patient and physician satisfaction, as well as the recovery time and complication rates. The statistical analysis was performed using SPSS statistical software and included the Mann-Whitney test,  $\chi^2$  test, measurement of analysis of variance, and the  $\kappa$  statistic.

**RESULTS:** The times to induction of sedation, recovery, and discharge were shorter in the propofol-fentanyl group than the midazolam-fentanyl group. According to the OAA/S score, deep sedation events occurred in 25% of the propofol-fentanyl group and 11% of the midazolam-fentanyl group ( $P = 0.014$ ). Additionally, deep sedation events occurred in 19% of the propofol-fentanyl group and 7% of the midazolam-fentanyl group according to the BIS scale ( $P = 0.039$ ). There was good concordance between the OAA/S score and BIS for both groups ( $\kappa = 0.71$  and  $\kappa = 0.63$ , respectively). Oxygen supplementation was required in 42% of the propofol-fentanyl group and 26% of the midazolam-fentanyl group ( $P = 0.025$ ). The mean time to recovery was 28.82 and 44.13 min in the propofol-fentanyl and midazolam-fentanyl groups, respectively ( $P < 0.001$ ). There were no severe complications in either group. Although patients were equally satisfied with both drug combinations, physicians were more satisfied with the propofol-fentanyl combination.

**CONCLUSION:** Deep sedation occurred with propofol-fentanyl and midazolam-fentanyl, but was more frequent in the former. Recovery was faster in the propofol-fentanyl group.

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**Key words:** Endoscopy; Deep sedation; Anesthetic administration; Anesthetic dose; Adverse effects

Lera dos Santos ME, Maluf-Filho F, Chaves DM, Matuguma SE, Ide E, Luz GO, de Souza TF, Pessorusso FCS, de Moura EGH, Sakai P. Deep sedation during gastrointestinal endoscopy: Propofol-fentanyl and midazolam-fentanyl regimens. *World J Gastroenterol* 2013; 19(22): 3439-3446 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3439.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3439>

## INTRODUCTION

The routine use of sedation during endoscopic procedures is increasing worldwide<sup>[1,2]</sup>. In a survey conducted in the United States in 2006, > 98% of endoscopies and colonoscopies were performed under sedation<sup>[1,3]</sup>. A similar trend has been observed in Switzerland, Germany, and Australia<sup>[2,4-7]</sup>. The combination of a benzodiazepine and opioid is reportedly used at approximately 75% of all healthcare facilities in the United States<sup>[1,3]</sup> and considered the combination of choice by most endoscopists worldwide<sup>[1,2,4-9]</sup>. As a result of its anxiolytic and sedative properties, its ability to provide anterograde amnesia, and its short half-life, midazolam is the most widely used benzodiazepine. Fentanyl is the most widely used opioid, although meperidine is still frequently used<sup>[2-4,8,9]</sup>.

Propofol is a hypnotic agent that induces anesthesia almost immediately and has a short half-life. It also allows the patient to recover rapidly and be discharged. Patient and physician satisfaction is high with propofol. As a result of these properties, the use of propofol has been adopted at endoscopy centers worldwide<sup>[1-7,10]</sup>. However, its use has also been associated with deep sedation<sup>[11-14]</sup>.

During endoscopy, sedation and analgesia improve the efficiency of the procedure, quality of the results, and comfort of the patient<sup>[5,8]</sup>. However, sedation is also responsible for the majority of complications related to diagnostic endoscopy<sup>[15]</sup>. During sedation and analgesia, there is a continuum of states, ranging from mild sedation to general anesthesia. In the middle of this continuum is conscious sedation, which is the target level of sedation for patients undergoing upper or lower gastrointestinal endoscopy<sup>[4,5,10,11,13,14,16-19]</sup>. A level of sedation deeper than that intended is associated with a higher rate of complications<sup>[11,15,20]</sup>. The guidelines established in 2002 by the American Society of Anesthesiologists (ASA) task force on sedation and analgesia by non-anesthesiologists, which have also been endorsed by the American Society for Gastrointestinal Endoscopy, recommend that a distinction be made between conscious sedation and deep sedation and that one professional be dedicated to the assessment and monitoring of patients during sedation<sup>[11-13]</sup>.

The level of consciousness is typically assessed with the subjective clinical scale known as the observer's assessment of alertness/sedation (OAA/S) score, as validated by Chernik *et al*<sup>[21]</sup>, which ranks sedation as mild, conscious, or deep. Another means of assessing the level of consciousness is the calculation of the bispectral index

(BIS). Through the use of a BIS monitor, complex mathematical calculations of electroencephalography waves are transformed into numbers ranging from 0 (no brain activity) to 100 (fully conscious). This provides an objective measure of the level of consciousness. The BIS is considered a viable alternative for monitoring the level of consciousness in patients submitted to general anesthesia. Although its use in endoscopy is controversial, it has been investigated with increasing frequency, and further studies are recommended<sup>[22-27]</sup>.

In the past 10 years, new sedation guidelines have been established. Many of those guidelines state that propofol can be safely administered by an endoscopist or nurse under the supervision of a physician<sup>[1,3-5,8,10,12,14,17,25,28,29]</sup>. However, because propofol has been associated with deep sedation events and complications, some have recommended that propofol be administered exclusively by anesthesiologists<sup>[5,11-13,17]</sup>.

In view of these facts, we evaluated the use of propofol-fentanyl versus midazolam-fentanyl for sedation of patients undergoing upper gastrointestinal endoscopy. The primary endpoint of this study was to compare the frequency of deep sedation in each group. We also compared the two drug combinations in terms of time to induction, time to recovery, time to discharge, efficacy, and safety, as well as patient and endoscopist satisfaction.

## MATERIALS AND METHODS

This was a prospective, single-blind, randomized controlled trial carried out between January 2007 and October 2010 at the Gastrointestinal Endoscopy Clinic of the Department of Gastroenterology at the University of São Paulo Medical School - Hospital das Clínicas, Brazil.

### Patients

We recruited 262 patients from those scheduled to undergo upper gastrointestinal endoscopy at the Gastrointestinal Endoscopy Unit. The inclusion criteria were age > 18 years, physical status classified as ASA I, II or III, and having a contact telephone number. The exclusion criteria were as follows: pregnancy; a history of allergy to the medications to be administered; a history of allergy to soy or eggs; a psychotic disorder; being under treatment with psychoactive medications; being an illicit drug user or a heavy consumer of alcohol; Child-Pugh class C cirrhosis; presence of chronic kidney disease (being on dialysis); and being submitted to endoscopy as an emergency procedure. Of the 262 patients recruited, 62 were excluded (Figure 1). The final sample comprised 200 patients. Through a drawing of sealed envelopes, patients were randomized to two groups of 100: propofol-fentanyl and midazolam-fentanyl. The endoscopists scheduled to perform the procedures had no access to the envelopes.

### Drug administration

Drug infusion was performed by the nursing staff and attending endoscopist. In both groups, the objective was to

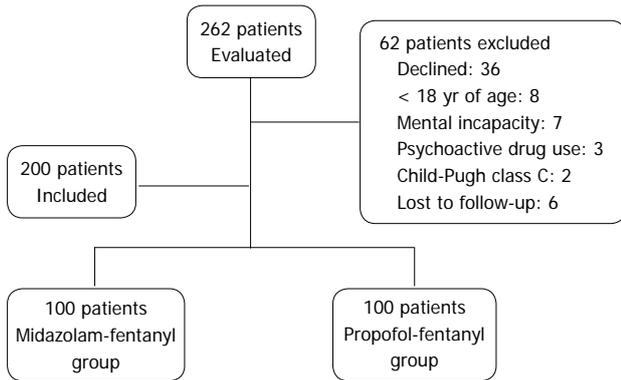


Figure 1 Flowchart of the study design.

achieve conscious sedation by using the dose calculated for that purpose (Table 1). The propofol and midazolam were administered by intravenous bolus and supplemented as necessary by the endoscopist to maintain the desired level of sedation. A single dose of fentanyl was used in both groups. The fixed maximum dose for midazolam was 10 mg or 0.1 mg/kg of body weight. If additional sedation became necessary, the endoscopist had the option of suspending the initial regimen and adding propofol.

### Patient evaluation and monitoring

Prior to the procedure, a clinical history and physical examination was performed for each patient. Additionally, the anesthetic risk was assessed with the ASA classification of physical status, and the patients completed a demographic questionnaire. Continuous monitoring during the procedure included the noninvasive measurement of blood pressure, heart rate, respiratory rate (thoracic excursion measurement), and oxygen saturation (SpO<sub>2</sub>). We defined the following evaluation time points: “baseline” (immediately before the procedure); “duodenum” (the approximate midpoint of the procedure, when the endoscopist was evaluating the duodenum or the jejunal loop in post-gastrectomy patients); and “recovery” (when the patient was awake and underwent the final evaluation).

### Evaluation of the level of sedation

During the procedure, the level of sedation was evaluated in two ways. We applied the OAA/S scale<sup>[21]</sup>, which is scored as 1 for deep sedation, 2-4 for conscious sedation, and 5 for mild sedation. In addition, after cleaning the skin with gauze and alcohol, we applied disposable electrodes to the forehead and connected the leads to a BIS monitor (A-2000 BIS XP; Aspect Medical Systems, Newton, MA, United States) (Figure 2). The BIS monitor output was evaluated continuously throughout the procedure and recovery period. BIS ≤ 65 indicates deep sedation, 66-85 indicates conscious sedation, and BIS > 85 indicates mild sedation. The OAA/S scale and BIS were determined simultaneously every 2 min.

An independent observer was responsible for the mo-



Figure 2 Bispectral index monitor.

onitoring, which included evaluating the level of consciousness, readout of other vital signs, collection of data regarding drugs and doses used, use of benzodiazepine or opioid antagonists, and occurrence of cardiorespiratory events, such as hypoxemia (defined as SpO<sub>2</sub> < 90% for > 30 s after application of the jaw thrust maneuver), hypotension (defined as ≥ 20% decrease in systolic or diastolic blood pressure), and bradycardia (heart rate < 50 bpm). Hypoxemia was classified as mild if it responded to supplemental oxygen delivered at 3-4 L/min; it was classified as severe if it did not respond to supplemental oxygen and the patient required noninvasive ventilatory support (*e.g.*, bag-mask ventilation) or intubation. The same observer also reported any other adverse events that occurred secondary to sedation. The observer was blinded to the randomization.

As described by Cohen *et al.*<sup>[30]</sup>, we compared the two groups in terms of the time to induction (interval between the first drug bolus administration and initiation of the procedure), time to recovery (interval between removal of the endoscope and final evaluation), and time to discharge (interval between removal of the endoscope and departure from the endoscopy unit). The final evaluation began when the BIS monitor indicated at least 90. Patients were discharged only when they had achieved an OAA/S score of 5 (the maximum), a BIS > 90, and reported no pain or any other type of discomfort.

### Visual analog scale and questionnaires

At discharge, patient satisfaction was assessed with a 10-point visual analog scale (1 = least satisfied and 10 = most satisfied). The patients also completed a satisfaction questionnaire before leaving the facility. At 24 h after discharge, the same observer who was responsible for the monitoring contacted the patients by telephone to administer a questionnaire that evaluated patient satisfaction with the procedure, adverse events and the resumption of domestic activities. All 200 patients completed both questionnaires. The visual analog scale was also applied to the endoscopists who performed the procedures to assess their level of satisfaction with the sedation regimen and was scored as follows: 1-3 = considerable difficulty in performing the procedure; 4-7 = minor difficulty in

**Table 1 Sedation regimens**

|                    |  |
|--------------------|--|
| Midazolam-fentanyl |  |
| Midazolam          |  |
| Initial dose       |  |
| ASA I              | 3-5 mg   |
| ASA II or III      | 2-3 mg   |
| Maintenance        | 0.5-1.0 mg every 2-3 min, up to a maximum cumulative dose of 10 mg or 0.1 mg/kg of body weight |
| Fentanyl           |  |
| Single dose        |  |
| ASA I              | 50 µg  |
| ASA II or III      | 20-30 µg   |
| Propofol-fentanyl  |  |
| Propofol           |  |
| Initial dose       |  |
| ASA I              | 0.5 mg/kg  |
| ASA II or III      | 0.25 mg/kg   |
| Maintenance        | 10-20 mg bolus at 60 s intervals   |
| Fentanyl           |  |
| Single dose        |  |
| ASA I              | 50 µg  |
| ASA II or III      | 20-30 µg   |

ASA: American Society of Anesthesiologists.

performing the procedure (patient moved at the beginning or end of the procedure); and 8-10 = no difficulty in performing the procedure.

### Ethics

This work was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The study was approved by the Hospital das Clínicas Research Ethics Committee, and all participating patients provided written informed consent.

### Statistical analysis

To calculate the sample size, we estimated the proportion of patients who received deep sedation by analyzing the BIS curve. We hypothesized that this proportion would be 10% for the midazolam-fentanyl group and 25% for the propofol-fentanyl group. By adopting an  $\alpha$  error tolerance (false-positive risk) of 5% and  $\beta$  error tolerance (false-negative risk) of 20%, we determined that 112 patients per group would be required to provide sufficient power to detect significant differences. It was agreed that we would perform an interim analysis involving 100 patients in each group.

The data were entered into a Microsoft Excel spreadsheet and analyzed with the assistance of the Statistics Sector of the University of São Paulo Medical School, Department of Gastroenterology. The statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, United States). We applied the Mann-Whitney test to evaluate continuous variables and used the  $\chi^2$  test to evaluate categorical variables. To study the effects of the variable "group" at the various time points, we used repeated measures analyses of variance. We used the  $\kappa$  statistic to evaluate the degree of concordance between the OAA/S scale and BIS.

Data were collected by a researcher who was blinded

**Table 2 Patient characteristics**

| Variable                 | Group              |                   | Total      | P value |
|--------------------------|--------------------|-------------------|------------|---------|
|                          | Midazolam-fentanyl | Propofol-fentanyl |            |         |
| Sex                      |                    |                   |            |         |
| Female                   | 66 (60.0)          | 71 (71.0)         | 137 (68.5) | 0.543   |
| Male                     | 34 (34.0)          | 29 (29.0)         | 63 (31.5)  |         |
| ASA                      |                    |                   |            |         |
| I                        | 63 (63.0)          | 55 (55.0)         | 118 (59.0) | 0.316   |
| II                       | 37 (37.0)          | 44 (44.0)         | 81 (40.5)  |         |
| III                      | 0 (0.0)            | 1 (0.5)           | 1 (0.5)    |         |
| Age (yr)                 | 52.14 ± 15.01      | 54.40 ± 15.44     |            | 0.352   |
| Weight (kg)              | 67.45 ± 11.28      | 70.93 ± 17.64     |            | 0.242   |
| Height (cm)              | 1.62 ± 0.09        | 1.61 ± 0.10       |            | 0.546   |
| BMI (kg/m <sup>2</sup> ) | 25.91 ± 4.54       | 27.39 ± 6.59      |            | 0.251   |
| DM proportion            | 4.5%               | 7.5%              |            | 0.276   |
| SH proportion            | 16.0%              | 18.0%             |            | 0.640   |
| Cardiopathy proportion   | 0.5%               | 0.0%              |            | 0.999   |
| Other proportion         | 11.5%              | 9.0%              |            | 0.404   |

Data are expressed as absolute numbers (percentage) or mean ± SD. BMI: Body mass index; DM: Diabetes mellitus; SH: Systemic hypertension.

to each patient's group. However, such masking was not possible when we assessed endoscopist satisfaction with the sedation regimen.

## RESULTS

### Patient characteristics

Most patients presented with a low anesthetic risk (ASA class I or II), although one patient in the propofol-fentanyl group was classified as ASA III. There were no significant differences between the two groups regarding demographics, weight, height, body mass index, level of education, or ASA class (Table 2). All of the patients completed the procedure with adequate sedation throughout. None of the procedures were suspended or halted prematurely.

### Drug doses

Patients in the midazolam-fentanyl group received midazolam and fentanyl at mean doses of  $5.25 \pm 1.7$  mg and  $43.1 \pm 9.87$  µg, respectively. Patients in the propofol-fentanyl group received propofol and fentanyl at mean doses of  $70.3 \pm 38.9$  mg and  $41.0 \pm 10.25$  µg, respectively. Sixty minutes after the end of the procedure, a patient in the midazolam-fentanyl group presented with persistent drowsiness, despite normal cardiorespiratory function, and was given 0.2 mg flumazenil.

### Level of sedation

As seen in Table 3, the OAA/S classification of sedation in the midazolam-fentanyl group was mild in 1%, conscious in 88%, and deep in 11%, compared with 2%, 73%, and 25%, respectively, in the propofol-fentanyl group. There was a statistically significant difference between the two groups in terms of the OAA/S score ( $P = 0.014$ ). Based on the BIS values, sedation in the midazolam-fentanyl and propofol-fentanyl groups was classified

**Table 3** Level of sedation *n* (%)

| Variable    | Group              |                   | Total      | P value |
|-------------|--------------------|-------------------|------------|---------|
|             | Midazolam-fentanyl | Propofol-fentanyl |            |         |
| OAA/S score |                    |                   |            |         |
| 1           | 11 (11.0)          | 25 (25.0)         | 36 (18.0)  | 0.014   |
| 2-4         | 88 (88.0)          | 73 (73.0)         | 161 (80.5) |         |
| 5           | 1(1.0)             | 2 (2.0)           | 3 (1.5)    |         |
| BIS         |                    |                   |            |         |
| ≤ 65        | 7 (7.0)            | 19 (19.0)         | 26 (13.0)  | 0.039   |
| 66-85       | 75 (75.0)          | 67 (67.0)         | 142 (71.0) |         |
| > 85        | 18 (18.0)          | 14 (14.0)         | 32 (16.0)  |         |

OAA/S: Observer's assessment of alertness/sedation score; BIS: Bispectral index.

as mild in 18% and 14%, as conscious in 75% and 67%, and as deep in 7% and 19%, respectively ( $P = 0.039$ ). Comparing the BIS values obtained before, during, and after the procedure, we found that there was a trend toward a return to its initial value more rapidly in the propofol-fentanyl group than in the midazolam-fentanyl group (Figure 3). The OAA/S score showed good concordance with the BIS in the midazolam-fentanyl group ( $\kappa = 0.635$ ,  $P < 0.001$ ), the propofol-fentanyl group ( $\kappa = 0.710$ ,  $P < 0.001$ ), and the sample as a whole ( $\kappa = 0.696$ ,  $P < 0.001$ ).

### Satisfaction

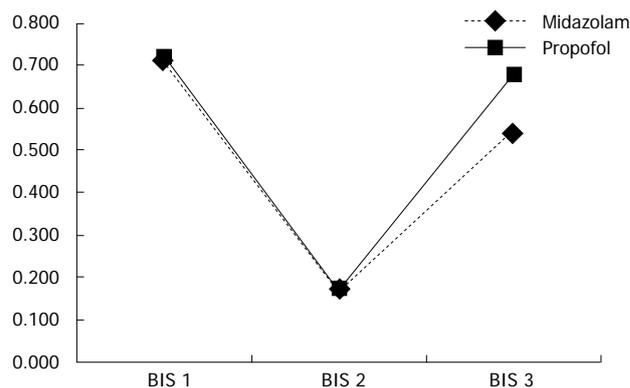
The mean score on the visual analog scale of patient satisfaction was  $9.84 \pm 0.4$  in the midazolam-fentanyl group and  $9.64 \pm 0.8$  in the propofol-fentanyl group ( $P = 0.178$ ). The mean score on the visual analog scale for endoscopist satisfaction was  $8.90 \pm 1.2$  for the midazolam-fentanyl regimen and  $9.30 \pm 0.9$  for the propofol-fentanyl regimen ( $P = 0.012$ ). The time to induction was significantly shorter in the propofol-fentanyl group ( $2.63 \pm 1.62$  min *vs*  $2.96 \pm 1.5$  min,  $P = 0.012$ ). The times to recovery and discharge were also shorter in the propofol-fentanyl group ( $P < 0.001$  and  $P < 0.001$ , respectively).

### Pre- and post-discharge questionnaires

We found no statistically significant differences between the two groups in terms of the patient-reported quality of sedation or pain/discomfort related to the procedure. The proportion of patients who remembered being awake at the beginning, middle, and end of the procedure was greater in the propofol-fentanyl group than the midazolam-fentanyl group ( $P < 0.001$  for all three time points). According to the results of the post-discharge questionnaire, none of the patients experienced any adverse reactions within the first 24 h after discharge. On average, the patients in the propofol-fentanyl group reported having resumed their domestic activities 60 min after discharge compared with 80 min after discharge for the midazolam-fentanyl group ( $P < 0.001$ ).

### Safety and complications

No serious complications were observed in either group. During the procedure, 42% of the propofol-fentanyl



**Figure 3** Bispectral values before, during, and after diagnostic upper gastrointestinal endoscopy according to group. Bispectral (BIS) 1: Before the procedure; BIS 2: During the procedure; BIS 3: After the procedure.

group patients developed mild transient hypoxemia ( $SpO_2$  of 85%-90% for  $> 30$  s after the jaw thrust maneuver), which also occurred in 26% of the midazolam-fentanyl group patients ( $P = 0.025$ ). In all of those cases, the hypoxemia responded to supplemental oxygen delivered by nasal cannula at 3-4 L/min. There were no instances of arrhythmia. Systolic hypotension was observed in 5% of the midazolam-fentanyl group and 10% of the propofol-fentanyl group, whereas diastolic hypotension was observed in 6% of the midazolam-fentanyl group and 16% of the propofol-fentanyl group. All of the variations in arterial blood pressure, heart rate, and respiratory rate were transient and required no mechanical or pharmacological intervention. There were no cases of perforation, bleeding, or death, and none of the patients required invasive ventilatory support, or hospitalization.

## DISCUSSION

Although there are abundant data in the literature and various guidelines on sedation during endoscopy<sup>[1-6,10,12,17,31]</sup>, few studies have compared propofol with midazolam for conscious sedation in patients undergoing diagnostic upper gastrointestinal endoscopy, especially when using the incidence of deep sedation events as the primary outcome. In the present study, we used the BIS and OAA/S scale in an innovative manner and assessed the frequency of deep sedation events for two sedation regimens frequently used during endoscopy<sup>[11,12,16,17,32,33]</sup>. In the midazolam-fentanyl group, deep sedation, defined according to the BIS and OAA/S score, occurred in 11% and 7% of the patients, respectively, compared with 25% and 19% of those in the propofol-fentanyl group. It was clear that despite the use of doses targeting conscious sedation, deep sedation events were common in the midazolam-fentanyl group. These findings are in agreement with those of Patel *et al*<sup>[20]</sup>, who evaluated the use of a benzodiazepine-opioid combination with the objective of achieving conscious sedation in patients undergoing diagnostic upper gastrointestinal endoscopy. The authors found that deep sedation, as evaluated by a modified OAA/S scale, occurred in 26% of the patients.

In the present study, the overall rates of deep sedation were 18% and 13% when assessed by the OAA/S score and BIS, respectively. However, we found that the frequency of clinically relevant complications was negligible, which is likely attributable to the relatively good overall health status of the patients. It is notable that the adverse effects arising from deep sedation were easily reversed with simple clinical maneuvers. There was a trend toward the BIS returning to its initial value more rapidly in the propofol-fentanyl group, which suggests faster recovery in those patients.

The principal complication that occurred in our study was hypoxemia, which was observed in 42% of the patients in the propofol-fentanyl group and 26% of the midazolam-fentanyl group. In all of those patients, hypoxemia responded to supplemental oxygen delivery. The close monitoring of the patients during sedation could explain the relatively high rates of transient mild hypoxemia found in both of the groups. These findings again raise the controversy regarding giving supplemental oxygen routinely during upper gastrointestinal endoscopy, which is our current practice. In addition, the small size of our sample prevented us from effectively evaluating the incidence of severe complications, which are rare in endoscopy.

In the pre-endoscopy, pre-medication period, patients typically have a BIS > 93, whereas a BIS of 60-70 is indicative of deep sedation<sup>[24]</sup>. There are some discrepancies between the data in the literature and information provided by the manufacturer in terms of the relationship between the numerical values and levels of consciousness<sup>[23,24,26,27]</sup>. Some authors define deep sedation as a BIS of 60-70 and conscious sedation as > 70<sup>[22]</sup>, while others define deep sedation as BIS < 75 in the presence of an OAA/S score of 1 or 2<sup>[27]</sup>. To improve the sensitivity and specificity of our evaluations, we defined deep sedation as BIS ≤ 65 and an OAA/S score of 1 and conscious sedation as BIS > 65 and an OAA/S score > 1. Bower *et al*<sup>[24]</sup> have suggested that a BIS of 75-85 indicates conscious sedation, which is an appropriate level of sedation for endoscopic procedures. In assessing the level of consciousness of patients undergoing endoscopic procedures, those authors demonstrated a strong temporal correlation between the BIS and OAA/S score ( $r = 0.59$ ,  $P < 0.0001$ ). In the present study, we observed good concordance between the BIS and OAA/S score ( $\kappa = 0.7$ ,  $P < 0.001$ ). However, the BIS has obvious advantages over the OAA/S. The primary advantage is that the BIS is a much simpler and more continuous measure. The BIS allows objective measurements of sedation in patients undergoing endoscopic procedures<sup>[26]</sup>.

The use of the BIS in monitoring the level of sedation of patients undergoing endoscopic procedures is controversial because its impact remains unclear<sup>[11,13,17,27]</sup>. In a study of patients undergoing colonoscopy, the sedation was administered by nurses under the supervision of gastroenterologists, and there was no reduction in the propofol dose used or the time to recovery<sup>[34]</sup>. Other au-

thors have shown that BIS monitoring leads to the use of a lower mean dose of propofol in endoscopic retrograde cholangiopancreatography<sup>[23]</sup>. In a study of BIS monitoring during sedation for endoscopic submucosal dissection, the propofol dose was not reduced, although there was increased satisfaction on the part of the patients and endoscopists<sup>[25]</sup>. Nevertheless, some authors question the accuracy of BIS monitoring in detecting deep sedation<sup>[27]</sup>. There is no evidence to support the routine use of BIS monitoring in the ambulatory setting of diagnostic endoscopic procedures. In the future, it may prove beneficial for more complex therapeutic endoscopic procedures.

The present study had some limitations. The fact that physical status was classified as ASA I or II in 99.5% of the patients might have limited the external validity of the study. However, the exclusion of patients with poor physical status allowed us to focus more closely on the relationship between propofol and deep sedation in the clinical setting most often encountered in endoscopy clinics. In fact, the inclusion of patients with more comorbidity who were undergoing endoscopic procedures of greater complexity would have shifted the discussion to the efficacy and safety of sedation in complex situations. Although that is an important topic and is currently being investigated by other authors<sup>[23,35]</sup>, it was not the focus of the present study. There were also some potential biases related to the difficulty of achieving full blinding of the sedation regimen. However, double blinding was achieved for the collection of data, such as the level of sedation (OAA/S and BIS), the time to induction, recovery, and discharge, and the level of patient satisfaction. All procedures were performed consecutively, respecting the sedation regimens initially proposed and with the observer present.

In the present study, we demonstrated that the times to induction, recovery, and discharge were significantly shorter in the propofol-fentanyl group. These findings replicate the results obtained by other authors who have demonstrated that propofol allows patients to resume their work activities sooner, thereby also increasing overall productivity<sup>[3,5,9,19,30,31,33,35-38]</sup>. We found no significant difference between the two regimens in terms of patient satisfaction, although there was a difference in terms of satisfaction on the part of endoscopists. The endoscopists expressed a preference for the propofol-fentanyl combination. This finding is in keeping with the global trend toward the use of propofol sedation by gastroenterologists and endoscopists<sup>[2,3]</sup>.

Our findings show that although the use of the midazolam-fentanyl regimen results in deep sedation less often than the propofol-fentanyl regimen does, the difference is not clinically relevant. In our opinion, there is little evidence to support the position that propofol should be administered only by anesthesiologists or that the use of propofol is disproportionately associated with the occurrence of unwanted deep sedation. In fact, deep sedation can also occur when the midazolam-fentanyl regimen is used. This underscores the importance of monitoring

the vital signs of patients under sedation. We could also add that both drug dosage and titration are crucial for the success of the sedation regimen.

In our opinion, patients classified as ASA I or II, if properly evaluated and monitored, can be safely subjected to diagnostic upper gastrointestinal endoscopy under sedation with the propofol-fentanyl combination at doses targeting conscious sedation. We also believe that the presence of an anesthesiologist is not mandatory in this setting. The use of this regimen can increase physician satisfaction and productivity.

## COMMENTS

### Background

The routine use of sedation during endoscopic procedures is increasing worldwide. The combination of a benzodiazepine (e.g., midazolam) and opioid (e.g., fentanyl) is reportedly used at approximately 75% of all healthcare facilities in the United States and considered the combination of choice by most endoscopists worldwide. Propofol is a hypnotic agent and its use has been adopted at endoscopy centers worldwide. However, its use has also been associated with deep sedation. In this study, authors evaluated the use of propofol-fentanyl versus midazolam-fentanyl for sedation of patients undergoing upper gastrointestinal endoscopy, comparing the frequency of deep sedation in each group. They also compared the two drug combinations in terms of time to induction, time to recovery, time to discharge, efficacy, and safety, as well as patient and endoscopist satisfaction.

### Research frontiers

Nowadays, propofol is only used routinely by anesthesiologists. The research hotspots are to find a secure way to provide safety and lower complications of propofol use by non-anesthesiologists.

### Innovations and breakthroughs

Although there are abundant data in the literature and various guidelines on sedation during endoscopy, few studies have compared propofol with midazolam for conscious sedation in patients undergoing diagnostic upper gastrointestinal endoscopy. In the present study, authors used the bispectral index (BIS) monitor and observer's assessment of alertness/sedation (OAA/S) scale in an innovative manner and assessed the frequency of deep sedation events for two sedation regimens frequently used during endoscopy.

### Applications

The results suggest that patients classified as low anesthetic risk, if properly evaluated and monitored, can be safely subjected to diagnostic upper gastrointestinal endoscopy under sedation with the propofol-fentanyl combination at doses targeting conscious sedation. Authors also believe that the presence of an anesthesiologist is not mandatory in this setting. The use of this regimen can increase physician satisfaction and productivity.

### Terminology

Deep sedation is represented by a BIS  $\leq$  65 and/or OAA/S scale = 1. The BIS monitors cerebral activity. BIS  $\leq$  65 indicates deep sedation, 66-85 indicates conscious sedation, and  $>$  85 indicates mild sedation. OAA/S scale was developed to measure the level of alertness in subjects who are sedated. It is scored as 1 for deep sedation, 2-4 for conscious sedation, and 5 for mild sedation.

### Peer review

An interesting study that assessed the differences and reliability between upper gastrointestinal endoscopies performed with midazolam-fentanyl versus propofol-fentanyl sedation. The main findings of the paper were that deep sedation was more frequent in the propofol-fentanyl group, however, this group had a more rapid recovery after the procedure.

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**P- Reviewers** Familiari L, Galloro G, Lakatos PL  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Xiong L



## Endoscopic gastrojejunostomy with a natural orifice transluminal endoscopic surgery technique

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Author contributions: Song TJ, Seo DW were responsible for the study concept and design, endoscopic procedures; Song TJ drafted the manuscript; Seo DW, Park DH, Lee SS, Lee SK and Kim MH critically revised the manuscript; Kim SH contributed to the material support and data acquisition.

Supported by A grant from the Asan Institute for Life Sciences, Seoul, South Korea, No. 2013-201

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Received: October 30, 2012 Revised: April 2, 2013

Accepted: April 18, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To determine the technical feasibility and safety of an endoscopic gastrojejunostomy with a pure natural orifice transluminal endoscopic surgery (NOTES) technique using a T-anchoring device in a porcine survival model.

**METHODS:** An endoscopic gastrojejunostomy with a pure NOTES technique using a T-anchoring device was performed on 10 healthy female minipigs weighing approximately 40 kg each under general anesthesia. All procedures were performed with a transgastric approach using a 2-channel therapeutic endoscope.

**RESULTS:** The transgastric gastrojejunostomy was technically successful in all cases. A total of four to six

stitched pairs of a T-anchoring device were used to secure the anastomosis. The median time required to enter the peritoneal cavity and pull the small bowel into the stomach was 34 min (range: 19-41 min); the median time required to suture the anastomosis was 67 min (range: 44-78 min). An obstruction of the efferent limb occurred in one case, and a rupture of the anastomosis site occurred in another case. As a result, the functional success rate was 80% (8/10). Small bowel adhesion to the stomach and liver occurred in one case, but the anastomosis was intact without leakage or obstruction.

**CONCLUSION:** A transgastric gastrojejunostomy with a T-anchoring device may be safe and technically feasible. A T-anchoring device may provide a simple and effective endoscopic suturing method.

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**Key words:** Natural orifice transluminal endoscopic surgery; Endoscopy; Pigs; Anastomosis

**Core tip:** Natural orifice transluminal endoscopic surgery (NOTES) have become part of the growing trend of minimally invasive surgery and have been gradually used in more diverse areas. An endoscopic gastrojejunostomy using a pure NOTES technique may be attractive because it can be a simple and less invasive method for bypassing a gastric outlet or duodenal obstruction. An endoscopic transgastric gastrojejunostomy with T-anchoring devices may be a technically feasible, useful alternative to invasive surgery. However, a great deal of care and further improvement is needed because of the risk of procedure-related complications.

Song TJ, Seo DW, Kim SH, Park DH, Lee SS, Lee SK, Kim MH. Endoscopic gastrojejunostomy with a natural orifice transluminal endoscopic surgery technique. *World J Gastroenterol* 2013; 19(22): 3447-3452 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3447.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3447>

## INTRODUCTION

Gastric outlet obstruction or duodenal obstruction is one of the most serious problems that frequently occur in patients with advanced malignancies of the stomach or periampullary region<sup>[1-3]</sup>. In cases in which an oral diet was impossible due to gastric outlet obstruction or duodenal obstruction, open surgery using hand-sewn techniques, staplers, or compression devices, as well as laparoscopic surgery using staplers, have been the major treatment modalities thus far<sup>[4-8]</sup>. However, these obstructions usually occur in patients with unresectable malignancies of an advanced stage, and invasive surgery may be impossible or quite burdensome for these end-stage patients<sup>[9]</sup>. Therefore, less invasive methods to address malignant obstructions may be attractive to these patients<sup>[10,11]</sup>.

Various procedures using natural orifice transluminal endoscopic surgery (NOTES) techniques are part of the growing trend of minimally invasive surgery, and these NOTES techniques have been gradually used in more diverse areas<sup>[12-16]</sup>. Because NOTES techniques have proven to have many advantages in terms of being less invasive compared with existing surgical methods, they are considered to be effective treatment measures that are not burdensome for terminal-stage patients, particularly those with advanced-stage malignancies<sup>[17,18]</sup>. The first NOTES anastomosis, a cholecystogastrostomy, was reported in 2005, and subsequent studies have demonstrated that various forms of intraperitoneal surgery performed using laparoscopy could be conducted using a flexible endoscope<sup>[19,20]</sup>. Performing a gastrojejunostomy using a NOTES technique is attractive because it can be a simple and less invasive method for bypassing a gastric outlet obstruction or duodenal obstruction.

The aim of these experiments was to assess the technical feasibility and safety of an endoscopic peroral transgastric gastrojejunostomy procedure with a prototype T-anchoring device in a porcine model.

## MATERIALS AND METHODS

### Subjects

For this experiment, 10 minipigs, which were breeds of pig developed for medical research, weighing approximately 40 kg each were used. The anesthesia was performed by one veterinarian, and the gastrojejunostomy was performed by two endoscopists and two nurses. Permission for this study was obtained from the Animal Experiment Review Board of Asan Medical Center.

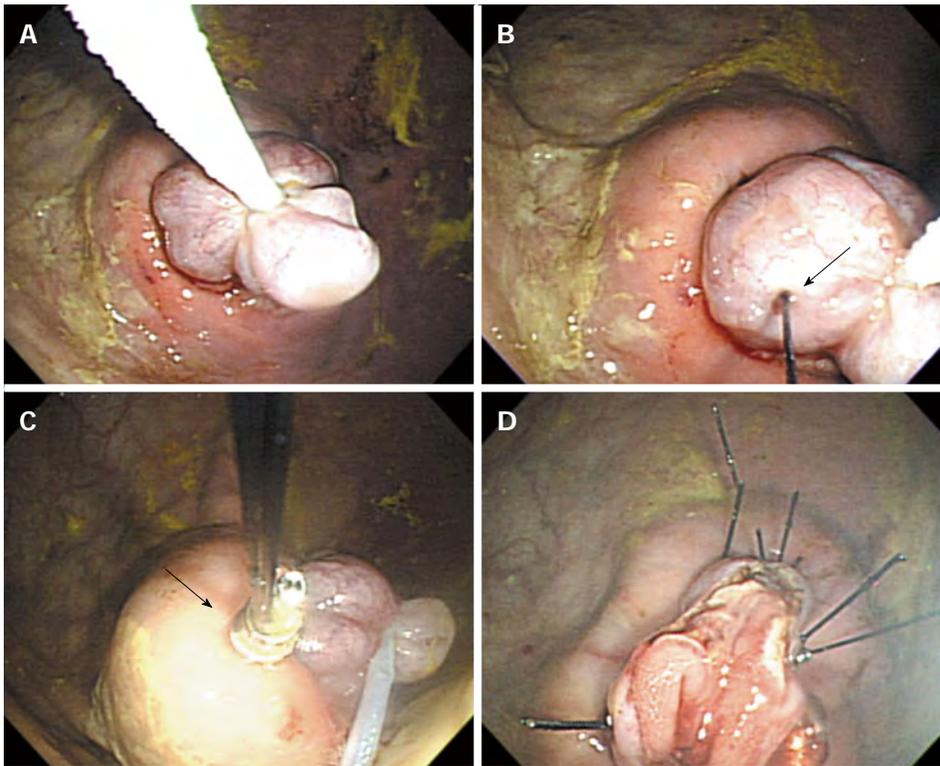
### Experiment method

**Pretreatment:** The animals were fed a soft liquid diet beginning 48 h before the procedure; then, they abstained from food-except for only a small quantity of water-beginning 24 h before the procedure. They were anesthetized with a combination of anesthetic agents, including tiletamine hydrochloride, zolazepam hypochloride (Zoletil<sup>®</sup>, Virbac do Brasil Ltda., Brazil), and xylazine (Rompun<sup>®</sup>, Bayer Korea Co. Ltd., South Korea) before the proce-

dures, and general anesthesia was maintained with 1.5% isoflurane (Forane<sup>®</sup>, Choongwae Pharma Co. Ltd., South Korea). Premedication was performed 30 min before anesthesia using an intramuscular injection of atropine sulfate (Bayer Korea Co. Ltd., South Korea).

**Procedure:** A multibending two-channel endoscope (2TQ260<sup>®</sup>, Olympus Optical Co. Ltd., Japan) was used for the procedures. The minipigs were placed in a supine position, and the endoscope was inserted into the stomach. The anterior wall of the body of the stomach was punctured with a needle knife (Micro knife<sup>®</sup>, Boston Scientific, Natick, MA, United States). After the puncture, a 0.035-inch guidewire (Jagwire<sup>®</sup>, Boston Scientific, Natick, MA, United States) was inserted into the peritoneal cavity through the needle knife, and the needle knife was then removed. A papillotome was inserted along the guidewire, and a 2-cm-long incision was made on the stomach wall. The incision was made in four directions-up, down, left, and right-while changing the direction of the papillotome. A dilating balloon with a diameter of 20 mm (CRE<sup>®</sup>, Boston Scientific, Natick, MA, United States) was inserted along the guidewire, and the puncture site was dilated twice for one minute each. After dilation, the dilating balloon was pushed inside the peritoneal cavity, together with the endoscope, while being deflated. After entry into the peritoneal cavity, the guidewire and inflating balloon were removed from the scope.

An appropriate loop of the small intestine was selected for the anastomosis. Usually, the mid-jejunum was selected using indications provided by the anatomic position relative to other structures. When the location of the anastomosis in the jejunum had been determined, grasping forceps were inserted into a channel of the endoscope, and a snare was inserted into another channel. After the snare was opened, the grasping forceps were pushed through the inside of the open snare, and the snare was slowly closed. Then, the predetermined antimesenteric site on the mid-jejunum was grasped using the grasping forceps, and the jejunum was drawn into the inside of the snare. Thereafter, the snare was closed to grasp the jejunum, which was brought into the stomach. The jejunum was grasped with care to avoid blocking the mesenteric vessel, and the air in the abdominal cavity was sufficiently aspirated before the jejunum was placed into the stomach. Because the small intestine would go back into the peritoneal cavity after being drawn into the stomach, the snare was held closed to hold the small intestine and prevent it from returning to the abdominal cavity while the grasping forcep was being removed. In the endoscopic channel (from which the grasping forceps had been removed), a needle, installed with the first T-anchoring tag, was inserted. Using the needle, the small intestine was punctured, and the first T-anchoring tag was detached through the inside of the needle. Then, the needle was removed, and the second T-anchoring tag was installed into the needle. This needle was inserted through the same channel, and the stomach wall (*i.e.*, the wall closest to the T-anchoring tag that was inserted first)



**Figure 1** Technique for endoscopic gastrojejunostomy using a T-anchoring device. A: After the gastric wall incision and balloon dilatation, the small intestine was held in the stomach; B: A needle installed with the first T-anchoring tag was used to start suturing. The first T-anchoring tag was attached to the small intestine (arrow), and then, the second was attached to the gastric wall; C: A pair of sutures was locked together using a locking device (arrow). The process was repeated four to six times to anchor the small intestine to the stomach; D: Finally, an incision using a needle knife was made on the jejunum.

was punctured. The second T-anchoring tag was detached through the inside of the needle, and the needle was removed. Then, a pair of T-anchoring tags was locked using a locking device. Thereafter, a pair of scissor forceps was inserted to cut the suture thread connected to the T-anchoring tag, thereby completing the suture. These processes were repeated four to six times to fix the jejunum to the stomach. Finally, the snare was opened, and an incision was made on the jejunum using a needle knife to open the jejunal lumen to the inside of the stomach (Figure 1).

**Postoperative care:** Third-generation cephalosporin and analgesics were administered intravenously after the procedures, and a liquid diet was administered after 24 h. All animals survived for seven days before they were euthanized. Then, autopsies were conducted. The health conditions and abnormal reactions of the animals were monitored for seven days. After seven days, the animals were euthanized, and the anastomosis site, afferent and efferent loops, and peritoneal cavity were observed.

## RESULTS

The detailed results of the endoscopic peroral transgastric gastrojejunostomy performed on the 10 animals are shown in Table 1. The transgastric gastrojejunostomy was technically successful in all cases (100%, 10/10). A total of four to six stitched pairs of T-anchoring devices were

used to secure the anastomosis. The median time from when the endoscope was inserted through the mouth to puncture the stomach, enter the peritoneal cavity, find an appropriate region of the small intestine, and draw it into the stomach was 34 min (range: 19-41 min). The median time required to complete the anastomosis using the T-anchoring devices was 67 min (range: 44-78 min). During the operations, there were no adverse events, such as bleeding or internal organ injury, and the vital signs of the animals were stable. On the postmortem examination, although the afferent limb was patent, the efferent limb was obstructed in one case. A rupture of the anastomosis site occurred in another case. As a result, the functional success rate was 80% (8/10). Mild adhesion of the short segment of the small intestine to the stomach and liver occurred in one case, but the anastomosis was intact without leakage or obstruction. In all other cases, the small intestine loops of the anastomosis reached the stomach without tension or abnormal rotation.

## DISCUSSION

In this study, the peroral transgastric gastrojejunostomy performed with a pure NOTES technique using a flexible endoscope and a T-anchoring device was technically feasible. Peroral transgastric gastrojejunostomy might be another potential therapeutic option for gastric outlet obstruction or duodenal obstruction in addition to surgical anastomosis. The method of using a pure NOTES

**Table 1** The results of an endoscopic peroral transgastric gastrojejunostomy on 10 animals *n* (%)

|  |            |
|--|------------|
| Technical success  | 10 (100)   |
| Functional success   | 8 (80)     |
| Number of stitched pairs of the T-anchoring device   |            |
| 4  | 4          |
| 5  | 4          |
| 6  | 2          |
| Median time required to enter the peritoneal cavity and pull the small bowel into the stomach (min, range) | 34 (19-41) |
| Median time required to suture the anastomosis (min, range)  | 67 (44-78) |
| Adverse events   |            |
| Rupture of anastomosis site  | 1          |
| Insufficient efferent limb opening   | 1          |
| Small bowel adhesion   | 1          |

technique to bypass a malignant obstruction is advantageous over surgical anastomosis because it does not require any abdominal incision; as a result, it induces less surgical stress<sup>[21]</sup>. Moreover, it can be easily performed using a flexible endoscope and accessories under a conscious sedation state without general anesthesia, which is required for the usual endoscopic procedures<sup>[22]</sup>. Therefore, this method is considered to be potentially applicable not only to the palliation of malignant obstructions but also to benign diseases in which conventional trans-abdominal and laparoscopic surgeries are unsuitable, such as bariatric surgery for morbidly obese patients<sup>[19]</sup>.

In this study, the anastomosis was performed using a T-anchoring device. Until recently, conventional hemoclips have been used for the closure of puncture sites during NOTES procedures<sup>[23]</sup>. Although existing hemoclips can be used easily in various types of conventional endoscopic procedures, they are disadvantageous as full-thickness sutures are impossible to secure, and the sutures may fail if two ends of an opening are distant from each other<sup>[24,25]</sup>. However, T-anchoring devices are advantageous because their use enables full-thickness sutures to be secured. Moreover, even in cases in which two ends of an opening are distant from each other, T-anchoring devices secure full-thickness sutures if only a needle puncture is possible. Therefore, T-anchoring devices can be used in diverse NOTES procedures, such as the closure of various incision sites or anastomosis, and also in the management of adverse events that may occur in conventional endoscopic procedures (*e.g.*, the closure of a bowel perforation and bleeding control).

In this study, room air, which is usually used in conventional endoscopic procedures, was used for the inflation of the stomach and the peritoneal cavity. During our pure NOTES procedures, air in the stomach may have leaked into the peritoneal cavity during the anastomosis to induce abdominal distension. Although air in the peritoneal cavity was sufficiently aspirated during the process of drawing the small intestine into the stomach, the air that leaked into the peritoneal cavity during the anastomosis was not removed by the suction of the stomach. Abdominal distension occurred immediately after the

anastomosis in two cases, and thus, percutaneous needle aspiration was performed after the procedures. When this procedure is performed on humans, CO<sub>2</sub> gas, which is easily absorbed and discharged through breathing, will be helpful in reducing this distension<sup>[26,27]</sup>. A great deal of care must be exercised to prevent pressure inside the stomach from rising too high during the procedure, and an endoscope installed with a pressure gauge at its fore-end should be developed to enable the easy measurement and constant maintenance of the pressure in the pneumoperitoneum<sup>[28,29]</sup>.

In our study, complications occurred with respect to the sizes of the initial incisions on the stomach wall. The case in which there was an obstruction in the efferent loop was the first, and we believe that the obstruction in the efferent loop occurred because the stomach wall incision was not sufficiently large. Although the incision site was further enlarged using a 20-mm dilatation balloon, openings enlarged by balloon dilatation may contract again, unlike direct incisions. Therefore, the initial incisions on the stomach wall are important. In one case in which a rupture occurred in the anastomosis site, the incision was large, exceeding 2.5 cm. Our speculation is that the suture site could not tolerate the pressure in the stomach after the diet because the incision was too large. Therefore, the size of the opening should be appropriate, and a great deal of care must be taken to ensure that it is not too small or too large. In our opinion, in the case of an endoscopic gastrojejunostomy, 2.0 cm is thought to be an appropriate incision size. In the event that a large incision exceeding 2.5 cm has been made, a greater number of sutures and a longer period of fasting after the procedure are necessary. In addition, the T-anchoring tag should be improved to ensure that the suture site can tolerate sufficiently high pressure.

The present T-anchoring device is a prototype. To secure one pair of sutures using this device, a needle should be inserted through an endoscopic channel at least twice, and then, a pair of scissor forceps should be inserted to cut the thread after installing two T-anchoring tags. Given these lengthy processes, coupled with the fact that more than four pairs of sutures are necessary for the anastomosis, the overall procedure time may become too long. In addition, the threads connected to the T-anchoring tags may occasionally become tangled, causing problems during the processes of inserting and removing the needle. Therefore, improving the T-anchoring device is necessary to simplify the suturing process. Many (but not all) of the limitations of the instruments that have been used for the NOTES technique can be overcome by innovative design and engineering improvements<sup>[20]</sup>.

In conclusion, a peroral transgastric gastrojejunostomy with T-anchoring devices may be a technically feasible, useful alternative to invasive surgery. However, a great deal of care is needed because of the risk of complications. The T-anchoring device is still in its early stage and needs further improvements; however, it may provide a simple and effective endoscopic suturing method.

## COMMENTS

### Background

Various natural orifice transluminal endoscopic surgery (NOTES) techniques have become part of the growing trend of minimally invasive surgery, and these NOTES techniques have been gradually used in more diverse areas.

### Research frontiers

Performing a gastrojejunostomy using a pure NOTES technique is attractive because it can be a simple and less invasive method for bypassing a gastric outlet obstruction or duodenal obstruction.

### Innovations and breakthroughs

The transgastric gastrojejunostomy was technically successful in all cases (100%, 10/10). A total of four to six stitched pairs of T-anchoring devices were used to secure the anastomosis. An obstruction of the efferent limb occurred in one case, and a rupture of the anastomosis site occurred in another case. As a result, the functional success rate was 80% (8/10). Small bowel adhesion to the stomach and liver occurred in one case, but the anastomosis was intact without leakage or obstruction in this animal.

### Applications

T-anchoring devices secure full-thickness sutures if only a needle puncture is possible. Therefore, T-anchoring devices can be used in diverse NOTES procedures, such as the closure of various incision sites or anastomosis, and also in the management of adverse events that may occur in conventional endoscopic procedures.

### Terminology

NOTES is a minimally invasive surgery technique that has been recently devised; the application of this technique has been gradually expanding.

### Peer review

The authors reported an animal study on the transgastric gastrojejunostomy using NOTES technique. This study provides a further potential advances in NOTES, with important potential advantages for use in human in due course.

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**P- Reviewers** Day AS, Marinho RT, Wig JD

**S- Editor** Huang XZ **L- Editor** A **E- Editor** Xiong L



## Incidental focal colorectal $^{18}\text{F}$ -fluorodeoxyglucose uptake on positron emission tomography/computed tomography

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Received: December 25, 2012 Revised: March 20, 2013

Accepted: March 28, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To assess the clinical significance of incidental focal colorectal  $^{18}\text{F}$ -fluorodeoxyglucose ( $^{18}\text{F}$ -FDG) uptake on  $^{18}\text{F}$ -FDG-positron emission tomography/computed tomography (PET/CT).

**METHODS:** The records of all the cases which had undergone colonoscopy after PET/CT within a two weeks interval were reviewed. Adenomas were considered advanced when they were villous,  $\geq 10$  mm in size, or had high-grade dysplasia. Colorectal cancers and advanced adenomas are collectively referred to as advanced colorectal neoplasms. Receiver-operating characteristic curve analysis was used to determine the

significant predictive maximum standardized uptake value (SUVmax) cutoff value for advanced colorectal neoplasms and cancer.

**RESULTS:** Ninety-five colorectal lesions matched the site of incidental focal colorectal  $^{18}\text{F}$ -FDG uptake on PET/CT and 146 did not. Colonoscopy showed advanced colorectal neoplasms corresponding to the site of  $^{18}\text{F}$ -FDG uptake in 49 of the 95 (51.5%) lesions with incidental uptake. Of the lesions without incidental uptake, only 6 of 146 (4.1%) had advanced colorectal neoplasms on colonoscopy, indicating a statistically significant difference between the two groups ( $P < 0.001$ ). The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of incidental focal  $^{18}\text{F}$ -FDG uptake in identifying advanced colorectal neoplasms were 89.1%, 75.3%, 51.6%, 95.9%, and 78.4%, respectively. In detecting only CRC, these values were 89.2%, 69.6%, 34.7%, 97.3%, and 72.6%, respectively. The significant SUVmax cutoff value for advanced colorectal neoplasms (area under the curve 0.755,  $P < 0.001$ ) was 4.35, with a sensitivity, specificity, PPV, NPV, and accuracy of 75.5%, 65.2%, 69.8%, 71.4% and 70.5%, respectively. For CRC, 5.05 was the significant SUVmax cutoff value (area under the curve 0.817,  $P < 0.001$ ), with a sensitivity, specificity, PPV, NPV, and accuracy of 84.8%, 71.0%, 80.9%, 89.8%, and 75.8%, respectively.

**CONCLUSION:** The presence of incidental focal colorectal  $^{18}\text{F}$ -FDG uptake on PET/CT with a SUVmax  $\geq 4.35$  increases the likelihood of an advanced colorectal neoplasm.

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**Key words:** Positron emission tomography; Adenomas; Computed tomography; Colorectal cancer; Colonoscopy

**Core tip:** Increased focal uptake of <sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG) in the colon and rectum can be found incidentally during <sup>18</sup>F-FDG-positron emission tomography/computed tomography (PET/CT). The presence of incidental focal colorectal <sup>18</sup>F-FDG uptake on PET/CT indicated advanced colorectal neoplasms in more than half of the cases. Our study confirms the necessity of colonoscopy when incidental FDG uptake is detected on PET/CT to allow the early diagnosis and management of advanced colorectal neoplasms.

Cho SH, Kim SW, Kim WC, Park JM, Yoo IR, Kim SH, Oh ST. Incidental focal colorectal <sup>18</sup>F-fluorodeoxyglucose uptake on positron emission tomography/computed tomography. *World J Gastroenterol* 2013; 19(22): 3453-3458 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3453.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3453>

## INTRODUCTION

<sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG)-positron emission tomography/computed tomography (PET/CT) is a technique used to reduce false-positive findings by combining two imaging modalities, and can distinguish physiological activity from pathology more readily than PET or CT alone. PET/CT is commonly used during the evaluation of malignant tumors, and an increased focal colorectal uptake of <sup>18</sup>F-FDG can be observed incidentally because of the capacity of PET/CT to detect the foci of tumors, which display increased glycolysis<sup>[1]</sup>.

Several previous studies have shown that not only is a high incidence of unexpected gastrointestinal malignancies associated with incidental <sup>18</sup>F-FDG avidity<sup>[2]</sup>, but also that the presence of these lesions can change patient management in up to 28% of cases<sup>[3]</sup>. And in symptomatic patients with proven or suspected colorectal cancer (CRC) recurrence PET detected additional sites of disease in up to 48.4% of patients<sup>[4]</sup>.

However, the significance of incidental focal colorectal <sup>18</sup>F-FDG uptake observed on PET/CT has not yet been fully clarified. Only one previous prospective study has demonstrated a requirement for colonoscopy when incidental FDG uptake was found with PET/CT imaging<sup>[5]</sup>. However, those researchers could not determine the statistically significant maximum standardized uptake value (SUV<sub>max</sub>) with which to detect advanced colorectal adenoma and cancer because of the broad overlapping SUV values among the patient groups.

As far as we know, we have evaluated the largest number of cases yet assessed to determine the value of colonoscopy when incidental FDG uptake is observed on PET/CT images. In this study, we investigated the clinical significance of incidental focal colorectal <sup>18</sup>F-FDG uptake detected on PET/CT, and determined the SUV<sub>max</sub> for detecting advanced adenoma and cancer.

## MATERIALS AND METHODS

### Patients

We analyzed the records of 583 consecutive colonoscopic lesions examined over a three-year period, from those who had undergone colonoscopy after PET/CT within a two weeks interval.

The exclusion criteria were an incomplete colonoscopic examination, insufficient biopsy specimen for pathological diagnosis, diffuse colorectal <sup>18</sup>F-FDG uptake on PET/CT, and proven or suspected CRC in a previous study. A surveillance PET/CT examination performed for clinical assessment after curative CRC resection was not an exclusion criterion. Based on these criteria, 342 lesions were excluded.

A total of 241 colonoscopic lesions (88 from women and 153 from men) were eligible for the study. The median age was 62 years (range: 29-86 years). The study was approved by the Institutional Review Board of Seoul St Mary's Hospital, Catholic University of Korea College of Medicine.

### PET/CT acquisition

The subjects were fasted for at least 6 h before the FDG injection. Their blood glucose levels were determined in capillary blood samples before the intravenous injection of FDG. At our institution, a cutoff blood glucose level of 8 mmol/L contraindicates FDG injection. PET/CT images were acquired 1 h after the injection of 370-570 MBq of <sup>18</sup>F-FDG. The subjects were scanned from the base of the skull to the upper thighs, with their arms raised above their heads.

The PET/CT scans (dual-section helical CT) were performed on a Biograph Duo scanner (Siemens Medical Solutions, Knoxville, TN, United States) with an axial spatial resolution of 6.5 mm. Neither intravenous contrast medium nor bowel preparations were used for the CT scan.

The PET/CT images were reviewed on a workstation with fusion software (Syngo, Siemens, Knoxville, TN, United States). An experienced nuclear medicine physician reviewed the PET/CT images. PET, CT, and fused whole-body images displayed in the axial, coronal, and sagittal planes were available for review. The PET data were also displayed in a rotating maximum-intensity projection. The intensity of the <sup>18</sup>F-FDG uptake can be analysed qualitatively and semiquantitatively by measuring the standardized uptake value, which is usually expressed as its maximum. The SUV is defined as the ratio of activity within the tissue (Bq/mL) and decay-corrected total activity injected divided by body weight (Bq/g) and it is calculated using the software provided by the workstation manufacturer. Abnormal FDG uptake, the SUV<sub>max</sub> of the primary tumor, and distant metastases were evaluated.

### PET/CT interpretation and analysis

PET studies showing focal, well-circumscribed foci of

**Table 1** Baseline characteristics and pathologic diagnoses of the 241 eligible colorectal lesions *n* (%)

| Variables   | Total<br>( <i>n</i> = 241) | Focal FDG uptake (+)<br>( <i>n</i> = 95) | Focal FDG uptake (-)<br>( <i>n</i> = 146) | <i>P</i> value |
|---|----------------------------|--|---|----------------|
| Baseline characteristics                            |                            |  |   |                |
| Age, yr <sup>1</sup> (range)                        | 62 (29-86)                 | 64 (31-86)                               | 59.5 (29-84)                              | 0.068          |
| Males   | 153 (64)                   | 57 (60)                                  | 96 (65)                                   | 0.366          |
| Overweight or obesity (BMI > 25 kg/m <sup>2</sup> ) | 22 (9.1)                   | 9 (9.5)                                  | 13 (8.9)                                  | 0.367          |
| F/U after complete CRC resection                    | 67 (28.2)                  | 16 (17.9)                                | 51 (34.9)                                 | 0.006          |
| Pathologic diagnoses                                |                            |  |   |                |
| Non specific  | 140 (58.1)                 | 26 (27.5)                                | 114 (78.1)                                | < 0.001        |
| Inflammation  | 24 (9.9)                   | 18 (18.9)                                | 6 (4.1)                                   | < 0.001        |
| Adenoma (any size)                                  | 40 (16.6)                  | 18 (18.9)                                | 22 (15.1)                                 | 0.430          |
| Advanced adenoma                                    | 18 (7.5)                   | 16 (16.8)                                | 2 (1.4)                                   | < 0.001        |
| Cancer  | 37 (15.4)                  | 33 (34.7)                                | 4 (2.7)                                   | < 0.001        |
| Advanced colorectal neoplasms                       | 55 (22.9)                  | 49/95 (51.5)                             | 6/146 (4.1)                               | < 0.001        |

<sup>1</sup>Median (range). FDG: <sup>18</sup>F-Fluorodeoxyglucose; BMI: Body mass index; F/U: Follow up; CRC: Colorectal cancer.

**Table 2** Positron emission tomography/computed tomograph indications of the eligible colorectal lesions

| Initial cancer staging<br>( <i>n</i> = 99) | After complete cancer resection<br>( <i>n</i> = 87) | After cancer chemotherapy<br>( <i>n</i> = 12) | Health screening<br>( <i>n</i> = 31) | Others<br>( <i>n</i> = 12) |
|--|---|---|--------------------------------------|----------------------------|
| Stomach: 33                                | Colorectum: 67                                      | Stomach: 2                                    |                                      |                            |
| MUO: 19                                    | Stomach: 8  | OBGY: 2                                       |                                      |                            |
| OBGY: 12                                   | Others: 12  | Others: 8                                     |                                      |                            |
| Lung: 10                                   |   |   |                                      |                            |
| Hepatobiliary: 9                           |   |   |                                      |                            |
| Others: 16                                 |   |   |                                      |                            |

MUO: Metastasis of unknown origin; OBGY: Obstetrics and gynecology.

increased abdominopelvic <sup>18</sup>F-FDG uptake, localized by PET/CT images to the colorectum, and distinguishable from the background colonic uptake, were reviewed for interpretation<sup>[6]</sup>. The incidence of incidental focally increased <sup>18</sup>F-FDG uptake in the colorectum was calculated and the intensity of the uptake was measured by calculating SUVmax from the attenuation-corrected PET data, using the software provided by the workstation manufacturer. Lesion size on PET/CT was not part of the inclusion criteria. The PET/CT findings were correlated with the various colonoscopic and histopathological results.

### Colonoscopy and surgery

Two hundred forty-one colorectal pathological specimens obtained from 212 subjects who had undergone a total colonoscopy were studied. All colorectal lesions that appeared to be of neoplastic origin were biopsied and removed by polypectomy and/or surgery. Colonoscopy with a pathological examination was considered the gold standard diagnostic method.

Adenomas were considered advanced when they were villous, ≥ 10 mm in size, or had high-grade dysplasia. Colorectal cancers and advanced adenomas are collectively referred to as advanced colorectal neoplasms. If a subject had more than one detectable colorectal lesion, then each lesion was analyzed individually.

### Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the incidental focal <sup>18</sup>F-FDG uptake in predicting advanced colorectal neoplasms and CRC were calculated. Receiver-operating characteristic (ROC) curve analysis was used to determine the significant predictive SUVmax cutoff value for advanced colorectal neoplasms and cancer. For all tests, a *P* value < 0.05 was considered statistically significant. All statistical analyses were performed with SPSS, version 16 (SPSS, Chicago, IL, United States).

## RESULTS

### Patient characteristics

Two hundred forty-one eligible colonoscopic lesions were obtained from 212 individual subjects. Of these, 95 lesions showed incidental focal colorectal <sup>18</sup>F-FDG uptake on PET/CT and 146 did not. The characteristics of the 241 colonoscopic lesions evaluated in this study are shown in Table 1. Of these, 153 were male and the mean age was 62 years (range: 29-86 years). The sex, age, and body mass index did not differ significantly between the <sup>18</sup>F-FDG positive and negative groups. The indications for PET/CT were as part of the initial staging of malignancies other than CRC in 99 cases, after the complete resection of malignancies other than CRC in 87 cases, and after chemotherapy for malignancies other than CRC in 12 cases. Thirty-one scanned cases were performed during health screening and 12 during screening for other purposes (Table 2).

The pathological diagnoses were 37 cancers, 24 acute or chronic inflammation, 140 non specific, and 18 advanced adenomatous lesions among a total of 40 adenomatous lesions (Table 1).

### Diagnostic accuracy of focal incidental uptake on PET/CT

In those lesions showing incidental uptake on PET/CT, 49 of 95 (51.5%) had advanced colorectal neoplasms on colonoscopy corresponding to the site of <sup>18</sup>F-FDG uptake, whereas in those lesions without uptake, only 6

of 146 (4.1%) were found to have advanced colorectal neoplasms on colonoscopy. This indicates a statistically significant difference between the two groups ( $P < 0.001$ ). Overall, the sensitivity, specificity, PPV, NPV, and accuracy of incidental focal <sup>18</sup>F-FDG uptake in predicting advanced colorectal neoplasms were 89.1%, 75.3%, 51.6%, 95.9%, and 78.4%, respectively. For detecting only CRC, the sensitivity, specificity, PPV, NPV, and accuracy of incidental focal <sup>18</sup>F-FDG uptake were 89.2%, 69.6%, 34.7%, 97.3%, and 72.6%, respectively.

### **SUVmax and colorectal neoplastic lesions**

The statistically significant SUVmax cutoff value that allowed the discrimination of advanced colorectal neoplasms from non advanced neoplastic and non neoplastic lesions was 4.35 (area under the ROC curve 0.755,  $P < 0.001$ ), with a sensitivity, specificity, PPV, NPV, and accuracy of 75.5%, 65.2%, 69.8%, 71.4%, and 70.5%, respectively. For CRC, 5.05 was the statistically significant SUVmax cutoff value (area under the curve 0.817,  $P < 0.001$ ), with a sensitivity, specificity, PPV, NPV, and accuracy of 84.8%, 71.0%, 80.9%, 89.8%, and 75.8%, respectively.

## **DISCUSSION**

PET/CT is a well-accepted technique for the diagnosis and staging of several malignancies because it provides more accurate functional and anatomical assessments than CT or other conventional imaging modalities<sup>[6]</sup>. Some researchers demonstrated that multidetector CT colonography has excellent sensitivity and specificity for lesions  $< 10$  mm. In fact, one study reported superior sensitivity compared to conventional colonoscopy for the detection of adenomas 10 mm or larger (93.8% *vs* 85.5%)<sup>[7]</sup>. A recent study looking at the feasibility of FDG-PET/CT colonography in patients with high clinical suspicion of colorectal carcinoma showed promising results in being an “all-in-one” staging modality, but was practically challenging and still required a formal colonoscopy for histopathological confirmation<sup>[8]</sup>.

Physiological <sup>18</sup>F-FDG uptake within the gastrointestinal tract, with variable intensities and localization patterns, has previously been described. Focal tracer uptake is frequently seen at the gastroesophageal junction, moderate uptake in the stomach, low-intensity uptake in the small bowel, and diffuse or focal uptake in the colon<sup>[9]</sup>. The mechanisms underlying this physiological activity are unclear. Muscular peristaltic activity, the presence of lymphoid tissue in the cecum, high concentrations of white blood cells in the bowel wall, and/or the presence within the bowel wall of cells secreting <sup>18</sup>F-FDG, especially in cases of cecum distention, have been hypothesized<sup>[10]</sup>. Increased FDG uptake can also be associated with inflammation, such as enterocolitis<sup>[11]</sup> or inflammatory bowel disease<sup>[12]</sup>. In our study, only focal or multifocal FDG findings were considered significant because diffuse uptake is considered to have a physiological origin.

Focal colorectal <sup>18</sup>F-FDG uptake indicates a high (70%-80%) probability of corresponding abnormal histopathological findings<sup>[13-15]</sup>. Despite this increase in confidence and reduction in the number of suggestive or equivocal lesions, the precise localization of increased <sup>18</sup>F-FDG foci using PET/CT cannot at present resolve the diagnostic dilemma of abnormal tracer uptake in the colorectum. For this reason, we assessed the clinical significance of incidental focal colorectal <sup>18</sup>F-FDG uptake on PET/CT and also determined a clinically useful SUVmax cutoff value for the detection of advanced adenoma and cancer. Our results are consistent with two large studies that showed that PET/CT is a sensitive tool with which to detect premalignant lesions<sup>[15,16]</sup>. In another previous study, PET/CT had a sensitivity of 74% and a specificity of 84% in the detection of colonic abnormalities<sup>[17]</sup>. However, that study was limited by its small sample size (39 patients).

Although previous studies have evaluated the etiology of incidental PET/CT findings in the colon<sup>[15,18]</sup>, we believe that our study contributes to the clinician's perspective by providing a SUVmax cutoff value that discriminates advanced colorectal neoplasms from non advanced lesions.

Larger series, with 3210 and 2000 patients, identified 20 (12 villous adenomas, six carcinomas, and two tubular adenomas) and 13 abnormalities (seven adenomatous polyps and six carcinomas), respectively, with colonoscopy<sup>[5,19]</sup>. However, they failed to determine a significant SUVmax cutoff value that would be clinically useful in discriminating advanced colorectal neoplasms from malignant carcinomas. In our study, the cutoff SUVmax value was higher in the CRC group than in the advanced neoplasms group (5.05 *vs* 4.35, respectively). We showed that the intensity of FDG uptake correlated with the severity of the lesion. This is consistent with the results of previous studies, although in those, the cutoff SUVmax value that allowed advanced and non advanced neoplasms to be distinguished was not determined, whereas this was one of the main goals of our study<sup>[3,20]</sup>. In the present study, a SUVmax  $\geq 4.35$  increased the likelihood of advanced colorectal neoplasms and a SUVmax  $\geq 5.05$  increased the likelihood of CRC.

One limitation of our study was a potential selection bias because we included those cases which had undergone PET/CT for the initial staging of a non colorectal malignancy and those in whom surveillance PET/CT had been performed for clinical assessments after curative CRC resection. However, we believe that our study more accurately reflects the real-life clinical situation by including them. The prevalence of focal colonic lesions in our study is also similar to that in previous studies that have used PET to detect colonic lesions<sup>[19,21,22]</sup>.

In conclusion, our study shows that advanced colorectal adenomas and malignant carcinomas should be suspected when focal <sup>18</sup>F-FDG uptake is detected by PET/CT and that this is clinically significant in most cases. Incidental focal colorectal <sup>18</sup>F-FDG uptake detected on PET/CT with a SUVmax  $\geq 4.35$  and a SUVmax

≥ 5.05 increases the likelihood of advanced colorectal neoplasms and CRC, respectively.

Our study confirms the necessity of colonoscopy when incidental FDG uptake is detected on PET/CT to allow the early diagnosis and management of advanced colorectal neoplasms.

## COMMENTS

### Background

Positron emission tomography (PET)/computed tomography (CT) is commonly used during the evaluation of malignant tumors. Increased focal uptake of <sup>18</sup>F-fluorodeoxyglucose (<sup>18</sup>F-FDG) in the colon and rectum can be found incidentally during FDG-PET/CT.

### Research frontiers

Several previous studies have shown that not only is a high incidence of unexpected gastrointestinal malignancies associated with incidental <sup>18</sup>F-FDG avidity, but also that the presence of these lesions can change patient management. However, the significance of incidental focal colorectal <sup>18</sup>F-FDG uptake observed on PET/CT has not yet been fully clarified.

### Innovations and breakthroughs

The authors have evaluated the largest number of cases yet assessed to determine the value of colonoscopy when incidental FDG uptake is observed on PET/CT images. In this study, the authors investigated the clinical significance of incidental focal colorectal <sup>18</sup>F-FDG uptake detected on PET/CT, and determined the maximal standardized uptake value (SUVmax) for detecting advanced adenoma and cancer.

### Applications

This study shows that advanced colorectal adenomas and malignant carcinomas should be suspected when focal <sup>18</sup>F-FDG uptake is detected by PET/CT and that this is clinically significant in most cases. This study confirms the necessity of colonoscopy when incidental FDG uptake is detected on PET/CT to allow the early diagnosis and management of advanced colorectal neoplasms.

### Terminology

FDG-PET/CT display increased sites of glycolysis and by increased focal colorectal uptake of <sup>18</sup>F-FDG and is capable to detect the foci of tumors. The SUVmax of <sup>18</sup>F-FDG is a way to measure the intensity of the <sup>18</sup>F-FDG uptake during FDG-PET/CT. It is measured using the software provided by the FDG-PET/CT workstation manufacturer.

### Peer review

The authors assessed the clinical significance of incidental focal colorectal <sup>18</sup>F-FDG uptake on FDG-PET/CT. The accuracy of incidental focal <sup>18</sup>F-FDG uptake in identifying advanced colorectal neoplasms and colorectal cancer (CRC) were 78.4% and 72.6%, respectively. In addition, they demonstrated higher cutoff SUVmax value in the CRC group than in the advanced neoplasms group (5.05 vs 4.35, respectively).

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**P- Reviewer** Assadi M **S- Editor** Huang XZ  
**L- Editor** A **E- Editor** Xiong L



## Perceptions about preventing hepatocellular carcinoma among patients with chronic hepatitis in Taiwan

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Author contributions: Chen YW and Perng DS designed and performed the research and analyzed the data; Chen YW, Liu CC and Perng DS wrote the paper.

Supported by E-Da Hospital, EDAH 99021

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Received: January 31, 2013 Revised: April 2, 2013

Accepted: April 10, 2013

Published online: June 14, 2013

HCC was significantly correlated with their age. The participants' perceptions were also associated with their educational levels, household incomes and knowledge of hepatitis. Older patients and those with a lower socioeconomic status tended to have negative perceptions and less knowledge of hepatitis. Multivariate logistic regression further indicated that the participants' age ( $B = -0.044$ ,  $SE = 0.017$ , odds ratio = 0.957,  $P = 0.008$ , 95%CI: 0.926-0.989) and perceived barriers ( $B = -0.111$ ,  $SE = 0.030$ , odds ratio = 0.895,  $P < 0.001$ , 95%CI: 0.845-0.949) were correlated with their willingness to receive antiviral therapy.

**CONCLUSION:** Healthcare professionals should provide appropriate and effective guidance to increase their patients' awareness and to decrease the perceived barriers for continuing surveillance and antiviral therapy.

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**Key words:** Antiviral therapy; Health perception; Hepatitis B; Hepatitis C; Hepatocellular carcinoma; Health knowledge

**Core tip:** Chronic hepatitis B/C carriers may benefit from regular surveillance for allowing an early diagnose of hepatocellular carcinoma (HCC). In addition, raising awareness of and health perceptions about HCC, and increasing willingness to receive antiviral therapy for preventing the development of HCC are crucial in patients with chronic hepatitis B/C, particularly in rural areas.

### Abstract

**AIM:** To measure patient perceptions about preventing hepatocellular carcinoma (HCC) and to predict the factors that influence patient willingness to receive therapy.

**METHODS:** A cross-sectional descriptive study was conducted at an outpatient clinic of a medical institution in southern Taiwan. Four hundred patients with chronic hepatitis B/C were recruited as participants. Two structured questionnaires based on the health belief model were utilized in this study, including the scales of perceptions about preventing HCC and knowledge of hepatitis B/C.

**RESULTS:** The statistical results demonstrated that the participants' perceived susceptibility ( $r = -0.22$ ,  $P < 0.001$ ), benefits ( $r = -0.11$ ,  $P = 0.028$ ) and cues to action ( $r = -0.12$ ,  $P = 0.014$ ) about the prevention of

Chen YW, Liu CC, Perng DS. Perceptions about preventing hepatocellular carcinoma among patients with chronic hepatitis in Taiwan. *World J Gastroenterol* 2013; 19(22): 3459-3465 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3459.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3459>

## INTRODUCTION

Chronic liver disease is the eighth most common cause of death, and hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in Taiwan<sup>[1]</sup>. More than 80% of primary HCC is attributed to chronic infection with hepatitis B/C viruses<sup>[2,3]</sup>. To prevent further development of HCC, patients infected with hepatitis B/C should maintain a healthy lifestyle, reduce alcohol consumption, undergo regular liver function tests and abdominal sonography, and receive antiviral therapy, if necessary<sup>[4,5]</sup>. Chronic infection with hepatitis B/C is the most important cause of HCC. The early development of HCC is asymptomatic, and patients with chronic hepatitis are usually unaware of their carrier status, the symptoms and signs of HCC, and the importance of regular surveillance and treatment. During the early stage of HCC, when tumors are less than 2 cm in diameter, even expert radiologists have difficulty differentiating a cirrhotic nodule from a malignant tumor<sup>[4]</sup>. These tumors are highly heterogeneous, and HCC is nearly always fatal once the tumors cannot be eradicated by surgical or ablative approaches<sup>[4]</sup>. In addition, patients with HCC detected at the early asymptomatic stage have better survival rates than those who are diagnosed when symptomatic<sup>[6]</sup>. Therefore, increasing awareness of and health perceptions about HCC are crucial in patients with chronic hepatitis B/C, particularly in rural areas.

### Perceptions about preventing HCC

Henchoz *et al.*<sup>[7]</sup> defined perceived health as “an individual’s evaluation of his or her own health” which can determine personal health values and influence health behaviors<sup>[8]</sup>. Furthermore, Kartal *et al.*<sup>[9]</sup> stated that “self-perceived health is a subjective measure that can be calculated at an individual level. It gives an indication of how an individual feels about the condition of his/her own health.” To examine patient perceptions about preventing HCC, a health belief model was utilized as a theoretical framework in the present study. The health belief model was formulated using five concepts to explain attitudes and behaviors of individuals, including perceived susceptibility, perceived severity, perceived benefits of action, perceived barriers to action, and cues to action<sup>[10,11]</sup>. The model was developed to predict compliance with preventive health recommendations<sup>[6,12]</sup> and to specify the individuals’ values and beliefs about health and their influence on choices, especially for explaining screening behaviors<sup>[2,13]</sup>.

Perceived susceptibility refers to a person’s experience with a potentially harmful condition<sup>[11]</sup>. This concept measures whether patients with asymptomatic hepatitis perceive themselves as being at high risk and believe that they could acquire HCC. Perceived severity indicates individual concerns about the seriousness and consequences to the quality of life upon development of cancer, including the physical, mental, and social perspectives<sup>[11]</sup>. This concept can measure the impact of chronic hepatitis on a person’s life and his or her belief that people infected

could possibly die from liver cancer.

Perceived benefits of action refer to whether one believes the potential effectiveness of recommended health actions in preventing or reducing the risk or seriousness of a disease<sup>[11]</sup>. Perceived benefits of action can assess one’s beliefs that regular screening and antiviral therapy reduce the risks of progressing to liver cirrhosis and cancer.

Perceived barriers to action are the negative aspects of anticipated health behaviors that patients adopt for prevention or early detection of hepatic cancer, such as the inconvenience of seeing a doctor, the waiting time and pain involved in giving blood samples, and the side effects from taking medications. Patients may believe that regular screening of liver function and abdominal sonography are not necessary if they do not experience any symptoms of discomfort. Moreover, if patients receive antiviral therapy, they may develop physical and psychological side effects, such as fever, headache, fatigue, nausea, vomiting, anxiety and even depression. These adverse events often make patients feel uncomfortable, resulting in early withdrawal from therapy.

Cues to action measure the perceived social and environmental influences that stimulate an individual’s desire to take health-related action<sup>[11]</sup>. The concept can assess whether individuals with hepatitis have any family members, relatives or friends who are infected with hepatitis or whether the patients have ever followed the advice of healthcare professionals about regular check-ups of liver function, abdominal sonography, and antiviral therapy. Therefore, the five concepts of the health belief model were applied in the present study to explore health perceptions about preventing HCC among patients with chronic hepatitis B/C.

### Knowledge of hepatitis

Most people are not aware of the routes of viral transmission of hepatitis B/C and that these viruses spread more easily than the human immunodeficiency virus<sup>[14]</sup>. Ma *et al.*<sup>[15]</sup> found that populations with minimal education and low socioeconomic status are vulnerable to hepatitis infection. If high-risk groups with chronic hepatitis are not aware of the importance of regular surveillance and antiviral therapy, then their condition could gradually progress to liver cirrhosis and hepatic cancer. Wai *et al.*<sup>[16]</sup> identified that those hepatitis B carriers with high knowledge were significantly younger, and more likely to have received college education in Singapore. Treloar *et al.*<sup>[17]</sup> conducted a cross-sectional survey and indicated that knowledge is a precursor to decisions about treatment of hepatitis C, particularly for those patients who are less engaged with hepatitis C treatment and those with lower literacy.

The prevention of HCC in high-risk populations depends on regular serum screening, abdominal sonography and antiviral therapy. Regular screening of liver function, including assays to measure serum aspartate aminotransferase and alanine aminotransferase levels,  $\alpha$ -fetoprotein assays and abdominal sonography are strongly recom-

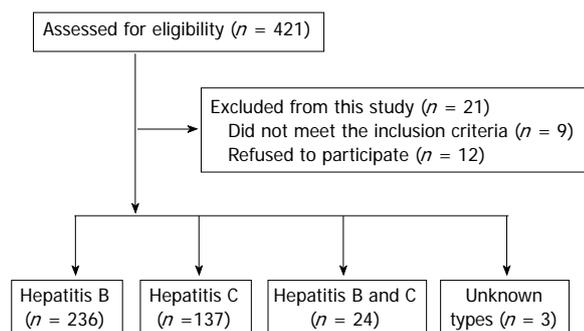


Figure 1 Sampling schema.

mended for patients with chronic hepatitis to ensure earlier detection of HCC and better survival<sup>[6,18]</sup>. Therefore, the purpose of the present study was to measure patient perceptions about the prevention of HCC, knowledge of hepatitis B/C, and frequency of hepatic check-ups, as well as to predict the factors that influence patient willingness to receive antiviral therapy.

## MATERIALS AND METHODS

### Participants

Four hundred patients with chronic hepatitis B/C from the outpatient clinic in a medical institution were recruited as participants. The teaching hospital is located in a rural area in southern Taiwan. The markers for hepatitis B are positive hepatitis B surface antigen (HBsAg) and positive antibody for hepatitis C (anti-HCV)<sup>[18]</sup>. The inclusion criteria for participants were (1) HBsAg (+) and anti-HBs (-), and/or (2) anti-HCV (+) in serum tests for more than 6 mo. The patients who were younger than 18 years and those who did not meet the inclusion criteria were excluded from this study. The sampling schema is shown in Figure 1.

### Instruments

This study had a cross-sectional, descriptive design. Two structured questionnaires developed by Chao *et al.*<sup>[19]</sup> and based on a health belief model were utilized in this investigation, namely, the scales of perceptions about preventing HCC and knowledge of hepatitis B/C. After obtaining permission from the original developers, a pilot test was conducted with 40 target adults (who did not participate in the main study) to examine whether there were any ambiguous statements in the questionnaires and to establish the reliability and validity of the instruments.

The scale of perceptions about preventing HCC consisted of five dimensions and 34 items, including the dimensions of perceived susceptibility (3 items), perceived severity (5 items), perceived benefits (3 items), perceived barriers (15 items), and cues to action (8 items). Each item was designed as a 5-point Likert scale, ranging from 1 “strongly disagree” to 5 “strongly agree.” Higher scores indicated greater degrees of perceived susceptibility, severity, benefits, barriers and cues to action. Cronbach’s alpha values were as follows: whole scale, 0.8; perceived suscep-

tibility, 0.71; perceived severity, 0.78; perceived benefits, 0.68; perceived barriers, 0.84; and cues to action, 0.77<sup>[19]</sup>. The internal consistency reliability with Cronbach’s alpha was 0.72 in this study. The values of Cronbach’s alpha for each dimension of perception indicated the reliability of the questionnaire utilized in this study.

The scale of knowledge of hepatitis B/C had 15 items, including liver function (2 items), blood tests for hepatitis (1 item), symptoms of hepatitis (2 items), definition of hepatitis (1 item), modes of viral transmission (4 items), screening to prevent liver cancer (2 items) and infectious status of hepatitis (3 items). Participants responded to these items using the following options: 0 = “do not know” or “false” and 1 = “true.” The total scores of 15 items represented a dimension of the participants’ knowledge. A higher score indicated a better understanding of hepatitis. The internal consistency with the Kuder-Richardson coefficient was previously determined to be 0.71<sup>[19]</sup>, whereas in the present study, the Kuder-Richardson coefficient was 0.62.

### Procedure

Approval to conduct this study was obtained from the institutional review board. This study was conducted between January and December of 2010. When patients visited the outpatient hepatic clinic and met the inclusion criteria, the researchers used convenient sampling to choose the participants. Subsequently, the participants were placed in another room to complete the questionnaires without interruption. All of the questionnaires were anonymous and separated from the consent forms. Prior to administering the questionnaires, the researchers informed the patients of the purpose of this study and explained that the responses would not influence their treatment.

### Statistical analysis

The Statistical Package for the Social Sciences (SPSS, Chicago, IL, United States) version 17.0 was used to analyze the data. The data analysis for this study included descriptive statistics, independent-samples *t*-tests, the chi-square test, a one-way analysis of variance (ANOVA), Pearson’s correlation, and logistic regression.

## RESULTS

### Participants’ demographics

Four hundred questionnaires were distributed and completed by 174 females (43.5%) and 226 males (56.5%). The average age of the respondents was 54 years (range: 18-89 years). The participants’ demographic information is summarized in Table 1.

### Participants’ hepatitis-related information

In this survey, 236 (59%) participants had hepatitis B, and 137 (34.3%) had hepatitis C. Twenty-four (6.0%) participants reported having both hepatitis B and C, and three (0.5%) did not know what types of hepatitis they had. In terms of vaccination, 298 patients (74.5%) responded

**Table 1** Participants' demographics (*n* = 400) *n* (%)

| Participants' demographics       | Value      |
|----------------------------------|------------|
| Gender                           |            |
| Female                           | 174 (43.5) |
| Male                             | 226 (56.5) |
| Marital status                   |            |
| Married                          | 353 (88.3) |
| Single                           | 27 (6.8)   |
| Widowed                          | 10 (2.5)   |
| Divorced/separated               | 9 (2.3)    |
| Educational level                |            |
| Illiterate                       | 41 (10.3)  |
| Elementary school                | 109 (27.3) |
| Junior high school               | 73 (18.3)  |
| High school                      | 104 (26)   |
| College/university               | 62 (15.5)  |
| Graduate school                  | 11 (2.8)   |
| Occupation                       |            |
| Laborers                         | 82 (20.5)  |
| Businessmen                      | 61 (15.3)  |
| Farmers                          | 36 (9.0)   |
| Fishermen                        | 9 (2.3)    |
| Military personnel               | 2 (0.5)    |
| Government employees             | 19 (4.8)   |
| Homemakers                       | 78 (19.5)  |
| Others                           | 113 (28.3) |
| Household income (NT dollars/mo) |            |
| Below 10000                      | 101 (25.3) |
| 10000-20000                      | 63 (15.8)  |
| 21000-30000                      | 78 (19.5)  |
| 31000-40000                      | 47 (11.8)  |
| 41000-50000                      | 35 (8.8)   |
| Above 50000                      | 76 (19.0)  |

that they had never been vaccinated for hepatitis B. Thirty-eight patients (9.5%) had received the vaccine, and 64 (16.0%) did not know if they had been vaccinated.

Most of the patients (*n* = 292, 73.0%) reported that the virus had been found during a regular physical examination, 45 (11.3%) through the screening process for blood donation, 21 (5.3%) upon health screening in the community and 42 (10.5%) in other ways. Moreover, 332 patients (83.0%) were willing to receive antiviral therapy, 35 patients (8.8%) did not want to receive the therapy, and 33 patients (8.3%) were unaware that this antiviral therapy was available or whether they needed to receive this therapy.

Regarding the sources of hepatitis-related health information, 282 participants stated that they obtained this information from healthcare professionals, 189 from their relatives and friends, 220 from television, 173 from newspapers and magazines, and 70 from educational pamphlets or brochures. In addition, over one-half of the participants underwent regular blood screening every 3 mo (*n* = 247, 61.8%) and abdominal sonography every 6 mo (*n* = 183, 45.8%) according to the statistical results.

### Health perceptions and knowledge of hepatitis

The mean scores for each dimension of the health perceptions were as follows: perceived susceptibility,  $10.26 \pm 2.18$ ; perceived severity,  $20.43 \pm 3.53$ ; perceived benefits,  $12.07 \pm 1.63$ ; perceived barriers,  $37.60 \pm 7.06$ ; and cues

to action,  $27.01 \pm 4.73$ . The mean score for knowledge of hepatitis B/C in this study was  $7.36 \pm 1.87$ .

### Relationships among the patients' demographics, health perceptions, knowledge, screening, and willingness to receive antiviral therapy

Pearson's correlation revealed significantly negative correlations between the participants' age and their health perceptions, except for perceived severity and barriers. Likewise, the participants' age was significantly and negatively correlated with their level of knowledge ( $\gamma = -0.20$ ,  $P < 0.001$ ). The statistical results also suggested that the participants' perceptions had a significantly positive correlation with their knowledge of hepatitis. In addition, the patients' gender was not significantly correlated with their perceptions (except for perceived barriers) and knowledge, as presented in Table 2.

Moreover, a one-way ANOVA indicated that there were significantly positive correlations between the participants' educational levels and their perceptions (except for perceived severity) and knowledge of hepatitis. Significant relationships were found between the patients' frequency of blood screening for HCC prevention and the perceived severity ( $F = 3.67$ ,  $P = 0.001$ ) as well as between the frequency of abdominal sonography and perceived barriers ( $F = 3.66$ ,  $P = 0.001$ ).

Furthermore, the frequency of abdominal sonography was significantly correlated with the patients' knowledge ( $F = 2.74$ ,  $P = 0.009$ ) and with being vaccinated ( $F = 13.88$ ,  $P < 0.001$ ). A significant correlation existed between the participants' willingness to receive antiviral therapy and perceived barriers ( $F = 3.55$ ,  $P < 0.001$ ). The researchers further divided the participants into three groups by age (18-39 years, 40-64 years, and 65-89 years old). A significantly positive correlation was found between the participants' age and their willingness to receive antiviral therapy ( $\chi^2 = 13.97$ ,  $P = 0.007$ ), but there was no correlation between the participants' gender and their willingness to receive therapy ( $\chi^2 = 2.35$ ,  $P = 0.309$ ).

### Predictors for willingness to receive antiviral therapy

The participants' age, five dimensions of health perceptions and knowledge of hepatitis were analyzed as independent variables in the multivariate logistic regression. The results indicated that the participants' age ( $B = -0.044$ ,  $SE = 0.017$ , odds ratio = 0.957,  $P = 0.008$ , 95%CI: 0.926-0.989) and perceived barriers ( $B = -0.111$ ,  $SE = 0.030$ , odds ratio = 0.895,  $P < 0.001$ , 95%CI: 0.845-0.949) were significantly associated with their willingness to receive antiviral therapy, as presented in Table 3. Adjusting for other effects in the logistic regression model, for every one-year increase in age, the participants were 0.957 times less likely to have received antiviral therapy.

## DISCUSSION

Most of the participants in this study had elementary and high school educations, and their household in-

**Table 2 Relationships between the participants' demographics, health perceptions, and knowledge of hepatitis (*n* = 400)**

| Variables                                   | Perceived susceptibility | Perceived severity | Perceived benefits | Perceived barriers | Cues to action | Knowledge       |
|---|--------------------------|--------------------|--------------------|--------------------|----------------|-----------------|
| Age <sup>1</sup>                            | -0.22 (< 0.001)          | -0.07 (0.162)      | -0.11 (0.028)      | 0.08 (0.121)       | -0.12 (0.014)  | -0.20 (< 0.001) |
| Gender <sup>2</sup>                         | 1.53 (0.135)             | 0.92 (0.362)       | 0.23 (0.819)       | 2.14 (0.039)       | -0.04 (0.967)  | -1.14 (0.254)   |
| Marital status <sup>3</sup>                 | 0.64 (0.589)             | 1.91 (0.127)       | 0.40 (0.753)       | 0.19 (0.905)       | 0.08 (0.973)   | 1.13 (0.336)    |
| Education level <sup>3</sup>                | 2.96 (0.008)             | 1.30 (0.257)       | 3.95 (0.001)       | 6.31 (<0.001)      | 2.27 (0.036)   | 9.17 (< 0.001)  |
| Occupation <sup>3</sup>                     | 2.52 (0.015)             | 0.92 (0.489)       | 0.92 (0.490)       | 2.55 (0.014)       | 0.59 (0.767)   | 2.61 (0.012)    |
| Monthly income <sup>3</sup>                 | 2.27 (0.047)             | 0.63 (0.679)       | 3.91 (0.002)       | 3.91 (0.002)       | 2.87 (0.015)   | 6.59 (< 0.001)  |
| Willingness to receive therapy <sup>3</sup> | -0.75 (0.453)            | -1.15 (0.252)      | -0.42 (0.675)      | 3.55 (< 0.001)     | -1.67 (0.096)  | -0.48 (0.633)   |
| Had received a vaccine <sup>3</sup>         | 2.98 (0.052)             | 0.07 (0.937)       | 2.04 (0.131)       | 1.94 (0.145)       | 3.14 (0.044)   | 13.88 (< 0.001) |
| Frequency of blood screening <sup>3</sup>   | 1.28 (0.265)             | 3.67 (0.001)       | 0.43 (0.859)       | 1.87 (0.084)       | 1.66 (0.129)   | 1.44 (0.198)    |
| Frequency of abd. sonography <sup>3</sup>   | 0.51 (0.830)             | 1.44 (0.188)       | 0.55 (0.798)       | 3.66 (0.001)       | 1.48 (0.173)   | 2.74 (0.009)    |

Data are presented as <sup>1</sup>*r* of Pearson's correlation/<sup>2</sup>*t* of independent *t*-test/<sup>3</sup>*F* of one-way analysis of variance (*P* value).

**Table 3 Predictors of receiving antiviral therapy: Logistic regression (*n* = 364)**

| Variables      | <i>B</i> | <i>SE</i> | <i>OR</i> | <i>P</i> | 95%CI       |
|----------------|----------|-----------|-----------|----------|-------------|
| Age            | -0.044   | 0.017     | 0.957     | 0.008    | 0.926-0.989 |
| Perceptions    |          |           |           |          |             |
| Susceptibility | 0.011    | 0.096     | 1.011     | 0.912    | 0.837-1.220 |
| Severity       | 0.061    | 0.053     | 1.063     | 0.255    | 0.957-1.180 |
| Benefits       | -0.156   | 0.130     | 0.856     | 0.232    | 0.663-1.105 |
| Barriers       | -0.111   | 0.030     | 0.895     | < 0.001  | 0.845-0.949 |
| Cues to action | 0.059    | 0.042     | 1.060     | 0.160    | 0.977-1.150 |
| Knowledge      | -0.102   | 0.117     | 0.903     | 0.383    | 0.719-1.135 |

comes were between \$10000 and \$ 30000 NT dollars. The patients in rural areas had lower educational levels and household incomes than those in urban areas. The results revealed that the participants' education levels were significantly correlated with their health perceptions and knowledge about hepatitis. This finding is consistent with the previous study<sup>[16,20]</sup>. The participants' education levels were not associated with the perceived severity in this study, which may be because the patients themselves might not have perceived the potentially severe consequences of being hepatitis carriers and might not have been aware of the asymptomatic development of HCC.

In addition, Hsu *et al.*<sup>[21]</sup> stated that educational attainment and knowledge of hepatitis B are associated with individual willingness to receive screening and vaccination. As previous studies have indicated, subjects with higher levels of knowledge were significantly more likely to receive blood screening for hepatitis B and to have received the vaccine<sup>[14,22-25]</sup>. However, the majority of the participants responded that they had never been vaccinated for hepatitis B prior to infection. Therefore, healthcare providers should educate patients with chronic hepatitis to increase their awareness and knowledge and to take action for prevention or early detection of HCC.

Likewise, household income was significantly and positively correlated with perceived susceptibility, benefits, barriers, cues to action, and knowledge. This finding revealed that patients with lower incomes perceived less susceptibility, benefits, and cues to action for preventing liver cancer, and they had less knowledge of hepatitis. These results were similar to those of a previous study<sup>[14]</sup>.

Thus, it is necessary to focus on the low socioeconomic population and to increase their perceptions and knowledge to reduce health disparities about liver cancer in the community.

Moreover, the vast majority of the participants usually obtained hepatitis-related health information and knowledge from healthcare professionals, public media (such as television, newspapers and magazines), and educational brochures and pamphlets. Cacoub *et al.*<sup>[26]</sup> indicated that the therapeutic education for those hepatitis patients can improve the probability of compliance with antiviral therapy. Hence, to raise perceptions and knowledge related to HCC, appropriate and effective instruction based on the individual's age and educational level is crucial, especially in rural areas.

Furthermore, the predictors for patient willingness to receive antiviral therapy were age and the perceived barriers. The participants' age was negatively associated with their health perceptions and knowledge of hepatitis. This finding indicates that elderly patients tend to have lower perceived susceptibilities, benefits, and cues to action for prevention of liver cancer; less knowledge about hepatitis; and less willingness to receive antiviral therapy. Similarly, Henchoz *et al.*<sup>[7]</sup> found that the paradox between health status and health perception diverges with advancing age.

Conversely, the participants' perceived barriers had a significantly negative relationship to their willingness to receive antiviral therapy. High barriers to screening and adverse consequences from antiviral therapy such as pain, high costs, and time expenditure may discourage patients from regular screenings and cause them to withdraw early from antiviral therapy. This finding is also consistent with previous studies<sup>[2,19]</sup>. Thus, identifying the patients' inherent barriers is the first crucial step for increasing participation in preventive behaviors<sup>[2]</sup>.

There were two limitations in the present study. Because the participants were restricted to an outpatient clinic of a rural medical institution, generalization beyond this population may be limited. Further research should be conducted in multiple diverse areas. In addition, this study had a cross-sectional descriptive design, and its analytic results do not indicate causality.

In conclusion, patient health perceptions about preventing HCC are related to their age, education levels, household income, and knowledge of hepatitis. Members of the high-risk population of hepatitis B/C carriers should be aware of the serious consequences of chronic active infection and should receive regular surveillance and treatment. To successfully complete a series of medical regimens, detailed explanation and communication regarding possible adverse effects are essential before starting antiviral therapy, especially in elderly individuals. In terms of the implications for clinical practice, health-care professionals should reduce patient discomfort and inconvenience as much as possible and should strive to remove barriers to screening and treatment. Moreover, clinicians may utilize educational programs as an intervention to increase patient perception, knowledge and willingness to receive antiviral therapy.

## ACKNOWLEDGMENTS

The authors sincerely appreciate the research assistants for data collection and entry and thank the participants who provided information.

## COMMENTS

### Background

Chronic liver disease is the eighth most common cause of death, and hepatocellular carcinoma (HCC) is the second leading cause of cancer-related death in Taiwan. More than 80% of primary HCC is attributed to chronic infection with hepatitis B/C viruses. The early development of HCC is asymptomatic, and patients with chronic hepatitis are usually unaware of their carrier status, the symptoms and signs of HCC, and the importance of regular surveillance and treatment.

### Research frontiers

The participants' educational levels were significantly correlated to their health perceptions (except for perceived severity) and knowledge about hepatitis. The elderly patients and those with lower household incomes tend to have lower perceived susceptibility, benefits, and cues to action for prevention of HCC, less knowledge about hepatitis and less willingness to receive antiviral therapy. The results indicated that the participants' age and perceived barriers were significantly associated with their willingness to receive antiviral therapy.

### Innovations and breakthroughs

Chronic hepatitis B/C carriers may benefit from regular surveillance for allowing an early diagnose of HCC. In addition, raising awareness of and health perceptions about HCC, and increasing willingness to receive antiviral therapy for preventing the development of HCC are crucial in patients with chronic hepatitis B/C, particularly in rural areas.

### Applications

In terms of the implications for clinical practice, health-care professionals should reduce patient discomfort and inconvenience as much as possible and should strive to remove barriers to screening and treatment. Moreover, clinicians may utilize educational programs as an intervention to increase patient knowledge and willingness to receive antiviral therapy.

### Terminology

Health perception is defined as "an individual's evaluation of his or her own health". Health perception can determine personal health values and influence health behaviors.

### Peer review

The authors conducted a cross-sectional descriptive study to identify several important factors of healthy perceptions about preventing HCC in Taiwan. The participants' age and perceived barriers were correlated with their willingness to receive antiviral therapy. The study was novel and well-constructed and the results have many implications in the field of preventive medicine.

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**P- Reviewers** Sakamoto N, Wang K **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Zhang DN



## Rockall score in predicting outcomes of elderly patients with acute upper gastrointestinal bleeding

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Received: March 7, 2013 Revised: March 27, 2013  
Accepted: April 3, 2013  
Published online: June 14, 2013

### Abstract

**AIM:** To validate the clinical Rockall score in predicting outcomes (rebleeding, surgery and mortality) in elderly patients with acute upper gastrointestinal bleeding (AUGIB).

**METHODS:** A retrospective analysis was undertaken in 341 patients admitted to the emergency room and Intensive Care Unit of Xuanwu Hospital of Capital Medical University with non-variceal upper gastrointestinal bleeding. The Rockall scores were calculated, and the association between clinical Rockall scores and patient outcomes (rebleeding, surgery and mortality) was assessed. Based on the Rockall scores, patients were divided into three risk categories: low risk  $\leq 3$ , moderate risk 3-4, high risk  $\geq 4$ , and the percentages of rebleeding/death/surgery in each risk category were compared. The area under the receiver operating characteristic (ROC) curve was calculated to assess the validity of the Rockall system in predicting rebleeding, surgery and mortality of patients with AUGIB.

**RESULTS:** A positive linear correlation between clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality was observed ( $r =$

0.962, 0.955 and 0.946, respectively,  $P = 0.001$ ). High clinical Rockall scores  $> 3$  were associated with adverse outcomes (rebleeding, surgery and death). There was a significant correlation between high Rockall scores and the occurrence of rebleeding, surgery and mortality in the entire patient population ( $\chi^2 = 49.29, 23.10$  and  $27.64$ , respectively,  $P = 0.001$ ). For rebleeding, the area under the ROC curve was 0.788 (95%CI: 0.726-0.849,  $P = 0.001$ ); For surgery, the area under the ROC curve was 0.752 (95%CI: 0.679-0.825,  $P = 0.001$ ) and for mortality, the area under the ROC curve was 0.787 (95%CI: 0.716-0.859,  $P = 0.001$ ).

**CONCLUSION:** The Rockall score is clinically useful, rapid and accurate in predicting rebleeding, surgery and mortality outcomes in elderly patients with AUGIB.

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**Key words:** Rockall score; Acute upper gastrointestinal bleeding; Prognosis; Elderly patients

**Core tip:** This study verified the advantages of the Rockall score in predicting the outcomes of the elderly patients with non-variceal upper gastrointestinal bleeding (UGIB) and assessed its clinical usefulness and prognostic value in rebleeding, surgery and mortality. The results suggest that the Rockall scoring system had satisfactory validity for the prediction of rebleeding, surgery and mortality in patients with acute non-variceal UGIB, and there was a positive linear correlation between the clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality.

Wang CY, Qin J, Wang J, Sun CY, Cao T, Zhu DD. Rockall score in predicting outcomes of elderly patients with acute upper gastrointestinal bleeding. *World J Gastroenterol* 2013; 19(22): 3466-3472 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3466.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3466>

## INTRODUCTION

Acute upper gastrointestinal bleeding (AUGIB) is common, costly, and potentially life-threatening and requires prompt assessment and aggressive medical management<sup>[1]</sup>. Despite changes in management, mortality has not significantly improved over the past 50 years. Elderly patients and those with chronic medical diseases withstand AUGIB less well than younger, fitter patients, and have a higher risk of death<sup>[2,3]</sup>. AUGIB is defined as hemorrhage that emanates proximal to the ligament of Treitz, which differentiates it from lower gastrointestinal bleeding involving the colon, and middle gastrointestinal bleeding involving the small intestine distal to the ligament of Treitz<sup>[4]</sup>. Clinically, AUGIB often causes hypodynamia, hematemesis (vomiting of blood), melena (passage of black tarry stools due to the presence of altered blood), and systemic shock typically ensues upon loss of 15% or more of the circulating blood volume. The color of the vomitus depends on its contact time with hydrochloric acid in the stomach<sup>[5,6]</sup>. If vomiting occurs early after the onset of bleeding, it appears red; with delayed vomiting, it is dark red, brown, or black. Coffee-ground emesis results from the precipitation of blood clots in the vomitus. Hematochezia (red blood per rectum) usually indicates bleeding distal to the ligament of Treitz. Occasionally, rapid bleeding from an upper gastrointestinal bleeding source may result in hematochezia<sup>[7]</sup>. The rate and extent of hemorrhage, coupled with the patient's comorbidities, determine the clinical presentation of UGIB. AUGIB is a common medical emergency, the annual incidence of hospitalization for AUGIB is 50-150 per 10000 people in China, and it has a mortality of 4%-14%<sup>[4]</sup>. Early predictors of adverse prognosis in AUGIB, include increasing age (above 60 years), an increased number of co-morbid conditions, the underlying cause of bleeding (*i.e.*, variceal), red blood cells (RBCs) in the emesis or stool, shock or hypotension on presentation, an increased number of units of blood transfused, active bleeding at the time of endoscopy, bleeding from large (> 2 cm) ulcers, onset of bleeding in the hospital, and emergency surgery<sup>[8-10]</sup>. Timely and effective assessment of the patient's condition and the degree of risk is very important, which could serve as a basis for the establishment of treatment procedures to reduce medical costs and improve prognosis<sup>[11]</sup>.

One of the major challenges in managing UGIB involves the identification of patients who are at high risk of rebleeding and death; conversely, the identification of patients who are suitable for early discharge and outpatient endoscopy is also important for effective resource use<sup>[12]</sup>. Similar to other common medical conditions, risk scores have been developed to try and identify those at lower or higher risk of poor outcome<sup>[13]</sup>. An ideal risk score is one that is easy to calculate, accurate for relevant outcomes and can be measured early after presentation with AUGIB. Several clinical scoring systems have been developed to help predict outcome in patients with a view to improving patient management and promoting

cost-effective use of resources. In most published scoring systems, a combination of clinical, laboratory, and endoscopic variables are weighted to produce a score that predicts the risk of mortality, recurrent hemorrhage, need for clinical intervention, or suitability for early discharge. The commonly used systems are the Rockall score, the Baylor bleeding score, the Cedars-Sinai Medical Centre Predictive Index, and the Blatchford score<sup>[14-17]</sup>. The most commonly used risk scoring system in UGIB is the Rockall score, which was described in 1996 following the analysis of data from a large English audit<sup>[12]</sup>. The score was developed to assess the risk of death following presentation with UGIB and incorporates patient age, hemodynamics, comorbidities and endoscopic findings. The clinical Rockall score, which relies on only clinical variables, is used to identify patients with AUGIB who have an adverse outcome, such as death or recurrent bleeding. The complete Rockall score, which relies on clinical and endoscopic variables, is also used to identify patients with AUGIB who died or have recurrent bleeding<sup>[18,19]</sup>. Rockall scores can be calculated both before and after endoscopy, but the post-endoscopic Rockall score provides a more accurate risk assessment. Patients at high-risk for rebleeding receive endoscopic therapy to achieve hemostasis and are subsequently treated with high-dose acid suppression to promote the formation of blood clots over the arterial defect responsible for bleeding. The aim of this study was to verify the advantages of the Rockall score in 341 patients with non-variceal upper gastrointestinal bleeding admitted to the emergency room and Intensive Care Unit (ICU) of Xuanwu Hospital, and to assess its clinical usefulness and prognostic value in rebleeding, surgery and mortality.

## MATERIALS AND METHODS

### Patients

This study included 341 patients with non-variceal UGIB admitted to the emergency room and ICU of Xuanwu Hospital of Capital Medical University. The median age of the patients was 72.85 ± 7.11 years (range: 60-85 years) and 181 were men and 160 were women. Patients admitted to hospital through the emergency department with a primary diagnosis of UGIB (hematemesis, melena or bloody nasogastric aspirate) were considered for inclusion, endoscopies were performed to confirm the diagnosis within 6-48 h after admission, and the characteristics of the patients are presented in Table 1. Patients were selected based on the following criteria: ≥ 60 years of age; patients with clinically significant UGIB (*i.e.*, signs of active UGIB including hematemesis, melena or hematochezia) confirmed by gastroscopy, surgery, blood or coffee grounds detected during nasogastric lavage; patients fulfilling the low-risk criteria such as having a low risk of requiring intervention (endoscopic therapy, blood transfusion, surgery) or death if they had a Rockall score ≤ 2 and were < 70 years old. Patients were excluded based on the following criteria: < 60 years of age; patients with a record of poor compliance, such as those

**Table 1** Classification of patients with acute upper gastrointestinal bleeding

| Classification of diseases   | <i>n</i> = 341 |
|------------------------------|----------------|
| Esophageal diseases          | 74             |
| Esophageal carcinoma         | 37             |
| Esophagitis                  | 25             |
| Mallory-Weiss syndrome       | 7              |
| Hiatus hernia                | 5              |
| Gastroduodenal disease       | 265            |
| Peptic ulcer                 | 151            |
| Stomach cancer               | 59             |
| Erosive gastritis            | 32             |
| Anastomotic                  | 12             |
| Acute gastric mucosal lesion | 11             |
| Other                        | 2              |

who did not undergo endoscopy; patients with acute variceal or obscure UGIB.

### Calculation of Rockall scores

The clinical Rockall score, which was calculated without endoscopic findings for each patient, was based on points assigned for clinical variables: patient age at presentation, shock status based on initial heart rate and systolic blood pressure, and presence of comorbid disease (Table 2). The associations between Rockall scores and rebleeding rate, mortality rate and surgical rate were evaluated. Scores ranged from 0 to 9 and were divided into three risk categories: low risk  $\leq 3$ , moderate risk 3-4, and high risk  $\geq 4$ . We used the observed percentages of rebleeding/death/surgery in each risk category in the original patient sample of Rockall as the predicted probabilities of rebleeding/mortality for both validation samples. Calibration and discrimination were assessed as measures of validity of the scoring system. Calibration was evaluated by a  $\chi^2$  goodness of fit test, and discrimination was evaluated by calculating the area under the receiver operating characteristic (ROC) curve.

Comorbidity was based on reference standard diagnostic criteria, including cardiovascular and cerebrovascular disease, chronic obstructive pulmonary disease, chronic liver disease and cancer.

Rebleeding or bleeding recurrence was defined as a separate episode of hematemesis or melena, or nasogastric evidence of new bleeding, occurring during admission and within 24 h of initial presentation, as witnessed by hospital staff. Hematemesis was defined as the vomiting of fresh or old blood, including "coffee grounds." Melena was defined as the passage of black or tarry stools. Mortality was defined as death occurring within 30 d of hospital admission.

Successful hemostasis was defined as endoscopic hemostasis or negative occult blood in the feces, and patients were hemostatically stable when no hematemesis or melena was observed.

Rebleeding manifestations were defined by at least one of the following: recurrent hematemesis or melena, bloody or red colored vomit, or bloody stools (blood in the stool that may appear as maroon or red), or the

patient had hyperactive bowel sounds; hemorrhagic peripheral circulatory failure (due to excessive blood loss and rapid bleeding) was not improved or hemodynamic status was temporarily improved after fluid infusion and blood transfusion, and the central venous pressure fluctuated and then decreased; RBC counts and hemoglobin levels continued to decline, and high reticulocyte count (increased RBC destruction such as bleeding or hemolysis) was observed; serum creatinine level increased when 24-h total volumes of fluid infusion and urinary output were normal; a relatively large amount of fresh blood was drained by nasogastric tube lavage.

Surgical treatment guidelines were as follows: conservative treatment was not sufficient and the bleeding continued, and patients suspected of having a perforated duodenal ulcer were transferred to the surgical ICU.

### Statistical analysis

SPSS statistical software version 19.0 (SPSS Inc., Chicago, IL, United States) was used for data analysis and management. The sensitivity and specificity of detecting patients who needed clinical intervention, had recurrent bleeding, or died were calculated for the clinical Rockall score and the complete Rockall score with confidence interval. The Rockall scores for all patients were calculated based on their pre-endoscopic variables. The correlation between the variables was analyzed using the Pearson product-moment correlation. Categorical variables were analyzed by  $\chi^2$  tests. We assessed the validity of the scoring systems by plotting ROC curves. A two-sided *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Rockall scores and clinical outcomes

Of 341 patients, 63 (18.47%) patients developed recurrent bleeding, 30 (8.79%) patients died and 31 (9.09%) patients required endoscopic treatment. The Rockall scores were calculated based on the collected data (Table 3). A positive linear correlation between the clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality ( $r = 0.962, 0.955$  and  $0.946$ , respectively,  $P = 0.001$ ) was observed. High clinical Rockall scores  $> 3$  were associated with adverse outcomes (rebleeding, surgery and death).

### Distribution of patients in the risk categories

The distribution of patients classified into the three risk categories (low, moderate, high), as determined by the Rockall risk scoring system, and the observed percentages of rebleeding, surgery and mortality in each risk category are shown in Table 4. The Rockall score identified 114 of 341 patients as low risk ( $\leq 3$ ), 110 of 341 patients as moderate risk (3-4) and 117 of 341 patients as high risk ( $\geq 4$ ). There were significant correlations between high Rockall scores and the occurrence of rebleeding, surgery and mortality in the entire patient population ( $\chi^2 = 49.29, 23.10$  and  $27.64$ , respectively,  $P = 0.001$ ).

**Table 2** Rockall scores in patients with upper gastrointestinal bleeding

| Variable             | Scores  |                                    |                                       |   |
|----------------------|---|------------------------------------|---------------------------------------|---|
|                      | 0   | 1                                  | 2                                     | 3   |
| Age (yr)             | < 60  | 60-79                              | ≥ 80                                  |   |
| Shock                | No shock;<br>SBP ≥ 100 mmHg;<br>pulse < 100 bpm | SBP ≥ 100 mmHg;<br>Pulse ≥ 100 bpm | SBP < 100 mmHg;<br>Pulse ≥ 100 bpm    |   |
| Comorbidity          | No major  |                                    | CHF, IHD, major morbidity             | Renal failure,<br>liver failure,<br>metastatic cancer |
| Diagnosis            | Mallory-Weiss syndrome                          | All other diagnoses                | GI malignancy                         |   |
| Evidence of bleeding | None  |                                    | Blood, adherent clot, spurting vessel |   |

CHF: Chronic hearth failure; IHD: Ischaemic heart disease; SBP: Systolic blood pressure; GI: Gastrointestinal.

**Table 3** Relationship between clinical Rockall scores and patient outcomes

| Variables  | Rockall score |    |    |    |    |    |    |     |
|------------|---------------|----|----|----|----|----|----|-----|
|            | 1             | 2  | 3  | 4  | 5  | 6  | 7  | ≥ 8 |
| Number     | 65            | 49 | 44 | 66 | 51 | 31 | 20 | 15  |
| Rebleeding | 2             | 3  | 4  | 9  | 14 | 11 | 10 | 10  |
| Mortality  | 0             | 1  | 2  | 4  | 7  | 8  | 4  | 4   |
| Surgery    | 0             | 1  | 3  | 5  | 9  | 6  | 4  | 3   |

**Table 4** Percentages of rebleeding/death/surgery in each risk category *n* (%)

| Category      | Cases | Outcome    |            |            |
|---------------|-------|------------|------------|------------|
|               |       | Rebleeding | Surgery    | Mortality  |
| Low-risk      | 114   | 5 (4.38)   | 1 (0.87)   | 1 (0.87)   |
| Moderate-risk | 110   | 13 (11.81) | 8 (7.27)   | 6 (5.45)   |
| High-risk     | 117   | 45 (38.46) | 22 (18.80) | 23 (19.65) |

### Predictive value of the Rockall score for rebleeding, surgery and mortality in patients with upper gastrointestinal bleeding

The discriminative ability of the Rockall score for the prediction of rebleeding and mortality were compared. The Rockall score was found to have good predictive value for rebleeding (area under the ROC curve was 0.788, 95%CI: 0.726-0.849,  $P = 0.001$ ), surgery (area under the ROC curve was 0.752, 95%CI: 0.679-0.825,  $P = 0.001$ ) and mortality (area under the ROC curve was 0.787, 95%CI: 0.716-0.859,  $P = 0.001$ ).

## DISCUSSION

Acute non-variceal UGIB remains a common and challenging emergency for gastroenterologists and general physicians<sup>[20]</sup>. The annual incidence is 50-150 per 100000 of the population, and although there have been significant improvements in endoscopic and supportive therapies, the overall mortality stubbornly remains around 10% (4%-14%), and may even reach 27% in hospitalized patients with serious co-morbidity<sup>[21]</sup>. AUGIB results in considerable patient morbidity and significant medical costs. Elderly patients (aged over 80 years) now account for around 25% of all AUGIB and 33% of AUGIB occurring in hospitalized patients, and therefore tend to account for much of the poor outcome associated with this condition<sup>[1]</sup>. Many risk factors are associated with bleeding, and these must be addressed. Pharmacists, physicians, and dentists should record patients' medical history and analgesic requirements. The initial evaluation of patients with AUGIB involves recognition of a range

of symptoms depending on the source, rate, and volume of blood loss<sup>[2]</sup>. Medical comorbidities and the use of antiplatelet medications can complicate the severity and management of bleeding, especially in the elderly. Symptoms of AUGIB include anemia, hematemesis (vomiting bright red blood or "coffee ground" material), and melena<sup>[5,6]</sup>. Other symptoms may include epigastric pain, dyspnea, and syncope (due to volume depletion). Bleeding may be obscure when the gastrointestinal blood loss is of unknown origin<sup>[7]</sup>. Certain prognostic factors in patients who present with AUGIB can increase the incidence of complications, including morbidity and mortality<sup>[22]</sup>. The patient should be admitted to the ICU if one or more of the following prognostic factors are present: age greater than 60 years; shock; comorbidities (*e.g.*, cardiac, renal and hepatic diseases); current bleeding; low systolic blood pressure; need for more than 6 units of blood; and endoscopy showing major signs of bleeding.

Several clinical scoring systems, *e.g.*, the Rockall score, the Blatchford score and Acute Physiology and Chronic Health Evaluation II score, have been developed to direct appropriate patient management and predict mortality as well as rebleeding. These systems weigh a combination of clinical, laboratory and endoscopic variables to produce a score that predicts the risk of mortality, recurrent hemorrhage, the need for clinical intervention or suitability for early discharge<sup>[23-25]</sup>. Factors commonly associated with poor outcome in patients with AUGIB may be related to presentation and co-morbidities, or to the behavior of the ulcer. Before endoscopy is performed, use of the Rockall risk scoring system is recommended. This assessment tool, which predicts the patient's outcome and estimates rebleeding risk, is the most widely

used scoring system and has been validated by several studies. The patient's age, systolic blood pressure, pulse rate and the presence of comorbidities are used for scoring. Patients with a score of 0 should be considered for non-admission or early discharge with outpatient follow-up; if the score is above 0, there is a significant risk of mortality, and endoscopy is recommended for a full assessment of bleeding risk<sup>[14-16]</sup>.

Rockall included 4185 cases of AUGIB from 74 hospitals in the United Kingdom over a four-month period in 1993. Their scoring system was based on multivariate analysis of information from history, examination, blood tests, and endoscopic investigation. The complete Rockall score makes use of both clinical and endoscopic criteria to predict the risks of rebleeding and death; the scale ranges from 0 to 11 points, with higher scores indicating higher risk. In the present study, we used Rockall's risk scoring system to classify patients and found that high clinical Rockall scores > 3 were associated with adverse outcomes (rebleeding, surgery and death), and the results obtained were widely corroborated in clinical practice. The complete Rockall score has been validated as a clinically useful score in predicting outcomes (rebleeding, mortality) of patients with acute non-variceal UGIB<sup>[26,27]</sup>. As the original study included only 180 of 4185 patients with esophagus-stomach fundus variceal hemorrhage, some investigators argued that the Rockall score might not be ideal or accurate in predicting rebleeding and mortality in patients with esophagus-stomach fundus variceal hemorrhage.

In the present study, we found that 63 (18.47%) patients developed recurrent bleeding, 30 (8.79%) patients died and 31 (9.09%) patients required endoscopic treatment. These results were consistent with earlier research<sup>[28]</sup>. A positive linear correlation between the clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality ( $r = 0.962, 0.955$  and  $0.946$ , respectively,  $P = 0.001$ ) was observed. High clinical Rockall scores > 3 were associated with adverse outcomes (rebleeding, surgery and death). Our results validated the clinical Rockall score in predicting patient outcome (*i.e.*, rebleeding, surgery and mortality) after acute non-variceal UGIB, which will help identify low-risk patients for delayed, elective or outpatient endoscopy, whereas those at high risk could have urgent endoscopy and a higher level of hospital care<sup>[29-33]</sup>.

Recurrence of bleeding is one of the most important factors affecting prognosis, and early prediction and treatment of rebleeding would improve the outcome in patients with acute non-variceal UGIB, as rebleeding is associated with high mortality<sup>[34-36]</sup>. The commonly used scoring systems are the Rockall score, the Baylor bleeding score, the Cedars-Sinai Medical Centre Predictive Index, and the Blatchford score<sup>[37]</sup>. The Cedars-Sinai Medical Centre Predictive Index was less accurate than the Rockall score in predicting patient outcome (*i.e.*, rebleeding, surgery and mortality), the Baylor bleeding score is commonly used in predicting the likelihood of rebleeding after endoscopic hemostasis of peptic ulcers, while the

complete Rockall score has been found to have good predictive value for mortality and in-hospital rebleeding. In this study, we showed that a low clinical Rockall risk score in patients with AUGIB without endoscopy was not associated with adverse outcomes (rebleeding or mortality), whereas a high clinical risk score was associated with adverse outcomes. A positive linear correlation between the clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality ( $r = 0.962, 0.955$  and  $0.946$ , respectively,  $P = 0.001$ ) was observed. The discriminative ability of the Rockall score for the prediction of rebleeding and mortality was compared. For rebleeding, the area under the ROC curve was 0.788 (95%CI: 0.726-0.849.  $P = 0.001$ ). For mortality, the area under the ROC curve was 0.787 (95%CI: 0.716-0.859.  $P = 0.001$ ). Our results were consistent with those of other studies and suggested that the Rockall score had good predictive value for mortality and in-hospital rebleeding, and was validated as a clinically useful scoring system for stratifying patients into high-risk and low-risk categories for mortality and in-hospital rebleeding<sup>[38,39]</sup>. However, other reports have suggested that the Rockall score showed inadequate sensitivity and poor specificity for outcome prediction in terms of rebleeding and mortality, thus, further clinical research is needed to confirm our observations<sup>[40]</sup>.

In conclusion, our results suggest that the Rockall risk scoring system had satisfactory validity for the prediction of rebleeding, surgery and mortality in patients with acute non-variceal UGIB, and a positive linear correlation between the clinical Rockall scores and patient outcomes in terms of rebleeding, surgery and mortality was observed. The problems associated with AUGIB are challenging for patients and physicians, and a combination of clinical and laboratory assessments (including the Cedars-Sinai Medical Centre Predictive Index and Baylor bleeding score) should be performed to comprehensively assess and correctly diagnose various conditions in patients in order to develop appropriate treatment programs and improve the prognosis of patients.

## COMMENTS

### Background

Acute upper gastrointestinal bleeding (AUGIB) is common, costly, and potentially life-threatening and requires prompt assessment and aggressive medical management. The most commonly used risk scoring system in UGIB is the Rockall score, which was described in 1996 following the analysis of data from a large English audit.

### Research frontiers

Rockall scores can be calculated both before and after endoscopy, but the post-endoscopic Rockall score provides a more accurate risk assessment. Patients at high-risk for rebleeding receive endoscopic therapy to achieve hemostasis and are subsequently treated with high-dose acid suppression to promote the formation of blood clots over the arterial defect responsible for bleeding.

### Innovations and breakthroughs

Several clinical scoring systems have been developed to help predict outcome in patients with a view to improving patient management and promoting cost-effective use of resources. In most published scoring systems, a combination of clinical, laboratory, and endoscopic variables are weighted to produce a score that predicts the risk of mortality, recurrent hemorrhage, need for clinical intervention, or suitability for early discharge. The commonly used systems are the Rockall score, the Baylor bleeding score, the Cedars-Sinai Medical Centre

Predictive Index, and the Blatchford score. This study verified the advantages of the Rockall score in predicting the outcomes of the elderly patients with non-variceal UGB and assessed its clinical usefulness and prognostic value in rebleeding, surgery and mortality.

### Applications

The authors found that the Rockall score is clinically useful, rapid and accurate in predicting rebleeding, surgery and mortality outcomes in elderly patients with acute upper gastrointestinal bleeding.

### Peer review

This manuscript is very interesting. The authors intended to validate the clinical Rockall score in predicting outcomes (rebleeding, surgery and mortality) in elderly patients with acute upper gastrointestinal bleeding. The study is well designed and the data well support the conclusion. Rockall score plays an important role in predicting the outcomes of the elderly patients with non-variceal upper gastrointestinal bleeding. This study will be informative for the readers.

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P- Reviewers Chung SCS, Shapira S S- Editor Wang JL  
L- Editor A E- Editor Xiong L



## Combination treatment with comprehensive cryoablation and immunotherapy in metastatic hepatocellular cancer

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Received: December 21, 2012 Revised: March 21, 2013

Accepted: May 9, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To retrospectively assess the effect of comprehensive cryosurgery (ablation of intra- and extra-hepatic tumors) plus dendritic cell-cytokine-induced killer cell immunotherapy in metastatic hepatocellular cancer.

**METHODS:** We divided 45 patients into cryo-immunotherapy (21 patients), cryotherapy ( $n = 12$ ), immunotherapy ( $n = 5$ ) and untreated ( $n = 7$ ) groups. Overall survival (OS) after diagnosis of metastatic hepatocellular cancer was assessed after an 8-year follow-up.

**RESULTS:** Median OS was higher following cryo-immu-

notherapy (32 mo) or cryotherapy (17.5 mo;  $P < 0.05$ ) than in the untreated group (3 mo) and was higher in the cryo-immunotherapy group than in the cryotherapy group ( $P < 0.05$ ). In the cryo-immunotherapy group, median OS was higher after multiple treatments (36.5 mo) than after a single treatment (21 mo;  $P < 0.05$ ).

**CONCLUSION:** Cryotherapy and, especially, cryo-immunotherapy significantly increased OS in metastatic hepatocellular cancer patients. Multiple cryo-immunotherapy was associated with a better prognosis than single cryo-immunotherapy.

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**Key words:** Cryoablation; Dendritic cell-cytokine-induced killer cell; Immunotherapy; Metastatic hepatocellular cancer; Survival time

**Core tip:** Forty-five patients of metastatic hepatocellular cancer were divided into cryo-immunotherapy, cryotherapy, immunotherapy and untreated groups. Median overall survival (OS) was higher following cryo-immunotherapy (32 mo) or cryotherapy (17.5 mo) than in the untreated group (3 mo); In the cryo-immunotherapy group, median OS was higher after multiple treatments (36.5 mo) than after a single treatment (21 mo). In a word, cryotherapy and, especially, cryo-immunotherapy significantly increased OS in metastatic hepatocellular cancer patients. Multiple cryo-immunotherapy was associated with a better prognosis than single cryo-immunotherapy.

Niu LZ, Li JL, Zeng JY, Mu F, Liao MT, Yao F, Li L, Liu CY, Chen JB, Zuo JS, Xu KC. Combination treatment with comprehensive cryoablation and immunotherapy in metastatic hepatocellular cancer. *World J Gastroenterol* 2013; 19(22): 3473-3480 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3473.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3473>

## INTRODUCTION

Hepatocellular carcinoma (HCC), which is the fifth most common cancer worldwide, is usually discovered late and has a poor prognosis<sup>[1]</sup>. In about 80% of patients, HCC is associated with chronic liver disease (*i.e.*, hepatitis and cirrhosis), with major implications for the prognosis and therapeutic options<sup>[2]</sup>. Many patients are unsuitable for tumor resection because of factors such as poor hepatic reserve (cirrhosis), multicentric tumors or extrahepatic disease<sup>[3,4]</sup>. Until recently, no systemic chemotherapy has significantly increased survival in patients with advanced HCC<sup>[5,6]</sup>. External beam radiation has had a limited role in the treatment of HCC because of radiation toxicity to the adjacent normal liver<sup>[7,8]</sup>. Percutaneous ablation is currently considered the best therapeutic modality for patients with early stage HCC who are not candidates for surgery; it principally involves percutaneous ethanol injection, radiofrequency ablation, microwave ablation, laser ablation or cryoablation<sup>[9]</sup>.

Because cryoablation forms an ice ball that can be visualized by many imaging methods, it has been an attractive option for reasons of safety. Technically, cryoablation of tumors in multiple organs (*i.e.*, liver, lung, kidney, breast, pancreas and prostate) has been proved to be safe and effective<sup>[10]</sup>. A long term study of medium to large tumors (more than 5 cm in diameter) treated with cryoablation and/or transarterial chemoembolization (TACE) showed a 5 year survival rate of 23% and local progression rate of 24%<sup>[11,12]</sup>. To our knowledge, there are currently no reports on the long term effects of simultaneous cryoablation of intra- and extra-hepatic tumors in metastatic HCC patients.

Another potential advantage of the *in situ* freezing of malignant disease is the cryo-immunologic response<sup>[13]</sup>, which is an antitumor immune response triggered by the natural absorption of malignant tissue<sup>[14]</sup>. Immunotherapy mediated by autologous dendritic cells (DCs) is a promising treatment option for long lasting control of unresectable HCC<sup>[15-17]</sup>. Increased knowledge regarding vaccination with DCs co-cultured with cytokine-induced killer (CIK) cells has led to improved clinical treatment strategies<sup>[18]</sup>. Whether slow release of tumor antigen after cryoablation can improve the effect of immunotherapy remains unknown.

Here, we retrospectively compared the effects of comprehensive cryosurgery (simultaneous cryoablation of intra- and extrahepatic tumors and of liver tumors of diameter greater than 5 cm, with TACE performed once or twice before cryoablation to reduce the tumor to 5 cm) and/or DC-CIK immunotherapy in patients with untreated metastatic HCC. To measure the survival time of patients with metastatic cancer, overall survival (OS) after diagnosis of metastatic disease was the main evaluation index.

## MATERIALS AND METHODS

### Patient selection

Between January, 2004 and October, 2011, 45 HCC pa-

tients with metastatic HCC met our inclusion criteria and were enrolled in this study. Surgery and chemotherapy were deemed unsuitable in any of the following situations: multifocal disease, unresectable HCC, patient refused to undergo surgery and chemotherapy or was seeking further treatment after failure of chemotherapy, severe complications (*i.e.*, hypertension and ascites) and advanced age. Ideal patients for comprehensive cryoablation are those with: Karnofsky performance status (KPS) score  $\geq 70$ ; platelet count  $\geq 80 \times 10^9/L$ ; white blood cell count  $\geq 3 \times 10^9/L$ ; neutrophil count  $\geq 2 \times 10^9/L$ ; hemoglobin  $\geq 90$  g/L; prothrombin time international normalized ratio  $\geq 1.5$ ; hepatic tumor not obviously invading the gallbladder, diaphragm or large vessels; absence of level 3 hypertension, severe coronary disease, myelosuppression, respiratory disease and acute or chronic infection; and adequate hepatic function (bilirubin  $< 30$   $\mu\text{mol/L}$ , aminotransferase  $< 60$  U/L and Child-Pugh score A or B) and renal function (serum creatinine  $< 130$   $\mu\text{mol/L}$ , serum urea  $< 10$  mmol/L).

The diagnosis of HCC was confirmed by liver pathology in 41 patients; in the remaining cases, HCC was diagnosed by classical imaging methods, including computed tomography (CT) or magnetic resonance imaging, or by biochemical markers such as increased alpha-fetoprotein. Twenty-four patients had only one mass in the liver, of 3.8-15 cm in diameter with an average of 6.5 cm. Twenty-one patients had two to four masses of 4.5-13 cm in diameter. There were a total of 71 masses in 45 patients. All except two cases had cirrhosis. Using the Child-Pugh score to assess the severity of cirrhosis, 25 patients were class A and 18 were class B. All patients received their final treatment in our hospital within an 8 year follow-up period.

### TACE

The preferred treatment for 25 patients with a hepatic tumor of long diameter  $\geq 5$  cm was TACE<sup>[19,20]</sup>, which was performed after cross-sectional imaging as previously described<sup>[21]</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 tumor were then located and the venous phase was examined carefully 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 tumor. A mixture of doxorubicin (50 mg), mitomycin (10 mg) and lipiodol (4-15 mL) was injected into the arterial branches until hemostasis was achieved. If the tumor showed no shrinkage 2 wk after the procedure, a second TACE was performed.

### Cryoablation procedure

Comprehensive cryosurgery was performed on 33 patients, with complete cryoablation of obvious intra- and extrahepatic masses. Each procedure comprised two freeze/thaw cycles accomplished using an argon gas-

based cryosurgical unit (Endocare, Irvine, CA, United States). Cryoprobes (3, 5 or 8 mm) were inserted into the center of the tumor mass under ultrasonographic guidance and two freeze/thaw cycles were performed, each reaching a temperature of  $-180^{\circ}\text{C}$  at the tip of the probe. The duration of freezing was dependent on the achievement of an ice ball, visible as a hypoechoic region on ultrasonography. Generally, the maximal freezing time was 15 min, followed by thawing for 5 min; this cycle was then repeated. A margin of at least 1 cm of normal hepatic tissue was frozen circumferentially around the tumor. For masses larger than 5 cm in diameter, two or three cryoprobes were placed within the center and periphery of the tumor, to ensure freezing of the entire mass. The tracts formed were sealed with fibrin glue immediately after removal of the cryoprobes to ensure hemostasis.

### Immunotherapy

Twenty-six patients opted for immunotherapy (adoptive transfer of DC-CIK cells performed four times). DC-CIK cells were generated according to previously published protocols<sup>[22,23]</sup>; 70 mL peripheral blood was drawn before cryosurgery and the treatment was given 3-5 d after cryosurgery. Using Ficoll-Hypaque density centrifugation, we harvested peripheral blood mononuclear cells (PBMCs) from peripheral blood samples (80 mL) collected from the 48 patients 2 d before cryosurgery.

For DC culture, PBMCs were resuspended in DC medium [X-VIVO 15 (Lonza, Basel, Switzerland), 25 ng/mL interleukin (IL)-4 (Peprotech, Rocky Hill, NJ, United States) and 30 ng/mL granulocyte macrophage colony stimulating factor (GM-CSF; Peprotech)], at a concentration of  $1 \times 10^6$  to  $2 \times 10^6$ /mL. The cells were then allowed to adhere in two plastic flasks (T75; Corning Costa, Cambridge, MA, United States), each containing 50 mL DC medium and approximately  $10^8$  cells. After overnight culture at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$ , the suspended cells were transferred to two fresh flasks. The cells sticking to the initial two flasks were continuously cultured in DC medium and a small amount of fresh medium was added daily to the cultures.

For culture of CIK cells, PBMCs were suspended in CIK medium [X-VIVO 15 (Lonza), 1000 U/mL IL-2 (Peprotech), 2.5  $\mu\text{g}/\text{mL}$  monoclonal antibody to CD3 (OKT-3; Jansen-Kyowa, Tokyo, Japan), 25  $\mu\text{g}/\text{mL}$  phytohemagglutinin (Peprotech) and 1000 U/mL interferon gamma (Peprotech)]. The CIK cells were allowed to grow and then continuously passaged. At approximately 7 d of culture, the CIK cells were passaged to fourteen T225 flasks. Cells adhering to the flasks were removed with a cell spatula, centrifuged and resuspended in DC-CIK medium [X-VIVO 15 (Lonza), 400 U/mL IL-2 and 0.5  $\mu\text{g}/\text{mL}$  monoclonal antibody to CD3]. All DCs were distributed evenly in the 14 T225 flasks containing CIK cells (approximately  $10^8$  DCs per flask). After co-culture for 24-48 h, almost 1 wk after cryosurgery, the DC-CIKs were harvested and suspended in 100 mL saline for intravenous injection (cells were collected on four consecutive days;  $6 \times 10^9$  to  $10 \times 10^9$  cells were collected on each

day). The final cell products were assessed for viability by the dye-exclusion test and checked twice for possible contamination by bacteria, fungi and endotoxins. All cell preparation processes were performed by the same technician and assessed by another technician.

Seven patients refused to undergo cryo- or immunotherapy owing to its cost or their health or age. These patients received no treatment and left the hospital.

### Ethics

The study protocol received ethical approval from the Regional Ethics Committee of Guangzhou Fuda Cancer Hospital and conformed to the provisions of the World Medical Association's Declaration of Helsinki in 1995 (as revised in Tokyo in 2004). Written informed consent was obtained from each participant.

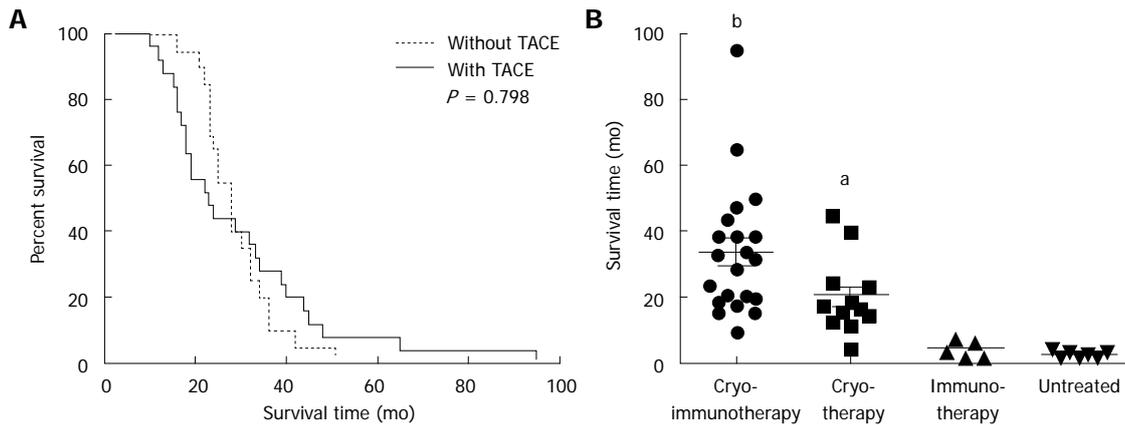
### Statistical analysis

Complications were recorded and classified in accordance with the Common Terminology Criteria of Adverse Events v4.0. Local tumor control and OS were also evaluated. Radiographic local tumor control was assessed using image-guided tumor ablation criteria<sup>[24]</sup>. Thoracic and/or abdominal ultrasonography was performed both 1 d and 1 wk after the minimally invasive treatment of primary and metastatic tumors. Follow-up dynamic CT was performed at 1 mo and then at 3-4 mo intervals. The revised Response Evaluation Criteria in Solid Tumors v1.1 were used to assess the response of the thoracic and abdominal tumors<sup>[25]</sup>. Three diagnostic radiologists reviewed CT scans for every case to determine whether progression or recurrence had occurred. Diagnoses were made independently, although the radiologists discussed cases over which they disagreed. Using the Dunnett test, we compared the OS of patients who had received cryo- and/or immunotherapy with that of untreated patients. The Kaplan-Meier test with log-rank analysis was used for comparison of OS between two groups. Significant differences were indicated by  $P < 0.05$  or  $P < 0.01$ . All analyses were conducted using GraphPad software (San Diego, CA, United States).

## RESULTS

### Clinical data

Twenty-eight men and five women underwent comprehensive cryoablation and/or TACE. Their ages ranged from 29 to 79 years, with a mean age of 53 years. Twenty-eight patients had histories of hepatitis B infection and two had hepatitis C infection. Fifteen patients were from China and 18 were from Southeast Asia. Of these patients, 18 had initially been treated with surgery and 13 with systemic chemotherapy in other centers; a total of 22 patients came to our hospital for further treatment 1-7 mo after metastases were found and 11 patients came to our hospital for first treatment. Bone metastases (17 lesions) were found in 11 patients, lung metastases (21 lesions) in 15 and multiple organ metastases (18 lesions) in seven. Moderate/severe abdominal pain, evaluated as 5-10



**Figure 1** Correlation of overall survival with type of treatment. All 45 patients with metastatic hepatocellular carcinoma died before June, 2012. There were 21 patients in the cryo-immunotherapy group, 12 in the cryotherapy group, five in the immunotherapy group and seven in the untreated group. The overall follow-up period was 8 years. **A:** Overall survival (OS) of patients who underwent comprehensive cryosurgery with or without transarterial chemoembolization (TACE). Thirty-three patients were enrolled; based on the long diameter of their hepatic tumors ( $\geq 5$  cm), 25 underwent TACE before hepatic cryoablation. Kaplan-Meier test with long-rank analysis; **B:** OS in the cryo-immunotherapy, cryotherapy and/or immunotherapy groups vs that in the untreated group using the Dunnett test. Horizontal lines represent the average and standard deviation. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs untreated group.

on a visual analog scale (VAS) (17 patients), and mild/moderate ascites (15 patients) were common complaints. For metastasis or recurrence of HCC after treatment, 16 patients received multiple treatments (10 in the cryo-immunotherapy group and 6 in the cryotherapy group); 17 patients refused to continue treatment (11 in the cryo-immunotherapy group and 6 in the cryotherapy group).

The untreated group (those who refused cryoablation, TACE and immunotherapy for reasons of treatment concept, age or economic ability) comprised 12 patients (47-77 years of age, median age 63 years; 8 male, 4 female). All of these patients had histories of hepatitis B or C infection. Five patients were from China and seven were from Southeast Asia. Of these patients, eight had initially been treated with surgery or systemic chemotherapy in other centers; a total of seven patients came to our hospital for further treatment 1-6 mo after metastases were found and five patients came to our hospital for first treatment. Bone metastases (5 lesions) were found in three patients, lung metastases (12 lesions) in seven and multiple organ metastases (6 lesions) in two. These patients had complaints similar to those of the comprehensive treatment group.

**Perioperative outcomes**

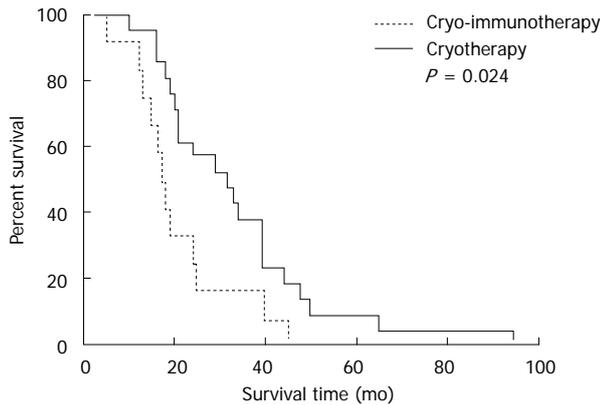
Percutaneous cryoablation of primary and metastatic HCC was successful in every case. No severe complications, such as liver cracking and failure or acute renal failure with myoglobinuria, were discovered post-cryoablation. After the first comprehensive cryosurgery in 33 patients, many slight side effects of cryoablation were observed but recovered with or without symptomatic treatment. Slightly hepatorrhagia was found in six patients (18%) but all healed within 5 d, after injection of a hemostatic agent. Liver capsular cracking was found in one patient (3%) who recovered after blood transfusion. Transient thrombocytopenia occurred in seven patients (21%) within 1 wk after cryoablation; two received

platelet transfusions. Two patients (6%) had tumor in the right lobe and developed asymptomatic right-sided pleural effusions close to the dome of the diaphragm; these disappeared spontaneously within 2-3 wk. Two patients (6%) developed liver abscess at the previous cryoablation site 2 and 4 d respectively following cryoablation, but recovered after antibiotic and drainage treatment. Four patients were found to have slight fever (body temperature less than 39 °C). No obvious side effects associated with TACE were found during the perioperative stage. In the first 2 wk after comprehensive cryosurgery, the VAS pain score decreased to 0-3 in 13 patients (76%) who had suffered pretreatment abdominal pain, with consumption of analgesics decreased by 50% and KPS score increased by  $\geq 20$ .

**Influence of treatment method and frequency on OS**

In our therapeutic protocol, large hepatic tumors (long diameter  $\geq 5$  cm) were treated by TACE first and considerably reduced in size before cryoablation. Whether patient life span is significantly affected by liver tumor size and additional TACE treatment remains to be determined. Of the 33 patients who received comprehensive cryosurgery, the median OS of those who underwent TACE first was 29 mo; those who received cryoablation directly had a median OS of 26 mo. There was no difference in the OS of these two groups according to the log-rank test ( $P = 0.798$ , Kaplan-Meier test with log-rank analysis; Figure 1A). Thus, a large hepatic tumor successfully shrunk by TACE can be treated as a small tumor, with no difference in the results of cryoablation.

To the date of the last follow-up, the median OS of all patients was 18 mo (25% percentile, 6 mo; 75% percentile, 33.5 mo). Median OS in the cryo-immunotherapy, cryotherapy, immunotherapy and untreated groups was 32, 17.5, 4 and 3 mo, respectively. OS was significantly higher in the cryo-immunotherapy ( $P < 0.01$ ) and cryotherapy ( $P < 0.05$ ) groups than in the untreated group (by



**Figure 2** Overall survival of patients who underwent comprehensive cryosurgery with or without immunotherapy. The Kaplan-Meier test with log-rank analysis was used to compare the overall survival of 21 patients in the cryo-immunotherapy group with that of 12 patients in the cryotherapy group.

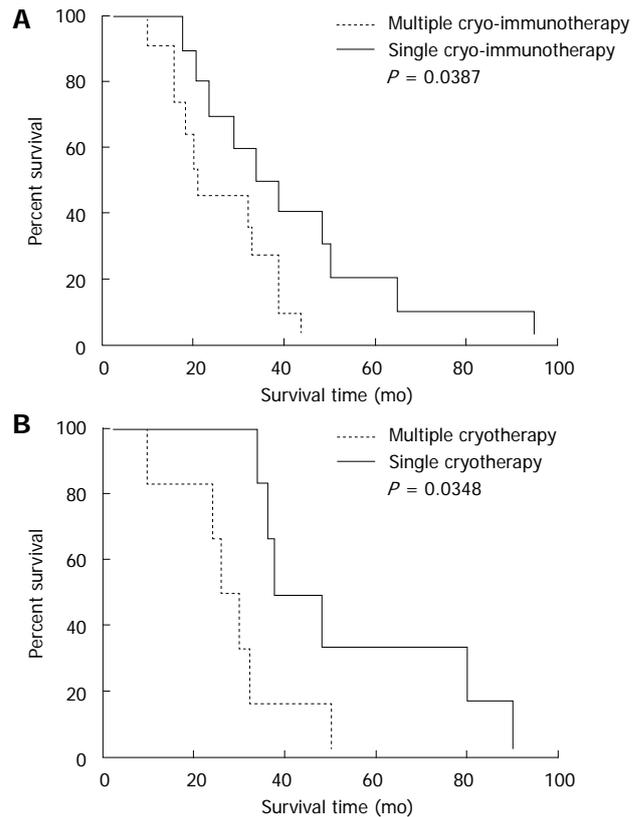
the Dunnett test, with the untreated group as the control group; Figure 1B).

Comparing the two groups in which there were obvious therapeutic effects, OS was higher in the cryo-immunotherapy group than in the cryotherapy group ( $P = 0.024$ , Kaplan-Meier test with log-rank analysis; Figure 2).

Repeated cryo- and immunotherapy for tumor progression and/or recurrence was performed in 10 patients in the cryo-immunotherapy group (twice in five patients, thrice in four patients and four times in one patient) and 6 patients in the cryotherapy group (twice in four patients and thrice in two patients); the remaining patients refused repeat treatments. Due to the shorter survival time, all patients in the immunotherapy group received one treatment. In the cryo-immunotherapy group, the median OS of the patients who underwent repeated treatment (36.5 mo) was higher than that of those who underwent a single treatment (21 mo;  $P = 0.039$ , Kaplan-Meier test with log-rank analysis; Figure 3A). In the cryotherapy group, the median OS after repeated treatment was 21.5 mo, whereas that after a single treatment was 14 mo ( $P = 0.035$ , Kaplan-Meier test with log-rank analysis; Figure 3B).

## DISCUSSION

In this study, we retrospectively reviewed our hospital's database to evaluate the survival time of patients with metastatic HCC. These patients had received various therapies in different medical centers before the metastases were found, and our treatment program directly determined their survival time in the metastatic stage. Increasing numbers of patients are undergoing cryoablation of their primary tumor and metastases, termed comprehensive cryoablation. With skilled operators and strict patient selection, this combined technology can be effective in preventing the occurrence of severe complications (*i.e.*, liver cracking and failure, acute renal failure with myoglobinuria), reducing the probability of side effects (*i.e.*, hepatorrhagia, liver capsular cracking, thrombocytopenia and liver abscess) and provide guar-



**Figure 3** Correlation of overall survival with number of cryo- and/or immunotherapy procedures, using the Kaplan-Meier test with long-rank analysis. A: Comparison of overall survival (OS) between 10 patients who underwent repeated treatments and 11 patients who underwent a single treatment in the cryo-immunotherapy group; B: Comparison of OS between six patients who underwent repeated cryoablation and six who underwent a single cryoablation in the cryotherapy group.

antee for the success of cryotherapy. Theoretically, most of the side effects can be further reduced, with the exception of thrombocytopenia. Development of systemic thrombocytopenia after cryosurgery is associated with excessive platelet trapping and destruction within the cryolesion<sup>[26]</sup>. This symptom is difficult to avoid simply through improved care, but can heal spontaneously or with platelet supplements.

It is increasingly clear that immunotherapy can be useful in cancer therapy, but there are also obstacles that need to be overcome. Due to their organ-like structural environment, these tumors are able to escape immune surveillance<sup>[27]</sup>, and immunotherapy for HCC must therefore be combined with additional therapy to disrupt this structure. Adoptive transfer of CIK cells along with DCs has been shown to be efficacious when the tumor burden is relatively low or when used as an adjuvant therapy rather than as a treatment for bulky tumors<sup>[18]</sup>, indicating the importance of cytoreductive cryoablation before immunotherapy. DCs have been the subject of much research in the last decade and are widely used in immunotherapy protocols. These bone marrow-derived cells have been identified as the most potent immune-stimulatory cells known and are specialized for the initiation and shaping of immune responses. Activated DCs after cryoablation

are potent stimulators of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as supported by evidence from experimental<sup>[28]</sup> and human<sup>[29,30]</sup> studies. DCs are often pulsed with synthetic peptides derived from known tumor antigens<sup>[31]</sup>, tumor cell lysates<sup>[32]</sup>, apoptotic tumor cells<sup>[33]</sup> or RNA derived from tumor antigens<sup>[34]</sup> and transfected with whole tumor cell DNA<sup>[35]</sup> or RNA<sup>[36]</sup>. Moreover, DCs have been fused with tumor cells to induce antigen-specific, polyclonal cytotoxic T lymphocyte responses<sup>[37]</sup>.

On account of continued antigen release after cryoablation<sup>[14]</sup>, *in vitro* activation of DCs was omitted and the DCs were stimulated *in vivo* in the present study. We found that combined cryo- and immunotherapy extended the median OS of metastatic HCC patients from 3 to 32 mo (Figure 1B). Desirable results were achieved, and OS was longer in the cryo-immunotherapy group than in the cryotherapy group, demonstrating the synergistic effect of these two therapies (Figure 2). Owing to procedural costs, age or health, some of our patients underwent cryo-immunotherapy only once. We found that, compared with a single treatment, multiple cytoreductive cryoablation combined with immunotherapy was therapeutically valuable (Figure 3A) and prolonged survival time. Continued cryotherapy delayed disease progression, maintained function of multiple organs and improved quality of life and KPS scores, thereby achieving a better effect than single cryotherapy (Figure 3B).

In studies of the sequential use of TACE and percutaneous cryosurgery for unresectable HCC, pre-cryosurgical TACE was shown to increase the efficacy of cryoablation and decrease its adverse effects in patients with large HCCs (> 5 cm in diameter)<sup>[19]</sup>. It is well known that the presence of large HCCs often predicts rapid loss of liver function and a poor prognosis, and reducing their size before treatment is more effective than direct treatment of a large tumor. Data are available from two studies on the possible effect of TACE on immune stimulation<sup>[38,39]</sup>, which may further increase the therapeutic effect of combination therapy. Depending on whether single or multiple TACE is performed, a large HCC can first be reduced to 5 cm in diameter and then completely ablated by the combined application of multiple cryoprobes<sup>[19]</sup>. Consistent with the 2009 report of Shibata *et al.*<sup>[40]</sup>, treatment of larger tumors with sequential TACE and cryoablation can achieve significantly better effects than TACE or cryoablation alone. The findings of these authors and our own results indicate that not the frequency of TACE but the shrinkage of large HCCs contributed to the increase in median OS of about 30 mo, and differences due to HCC diameter can be eliminated by additional TACE procedures (median OS was 29 and 26 mo, respectively;  $P = 0.798$ ; Figure 1A).

In conclusion, we combined a minimally invasive procedure (percutaneous cryoablation of primary and metastatic tumors) with a common immunotherapy method (DC-CIK) to treat metastatic HCC. This new strategy extended the median OS from 3 to 32 mo. Better outcomes are expected as more patients undergo cryo-immunotherapy.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC), which is the fifth most common cancer worldwide, is usually discovered late and has a poor prognosis. In about 80% of patients, HCC is associated with chronic liver disease (*i.e.*, hepatitis and cirrhosis), with major implications for the prognosis and therapeutic options. Many patients are unsuitable for tumor resection because of factors such as poor hepatic reserve (cirrhosis), multicentric tumors or extrahepatic disease. Until recently, no systemic chemotherapy has significantly increased survival in patients with advanced HCC. External beam radiation has had a limited role in the treatment of HCC because of radiation toxicity to the adjacent normal liver

### Research frontiers

The effects of comprehensive cryosurgery (simultaneous cryoablation of intra- and extrahepatic tumors and of liver tumors of diameter greater than 5 cm, with transarterial chemoembolization (TACE) performed once or twice before cryoablation to reduce the tumor to 5 cm) and/or dendritic cells - cytokine-induced killer (DC-CIK) immunotherapy in patients with untreated metastatic HCC.

### Innovations and breakthroughs

Cryotherapy and, especially, cryo-immunotherapy significantly increased overall survival in metastatic hepatocellular cancer patients. Multiple cryo-immunotherapy was associated with a better prognosis than single cryo-immunotherapy.

### Applications

For metastatic HCC, comprehensive cryotherapy and cryo-immunotherapy can help patients improve symptoms, reduce pain and prolong life.

### Terminology

Comprehensive cryotherapy: simultaneous cryoablation of intra- and extrahepatic tumors and of liver tumors of diameter greater than 5 cm, with TACE performed once or twice before cryoablation to reduce the tumor to 5 cm; cryo-immunotherapy: Immunotherapy is performed shortly after comprehensive cryosurgery, breaking products of tumor may continually stimulate immune cells to clean up the systemic metastases lesions.

### Peer review

In this study, authors combined a minimally invasive procedure (percutaneous cryoablation of primary and metastatic tumors) with a common immunotherapy method (DC-CIK) to treat metastatic HCC. This new strategy extended the median overall survival from 3 mo to 32 mo. Better outcomes are expected as more patients undergo cryo-immunotherapy.

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**P- Reviewers** Gokhan K, Mario K **S- Editor** Zhai HH  
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## Efficacy of combined therapy in patients with hepatitis B virus-related decompensated cirrhosis

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Supported by The China National Science and Technology Major Project, No. 2010ZB063; Health Department of Zhejiang Province, China, No. 2010KYA067

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Received: November 17, 2012 Revised: March 23, 2013

Accepted: March 28, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To investigate the efficacy and safety of combined *de novo* lamivudine (LAM) and adefovir dipivoxil (ADV) therapy in hepatitis B virus (HBV)-related decompensated liver cirrhosis patients.

**METHODS:** One hundred and forty patients with HBV-related decompensated cirrhosis were recruited, 70 patients were treated with combined LAM and ADV *de novo* therapy, and the other 70 patients were treated with LAM alone as controls. The follow-up period was 144 wk. All patients with LAM resistance were shifted to ADV.

**RESULTS:** The percentage of HBV-related decompensated cirrhosis patients with undetectable HBV DNA in

*de novo* combination group was 51.6% (33/64), 84.2% (48/57), and 92.3% (49/53) by weeks 48, 96, and 144, respectively. In monotherapy group, HBV DNA negativity rate was 46.1% (30/65), 56.1% (32/57), and 39.2% (20/51) by weeks 48, 96 and 144, respectively. There was a significant difference between the two groups by weeks 96 and 144 ( $P = 0.012$  and  $0.001$ ). The hepatitis B e antigen seroconversion rate was 28.1% (9/32), 40.0% (12/30), and 53.6% (15/28) in the combination group by weeks 48, 96 and 144, respectively, and 24.2% (8/33), 31.0% (9/29), and 37.0% (10/27) by weeks 48, 96 and 144, respectively, in monotherapy group. A total of 68.6% (44/64), 84.2% (48/57), and 92.5% (49/53) patients achieved alanine aminotransferase (ALT) normalization by weeks 48, 96 and 144, respectively in the combination group. In monotherapy group, the ALT normalization rate was 64.6% (42/65) by week 48, 73.7% (42/57) by week 96, and 80.4% (41/51) by week 144. No patients in the combination group exhibited detectable resistance for at least 144 wk. The cumulative resistance rate in monotherapy group at weeks 48, 96, and 144 was 20.0%, 36.8%, and 56.9%. Both combination group and monotherapy group demonstrated an improvement in Child-Turcotte Pugh and Model for End-Stage Liver Disease scores at weeks 48, 96, and 144. All patients tolerated both combination and monotherapy. The ceratinine levels and glomerular filtration rate remained normal in all patients during the follow-up period.

**CONCLUSION:** In HBV-related decompensated liver cirrhosis patients, the combined *de novo* LAM and ADV therapy is more efficacious and safer compared to LAM alone.

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**Key words:** Liver cirrhosis; Lamivudine; Adefovir dipivoxil; Efficacy; Alanine transaminase

**Core tip:** Present treatment guidelines advocate oral nucleos(t)ide analogues in decompensated chronic hepatitis B (CHB) patients. Studies with lamivudine

(LAM) have demonstrated decreased mortality and improved liver function in CHB decompensated patients. However, LAM resistance mutations emerging during monotherapy can negate therapeutic benefit. Adefovir dipivoxil had no cross resistance with LAM. Consistent with outcomes in patients with LAM-resistance, no patient in *de novo* combination therapy group showed detectable resistance up to 144 wk in this study. The *de novo* combination therapy markedly improved liver function, reduced Child-Turcotte Pugh and Model for End-Stage Liver Disease scores in hepatitis B virus-related decompensated cirrhosis patients.

Lv GC, Yao JM, Yang YD, Zheng L, Sheng JF, Chen Y, Li LJ. Efficacy of combined therapy in patients with hepatitis B virus-related decompensated cirrhosis. *World J Gastroenterol* 2013; 19(22): 3481-3486 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3481.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3481>

## INTRODUCTION

It is estimated that over 350 million people worldwide are chronically infected with hepatitis B virus (HBV). The majority of these individuals reside in the Asia-Pacific region<sup>[1]</sup>. Chronic hepatitis B (CHB) infection is the principal cause of liver cirrhosis and hepatocellular carcinoma (HCC). In cirrhotic patients, the 5-year probability of decompensation is 15%-20%, and the risk increases as the HBV DNA level increases<sup>[2]</sup>. The 5-year survival rate in decompensated cirrhosis patients is 14%-35%, compared to 84% for those with compensated cirrhosis<sup>[3]</sup>.

Previous studies have shown that high serum HBV DNA is a major risk factor for disease progression to cirrhosis or HCC. Lamivudine (LAM) is the first nucleoside analog widely prescribed for CHB patients due to its antiviral efficacy and safety profile. LAM is found effective for patients with HBV-related decompensated liver cirrhosis<sup>[4,5]</sup>. However, LAM is associated with a high risk of drug resistance and virological breakthrough<sup>[6]</sup>. Adefovir dipivoxil (ADV) exhibits specificity, low drug resistance, and no cross resistance with other nucleoside analogs, which has been strongly considered as a rescue therapeutic agent to combat resistance<sup>[7,8]</sup>. In China, as the first approved drug for CHB patients, LAM has been widely prescribed for its clinical efficacy and low cost. Clinical trials have demonstrated the superiority of combined LAM and ADV therapy compared to ADV monotherapy in LAM resistant patients<sup>[9,10]</sup>. In this study, LAM and ADV are utilized as *de novo* combination treatment. To date, no study has been performed to systematically evaluate the efficacy and safety of *de novo* combined therapy in patients with HBV-related decompensated liver cirrhosis.

In this study, we aimed to compare the efficacy and safety of *de novo* combination therapy with monotherapy in patients with HBV-related decompensated liver cirrhosis.

## MATERIALS AND METHODS

### Study population

From January 2007 to December 2008, 140 consecutive nucleoside analogs treatment-naïve patients with HBV-related decompensated cirrhosis were enrolled in this study in the First Affiliated Hospital of Zhejiang University.

**Diagnostic criteria:** The diagnosis of decompensated liver cirrhosis was based on clinical, laboratory, previous histological, ultrasonographic and radiological signs of cirrhosis with Child-Turcotte-Pugh (CTP) score. The inclusion criteria for this study were as follows: aged 18-65 years, with HBV DNA  $\geq 10^3$  copies/mL, a CTP score of 7-12 (inclusive), calculated serum creatinine clearance  $\geq 50$  mL/min, hemoglobin  $\geq 75$  g/L, total white blood cell  $\geq 2.5 \times 10^9$ /L, platelet count  $\geq 30 \times 10^9$ /L,  $\alpha$ -fetoprotein  $\leq 20$  ng/mL, and no evidence of HCC.

**Exclusion criteria:** Patients were excluded for resistance to LAM, co-infection with hepatitis C virus, hepatitis D virus, hepatitis E virus or human immunodeficiency virus, and autoimmune hepatitis, alcoholic cirrhosis, hepatorenal syndrome, grade 3 or 4 hepatic encephalopathy, or spontaneous bacterial peritonitis, and severe heart, renal, brain diseases.

### Study design

Among 140 patients with HBV-related decompensated cirrhosis, 70 patients were treated with *de novo* combination therapy of 100 mg/d LAM and 10 mg/d ADV; the other 70 patients were treated with 100 mg/d LAM alone as controls. The duration of the treatment was 144 wk. All patients who exhibited LAM resistance were administered ADV.

All patients were followed up every 3-6 mo with examinations of liver and renal function, prothrombin time (PT), international normalized ratio (INR), serum HBV DNA, hepatitis B surface antigen (HBsAg), hepatitis B surface antibody (anti-HBs), hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe), and hepatitis B core antibody (anti-HBc), as well as ultrasonographic or computerized tomography examination. Routine biochemical and hematological tests, and clinical examination were performed at all clinical visits.

### Biochemical and virological analysis

Biochemistry, hematology, PT, INR, and urinalysis were analyzed immediately. Serum hepatitis B viral markers, including HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc were detected using commercially available enzyme immunoassays (Abbott Laboratories, Chicago, IL, United States). Serum HBV DNA was measured by polymerase chain reaction with a linear range between  $1 \times 10^3$  and  $5 \times 10^8$  copies/mL (Shanghai ZJ Bio-Tech Co., Ltd., China). LAM and ADV associated mutations were assessed by direct sequencing.

### Ethics

Informed consent for inclusion in the study was obtained

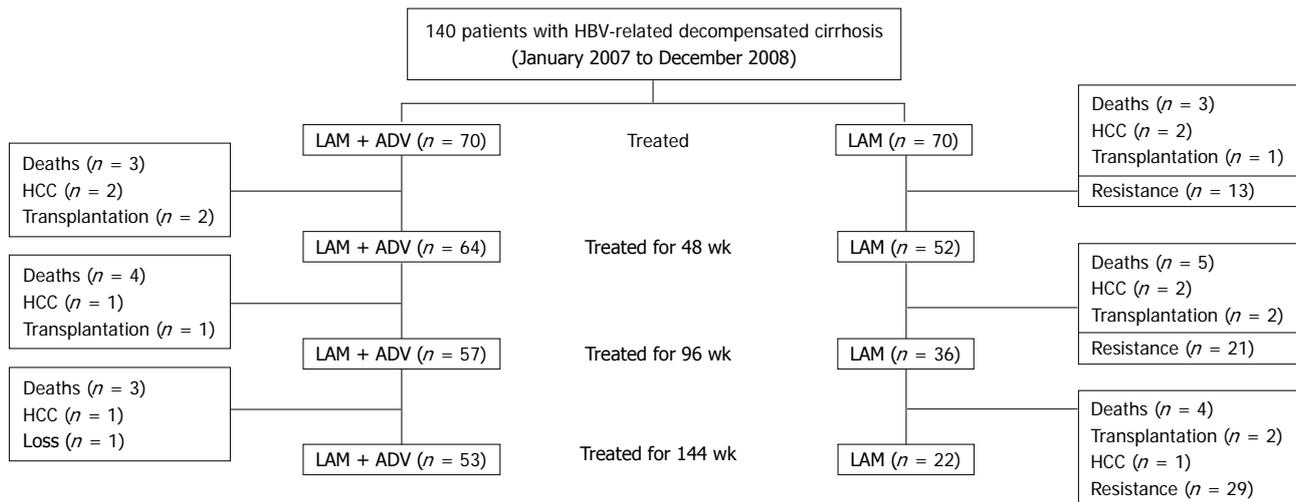


Figure 1 Flow chart of patients disposition in *de novo* lamivudine and adefovir dipivoxil combination group and lamivudine monotherapy group. HBV: Hepatitis B virus; HCC: Hepatocellular carcinoma; ADV: Adefovir dipivoxil; LAM: Lamivudine.

Table 1 Baseline characteristics of patients with hepatitis B virus-related decompensated cirrhosis (mean  $\pm$  SD)

| Characteristics                       | <i>De novo</i> combination group (n = 70) | Lamivudine group (n = 70) | P value |
|---------------------------------------|---|---------------------------|---------|
| Age (yr)                              | 46.8 $\pm$ 10.3                           | 47.1 $\pm$ 10.9           | 0.91    |
| Male/female                           | 41/29                                     | 40/30                     | 0.89    |
| ALT (IU/L)                            | 134.6 $\pm$ 101.3                         | 132.4 $\pm$ 120.8         | 0.78    |
| TBIL ( $\mu$ mol/L)                   | 38.5 $\pm$ 12.1                           | 37.8 $\pm$ 11.9           | 0.84    |
| Albumin (g/dL)                        | 3.2 $\pm$ 0.7                             | 3.3 $\pm$ 0.6 0.34        |         |
| Prothrombin time (s)                  | 17.1 $\pm$ 4.5                            | 17.6 $\pm$ 3.1            | 0.61    |
| HBV DNA (log <sub>10</sub> copies/mL) | 6.87 $\pm$ 1.21                           | 6.94 $\pm$ 1.15           | 0.73    |
| HBeAg positive                        | 34 (48.6)                                 | 33 (47.1)                 | 0.94    |
| Platelet count ( $\times 10^9$ /L)    | 78.9 $\pm$ 24.2                           | 76.7 $\pm$ 32.3           | 0.67    |
| Creatinine ( $\mu$ mol/L)             | 89.7 $\pm$ 12.3                           | 88.6 $\pm$ 13.1           | 0.45    |
| GFR (mL/min)                          | 115.2 $\pm$ 34.5                          | 113.5 $\pm$ 22.9          | 0.68    |
| CTP score                             | 8.9 $\pm$ 2.1                             | 8.8 $\pm$ 1.7             | 0.83    |
| CTP class                             |   |                           |         |
| A                                     | 6 (8.6)                                   | 7 (10)                    | 0.65    |
| B                                     | 48 (68.6)                                 | 49 (70)                   | 0.77    |
| C                                     | 16 (22.9)                                 | 14 (20)                   | 0.59    |
| MELD score                            | 12.4 $\pm$ 3.7                            | 11.9 $\pm$ 2.5            | 0.75    |

ALT: Alanine aminotransferase; TBIL: Total bilirubin; GFR: Glomerular filtration rate; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; CTP: Child-Turcotte-Pugh score; MELD: Model for End-Stage Liver Disease.

from all patients before recruitment. This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the First Affiliated Hospital of Zhejiang University.

### Statistical analysis

Statistical analysis was conducted using SPSS (version 16.0, IL, United States). Continuous variables were expressed as mean  $\pm$  SD. Continuous variables were examined using the Student's *t* test or Mann-Whitney *U* test. Categorical variables were compared by  $\chi^2$  test. A *P* value  $<$  0.05 was considered statistically significant.

## RESULTS

### Patients

A total of 140 patients were recruited: 70 received LAM and ADV *de novo* combination therapy and 70 received LAM monotherapy. Baseline characteristics were comparable between the two groups and are shown in Table 1. In *de novo* combination therapy group, 37 (52.9%) patients exhibited ascites, 9 (12.8%) exhibited episodes of hepatic encephalopathy, and 15 (21.4%) exhibited variceal bleeding. In LAM monotherapy group, 36 (51.4%) patients presented ascites, 8 (11.4%) presented episodes of hepatic encephalopathy, and 16 (22.9%) exhibited variceal bleeding. No patient in either group discontinued antiviral therapy during the study period. The characteristics of the patients during the 144 wk are shown in Figure 1.

### Virological, serological, and biochemical responses

The percentage of HBV related decompensated cirrhosis patients with undetectable HBV DNA in the *de novo* combination group was 51.6% (33/64), 84.2% (48/57) and 92.3% (49/53) by weeks 48, 96, and 144, respectively. In the monotherapy group, the rate of HBV DNA negativity was 46.1% (30/65), 56.1% (32/57) and 39.2% (20/51) by weeks 48, 96, and 144, respectively. A significant difference was observed between the two groups by weeks 96 and 144 (*P* = 0.012 and 0.001).

Patients who were HBeAg-positive at baseline, 34/70 (48.6%) in *de novo* combination group and 33/70 (47.1%) in monotherapy group, exhibited HBeAg seroconversion: 28.1% (9/32) *vs* 24.2% (8/33) by week 48 (*P* = 0.372), 40.0% (12/30) *vs* 31.0% (9/29) by week 96 (*P* = 0.021), and 53.6% (15/28) *vs* 37.0% (10/27) by week 144 (*P* = 0.014), respectively.

Of the 70 decompensated cirrhosis patients receiving *de novo* combination therapy, 68.6% (44/64), 84.2% (48/57) and 92.5% (49/53) of the patients achieved alanine aminotransferase (ALT) normalization by weeks 48, 96 and 144, respectively. In monotherapy group, the ALT

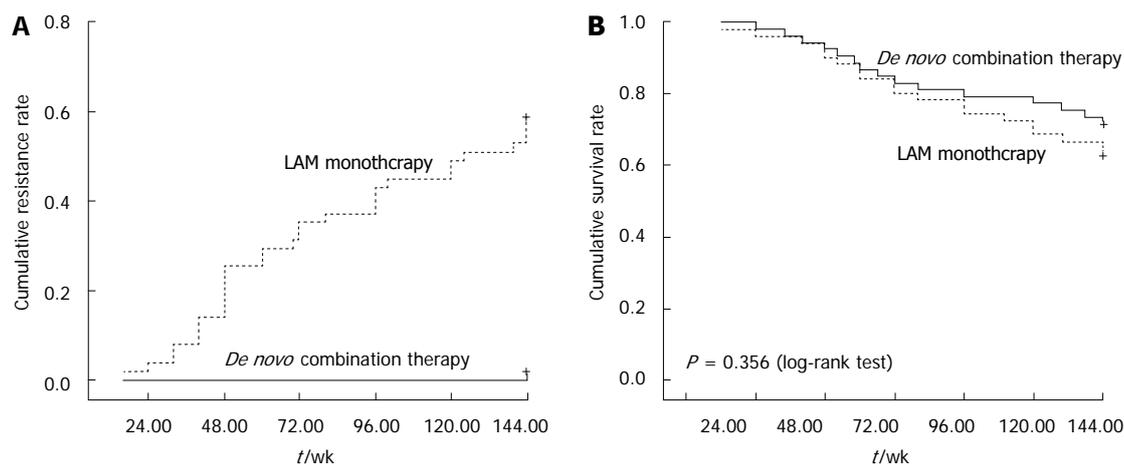


Figure 2 Kaplan-Meier analysis of cumulative resistance (A) and survival (B) rate in patients with hepatitis B virus-related decompensated cirrhosis on *de novo* lamivudine and adefovir dipivoxil combination therapy and lamivudine monotherapy. A: No resistance occurred in *de novo* combination group up to 144 wk. The cumulative resistance rate in lamivudine monotherapy group at weeks 48, 96 and 144 was 20%, 36.8% and 56.9%, respectively; B: The cumulative survival rate in *de novo* combination group was 91.4%, 81.6% and 75.7% at weeks 48, 96 and 144, respectively. The cumulative survival rate in lamivudine monotherapy group was 90.6%, 81.6% and 72.8%, respectively.

Table 2 Comparison of change in hepatic and renal function between two treatment groups (mean  $\pm$  SD)

| Characteristics     | <i>De novo</i> combination group |                  |                             |                             | Lamivudine monotherapy group |                  |                  |                             |
|---------------------|----------------------------------|------------------|-----------------------------|-----------------------------|------------------------------|------------------|------------------|-----------------------------|
|                     | Baseline                         | 48 wk            | 96 wk                       | 144 wk                      | Baseline                     | 48 wk            | 96 wk            | 144 wk                      |
| Hepatic function    |                                  |                  |                             |                             |                              |                  |                  |                             |
| Albumin (g/dL)      | 3.2 $\pm$ 0.7                    | 3.5 $\pm$ 0.4    | 3.8 $\pm$ 0.1               | 4.1 $\pm$ 0.3 <sup>a</sup>  | 3.3 $\pm$ 0.6                | 3.4 $\pm$ 0.3    | 3.6 $\pm$ 0.8    | 3.8 $\pm$ 0.3 <sup>c</sup>  |
| TBIL ( $\mu$ mol/L) | 38.5 $\pm$ 12.1                  | 24.3 $\pm$ 11.4  | 20.4 $\pm$ 10.8             | 16.1 $\pm$ 6.7 <sup>a</sup> | 37.8 $\pm$ 11.9              | 26.5 $\pm$ 12.3  | 23.2 $\pm$ 10.5  | 18.1 $\pm$ 12.3             |
| PT (s)              | 17.1 $\pm$ 4.5                   | 14.5 $\pm$ 5.3   | 13.3 $\pm$ 9.1 <sup>a</sup> | 11.4 $\pm$ 5.8 <sup>a</sup> | 17.6 $\pm$ 3.1               | 15.4 $\pm$ 3.5   | 14.5 $\pm$ 6.9   | 13.4 $\pm$ 5.8 <sup>c</sup> |
| CTP score           | 8.9 $\pm$ 2.1                    | 7.0 $\pm$ 1.2    | 6.3 $\pm$ 2.7 <sup>a</sup>  | 5.7 $\pm$ 1.9 <sup>a</sup>  | 8.8 $\pm$ 1.7                | 7.6 $\pm$ 2.4    | 6.6 $\pm$ 1.9    | 6.1 $\pm$ 3.8               |
| MELD score          | 12.4 $\pm$ 3.7                   | 10.0 $\pm$ 6.5   | 9.6 $\pm$ 5.3               | 8.8 $\pm$ 2.7 <sup>a</sup>  | 11.9 $\pm$ 2.5               | 10.8 $\pm$ 3.7   | 8.9 $\pm$ 4.8    | 8.9 $\pm$ 4.3               |
| Renal function      |                                  |                  |                             |                             |                              |                  |                  |                             |
| BUN (mmol/L)        | 6.7 $\pm$ 0.7                    | 6.8 $\pm$ 1.4    | 7.1 $\pm$ 1.1               | 6.9 $\pm$ 0.3               | 6.8 $\pm$ 0.6                | 7.4 $\pm$ 1.3    | 7.2 $\pm$ 0.8    | 7.0 $\pm$ 1.1               |
| Cr ( $\mu$ mol/L)   | 96.1 $\pm$ 12.3                  | 98.3 $\pm$ 8.9   | 97.8 $\pm$ 10.7             | 99.7 $\pm$ 9.6              | 95.6 $\pm$ 12.1              | 97.8 $\pm$ 7.9   | 98.6 $\pm$ 12.7  | 99.5 $\pm$ 9.7              |
| GFR (mL/min)        | 115.2 $\pm$ 34.5                 | 112.8 $\pm$ 56.3 | 103.4 $\pm$ 76.1            | 109.1 $\pm$ 21.5            | 113.5 $\pm$ 22.9             | 107.6 $\pm$ 13.4 | 112.4 $\pm$ 45.5 | 109.4 $\pm$ 13.4            |

<sup>a</sup> $P < 0.05$  vs baseline combination group; <sup>c</sup> $P < 0.05$  vs lamivudine monotherapy group. TBIL: Total bilirubin; PT: Prothrombin time; CTP: Child-Turcotte-Pugh score, MELD: Model for End-Stage Liver Disease; BUN: Blood urine nitrogen; Cr: Creatinine; GFR: Glomerular filtration rate.

normalization rate was 64.6% (42/65) by week 48, 73.7% (42/57) by week 96, and 80.4% (41/51) by week 144, respectively. Compared to *de novo* combination therapy group, ALT normalization rates were lower in monotherapy group by weeks 96 and 144 ( $P = 0.026$  and  $0.037$ ).

### Drug resistance

No patient in the *de novo* combination group exhibited detectable resistance within 144 wk. The cumulative resistance rate in monotherapy group at weeks 48, 96 and 144 was 20%, 36.8%, and 56.9%, respectively as shown in Figure 2A, significantly higher compared to *de novo* combination group.

### Change in hepatic function

Through week 144, both *de novo* combination group and monotherapy group achieved improvement in hepatic function, as evaluated by change from baseline in serum albumin, total bilirubin, prothrombin time; the degree of improvement in *de novo* combination group for albumin and prothrombin at week 144 was superior to mono-

therapy group, as shown in Table 2.

Both *de novo* combination and monotherapy groups demonstrated an improvement in CTP and Model for End-Stage Liver Disease (MELD) scores at weeks 48, 96 and 144. The mean change from baseline in CTP scores was -1.9, -2.6, and -3.2 at weeks 48, 96 and 144, respectively, for *de novo* combination group, and -1.7, -2.2 and -2.7 at weeks 48, 96, and 144, respectively, for monotherapy group. As a result, 45 (84.9%) of cirrhosis patients in *de novo* combination therapy group achieved CTP class A (score 5 or 6) after 144 wk of treatment, whereas in monotherapy group 68.6% (35/51) of patients achieved CTP class A after 144 wk.

The mean change from baseline in MELD scores was -2.4, -3.2, and -4.0 at weeks 48, 96, and 144, respectively, for *de novo* combination group, and -1.8, -2.3, and -3.0 at weeks 48, 96 and 144, respectively, for monotherapy group as shown in Table 2.

The 70 patients with decompensated cirrhosis in *de novo* combination therapy group exhibited a cumulative HCC incidence of 3.1% at week 48, 5.3% at week 96,

and 7.5% at week 144, respectively; four patients developed HCC during the follow-up period. In monotherapy group, the cumulative incidence of HCC was 3.1%, 7.0% and 9.8% at weeks 48, 96 and 144, respectively; five patients developed HCC during the follow-up period. In addition, the cumulative mortality or orthotopic liver transplantation rate was 8.6%, 18.4% and 24.3% at weeks 48, 96 and 144, respectively in *de novo* combination group, and 9.4%, 18.4% and 27.2% at weeks 48, 96 and 144, respectively, in the monotherapy group, as shown in Figure 2B.

### Side effects

All patients in this study tolerated both the *de novo* combination therapy and monotherapy. No patient in either group discontinued the treatment during the follow-up period. In the *de novo* combination group, the blood urine nitrogen (BUN) of three patients increased to 8.6, 9.1 and 9.7 mmol/L respectively, without a concomitant increase in creatinine levels. BUN level of these patients were normalized during the follow-up period. The creatinine levels and glomerular filtration rate remained normal in all patients during the follow-up period, as shown in Table 2.

## DISCUSSION

A consensus on the benefit of antiviral therapy for HBV-related cirrhosis has been achieved. Liaw *et al.*<sup>[11]</sup> reported that continuous treatment with LAM delays clinical progression in patients with CHB and advanced fibrosis or cirrhosis by significantly reducing the incidence of hepatic decompensation and risk of HCC. However, LAM exhibits a high incidence of resistance mutations compared to other nucleos(t)ide analogs. The current CHB treatment guidelines advocate initial combination therapy or agents with a high genetic barrier for patients with a high risk of developing drug resistance and potentially life-threatening associated diseases, such as HBV-related liver cirrhosis<sup>[12-14]</sup>. A combination with other drugs that do not share cross resistance may promote or exhibit synergistic antiviral effects; most importantly, it may exhibit the potential to prevent resistance. Ghany *et al.*<sup>[15]</sup> recently reported that extended combined therapy with LAM and ADV was associated with a high rate of long-term virological and biochemical response; none of the 22 combination therapy treated CHB patients developed resistance for up to 192 wk. Fan *et al.*<sup>[16]</sup> demonstrated that *de novo* combination therapy with LAM and ADV was superior to add-on combination therapy in terms of CTP score, virus inhibition, and renal function. In this study, for patients with HBV-related decompensated liver cirrhosis, *de novo* combination therapy resulted in a superior virological response than monotherapy at weeks 96 and 144. The cumulative tyrosinemethionine-aspartic acid-resistance rate was higher in monotherapy group by weeks 48, 96 and 144, whereas none of the *de novo* combination therapy treated patients developed re-

sistance during the follow-up period.

Higher HBeAg seroconversion rates and ALT normalization rates were also observed in *de novo* combination group compared to LAM monotherapy group at weeks 96 and 144. It is well known that HBeAg seroconversion is accompanied by biochemical and histological regression of liver diseases. Previous studies have indicated that combination therapy is efficacious in preventing drug resistance, but does not improve virological and serological response<sup>[17]</sup>. Our results and other studies suggest that *de novo* combination therapy can not only prevent drug resistance, but also improve virological and serological response<sup>[15,16]</sup>. Further elucidation concerning the mechanisms underlying the higher negative rates of HBV DNA and higher rates of HBeAg seroconversion by *de novo* combination therapy is warranted. In general, patients with CHB-related liver cirrhosis exhibit lower levels of HBV DNA and HBeAg compared with patients with CHB. Alternatively, during the course of HBV-related liver cirrhosis, the antiviral immune response is vigorously activated. In concordance with the findings of previous studies, the results that we present in this study suggest that *de novo* combination therapy is potentially suitable as an initial treatment for HBV-related decompensated liver cirrhosis in order to reduce resistance.

Previous clinical studies have demonstrated the positive effects of LAM therapy on the functional improvement in patients with HBV-related decompensated liver cirrhosis<sup>[4,5,18]</sup>. However, these benefits were offset due to drug-resistance. The CTP and MELD scores, both of which reflect components of liver function, including serum albumin, total bilirubin, and prothrombin time, were markedly improved during *de novo* combination therapy. These results clearly indicate that due to inhibition of HBV replication, *de novo* combination treatment potentially prevents clinical progression to liver failure, reduces complication risk, and delays or avoids the need for liver transplantation.

Renal impairment is a known risk of severe liver disease and is considered a potential side effect of *de novo* combination therapy. In this study, both *de novo* combination and monotherapy were well-tolerated, with no case of renal failure attributable to combination therapy.

This study has several limitations. It was not randomized or blinded, as this would be difficult for patients with CHB-related decompensated liver cirrhosis. This may contribute to the discord in results among previous studies.

In conclusion, this study demonstrated that *de novo* combination therapy is superior to monotherapy in suppressing HBV DNA and achieving ALT normalization and HBeAg seroconversion by weeks 96 and 144. Both treatments improved liver function as measured by reduction of CTP and MELD scores. In HBV-related decompensated liver cirrhosis patients, *de novo* combination therapy has also been proven safe. Our findings strongly support *de novo* combination therapy as a viable and effective therapeutic strategy in patients with HBV-related decompensated liver cirrhosis.

## COMMENTS

## Background

The mortality rate of hepatitis B virus (HBV)-related decompensated cirrhosis is very high. Recommended treatment options are nucleos(t)ide analogues. There are few reports regarding the issue of treatment for these patients with *de novo* combination therapy or monotherapy.

## Research frontiers

In China, tenofovir is not available yet and entecavir is expensive for most patients. Combination therapy with lamivudine (LAM) and adefovir dipivoxil (ADV) is better than ADV in LAM-resistance chronic hepatitis B (CHB) patients. But in patients with HBV-related decompensated liver cirrhosis, it remains unclear whether *de novo* combination therapy is better than monotherapy. In this study, the authors demonstrate that LAM and ADV *de novo* combination therapy is more effective than LAM monotherapy for patients with HBV-related decompensated liver cirrhosis.

## Innovations and breakthroughs

Many clinical studies showed that the combined LAM and ADV therapy is more effective than ADV monotherapy after LAM-resistance. However, the efficacy of *de novo* LAM and ADV combination therapy in patients with HBV-related decompensated liver cirrhosis is unclear. This is the first study to report that *de novo* LAM and ADV is more effective than LAM monotherapy, especially the combination therapy can reduce the drug resistance.

## Applications

By understanding that LAM and ADV *de novo* combination therapy is more effective than LAM monotherapy in patients with HBV-related decompensated liver cirrhosis, this study may represent a future strategy for therapeutic intervention in CHB patients with HBV-related decompensated liver cirrhosis.

## Terminology

*De novo* combination therapy means combination with two or more drugs from the beginning of the treatment. The diagnosis of decompensated liver cirrhosis was based on clinical, laboratory, previous histological, ultrasonographic and radiological signs of cirrhosis with Child-Turcotte-Pugh (CTP) score. The CTP score is a system to assess the disease stage for decompensated cirrhotic patients.

## Peer review

This is a good clinical study in which the authors compared the effect of *de novo* LAM and ADV combination therapy with LAM monotherapy. The authors concluded that LAM and ADV should be combined at the beginning for treatment of the patients with HBV-related decompensated liver cirrhosis.

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P- Reviewer Bruha R S- Editor Huang XZ  
L- Editor Ma JY E- Editor Xiong L



## Role of nesfatin-1 in a rat model of visceral hypersensitivity

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Supported by Natural Science Foundation of China, No. 81070308/H0308

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Received: January 13, 2013 Revised: May 15, 2013

Accepted: May 22, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To explore the role of nesfatin-1 on irritable bowel syndrome (IBS)-like visceral hypersensitivity.

**METHODS:** The animal model of IBS-like visceral hypersensitivity was induced by intracolonic infusion of 0.5% acetic acid (AA) in saline once daily from post-natal days 8-21. Experiments were performed when rats became adults. The visceral sensitivity of rats was evaluated by abdominal withdrawal reflex (AWR) and electromyographic (EMG) activity of the external oblique muscle to graded colorectal distension. The content of nesfatin-1 in serum was determined using enzyme-linked immunosorbent assay. After implantation of an intracerebroventricular (ICV) cannula and two electrodes into the external oblique muscle, model rats were randomly divided into four groups. Animals then received ICV injection of 8  $\mu$ g of anti-nesfatin-1/nucleobindin-2 (NUCB2), 50  $\mu$ g of  $\alpha$ -helical cortico-

tropin releasing factor (CRF) 9-41 (non-selective CRF receptor antagonist), 50  $\mu$ g of NBI-27914 (selective CRF1 receptor antagonist) or 5  $\mu$ L of vehicle. After 1 h of ICV administration, visceral sensitivity of each group was measured again, and comparisons between groups were made.

**RESULTS:** Rats treated with AA showed higher mean AWR scores and EMG activity at all distension pressures compared with controls ( $P < 0.05$ ). On histopathologic examination, no evidence of inflammation or abnormalities in structure were noted in the colon of either control or AA-treated groups. Myeloperoxidase values were not significantly different between the two groups. The level of nesfatin-1 in serum was significantly higher in the AA-treated group than in the control group ( $5.34 \pm 0.37$  ng/mL vs  $4.81 \pm 0.42$  ng/mL,  $P < 0.01$ ). Compared with rats injected with vehicle, rats which received ICV anti-nesfatin-1/NUCB2,  $\alpha$ -helical CRF9-41 or NBI-27914 showed decreased mean AWR scores and EMG activity at all distension pressures ( $P < 0.05$ ).

**CONCLUSION:** Nesfatin-1 may be associated with IBS-like visceral hypersensitivity, which may be implicated in brain CRF/CRF1 signaling pathways.

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**Key words:** Irritable bowel syndrome; Nesfatin-1; Visceral hypersensitivity; Corticotropin releasing factor; Intracerebroventricular injection

**Core tip:** This is a well conducted experimental study on the possible effect of nesfatin-1 in visceral hypersensitivity. Currently no reports have been published concerning the role of nesfatin-1 in irritable bowel syndrome (IBS). In a well-established visceral hypersensitivity animal model, we found an elevated nesfatin-1 level in the serum, and there was a reduction in evoked abdominal electromyography and abdominal withdrawal reflex scores after treatment with nesfatin-1 antibody, a non-selective corticotropin releasing factor (CRF) receptor antagonist, or a selective CRF1

receptor antagonist. These results suggest that nesfatin-1 may be associated with visceral hypersensitivity in model rats and implicated in brain CRF/CRF1 signaling pathways, which contribute to the visceral hypersensitivity of IBS.

Jia FY, Li XL, Li TN, Wu J, Xie BY, Lin L. Role of nesfatin-1 in a rat model of visceral hypersensitivity. *World J Gastroenterol* 2013; 19(22): 3487-3493 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3487.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3487>

## INTRODUCTION

Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder characterized by abdominal pain and alterations in bowel habits. The pathogenic mechanism of IBS remains incompletely understood. Visceral hypersensitivity is common in IBS patients and probably plays a major role in development of symptoms<sup>[1]</sup>. Nesfatin-1 is a newly discovered anorexigenic peptide derived from the precursor peptide nucleobindin-2 (NUCB2)<sup>[2]</sup>. Putative post-translational processing of NUCB2 by the enzyme pro-hormone convertase-1/3 results in nesfatin-1, nesfatin-2 and nesfatin-3<sup>[2]</sup>. Intracerebroventricular (ICV) injection of nesfatin-1 decreased dark phase food intake in freely fed rats, whereas injection of an antibody specific to nesfatin-1 potently stimulated appetite<sup>[2]</sup>. The hypothalamic selective corticotropin releasing factor-2 (CRF2) signaling system was also shown to be involved in the underlying mechanisms of nesfatin-1 induced reduction of dark phase food intake<sup>[3]</sup>. Nesfatin-1 is distributed in stress-related brain areas, such as the supraoptic nucleus, hypothalamic paraventricular nucleus (PVN), nucleus of the solitary tract, locus coeruleus and raphe pallidus nucleus<sup>[4,5]</sup>. Furthermore, nesfatin-1 is colocalized with CRF in the PVN<sup>[5]</sup>. The hormone CRF is the hallmark initiator of the stress response<sup>[6]</sup>. It exerts its biological actions by interacting with CRF1 and CRF2 receptors<sup>[7]</sup>. It has been well established that the brain CRF/CRF1 signaling system modulates pain responses although the exact sites mediating this modulation remain unidentified<sup>[8,9]</sup>. These observations suggest that nesfatin-1 may be involved in the autonomic regulation of visceral sensation. Given that visceral hypersensitivity is the major pathophysiology of IBS, nesfatin-1 may be an important contributor to the symptoms of IBS. Currently no reports have been published concerning the role of nesfatin-1 in IBS. The purpose of the present study, therefore, was to investigate the effect of nesfatin-1 on visceral sensitivity in IBS and the possible underlying mechanisms of this action. This was achieved by establishing a rat model of visceral hypersensitivity associated with IBS.

## MATERIALS AND METHODS

### Animals

Experiments were performed on male Sprague-Dawley

rats bought from the Experimental Animal Center of Nanjing Medical University (China). Rats were housed with *ad libitum* food and water in standard rodent cages at  $22 \pm 2$  °C in a 12-h light-dark controlled room. All animal procedures strictly adhered to the guidelines of the Institution Council of Animal Care and were approved by the Ethics Committee of Nanjing Medical University.

### Induction of chronic visceral hyperalgesia

Pups received an infusion of 0.3 mL of 0.5% acetic acid (AA) solution in saline into the colon 2 cm from the anus once daily on postnatal days 8-21<sup>[10]</sup>. Controls received an equal volume of saline. Experiments were conducted in these rats between 6 and 9 wk of age.

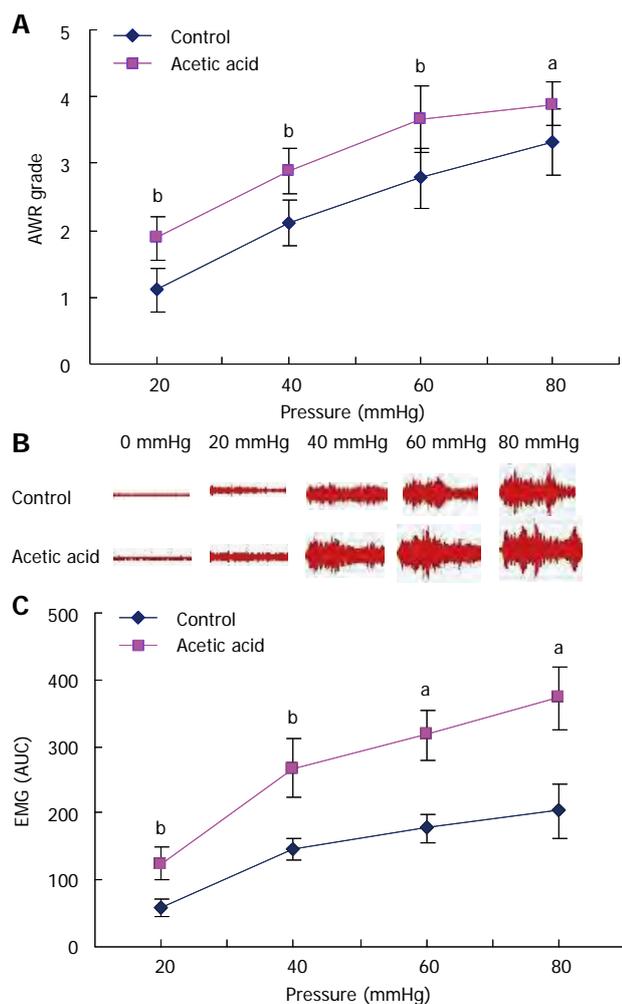
### Behavioral testing

We used a grading system based on the abdominal withdrawal reflex (AWR), as well as a measure of the electromyographic (EMG) activity of the external oblique muscle to evaluate visceral hypersensitivity 6 wk after treatment, by grading the response of rats to colorectal distention (CRD). Briefly, under mild sedation with ether, a flexible balloon (5 cm) constructed from a surgical glove finger attached to tygon tubing was inserted (8 cm) into the descending colon and rectum via the anus and held in place by taping the tubing to the tail. Rats were placed in small lucite cubicles (20 cm × 6 cm × 8 cm) and allowed to adapt for 30 min. CRD was performed by rapidly inflating the balloon to a constant pressure using a sphygmomanometer connected to a pressure transducer. The balloon was inflated to various pressures (20, 40, 60 and 80 mmHg) for 20 s followed by a 2-min rest. Behavioral responses to CRD were measured by visual observation of the AWR by a blinded observer. The assignment of an AWR score was as follows: 1 = normal behavior without response; 2 = contraction of abdominal muscles; 3 = lifting of abdominal wall; and 4 = body arching and lifting of pelvic structures<sup>[11]</sup>.

To obtain EMG measurements of visceromotor responses, two electrodes were implanted, under anesthesia with pentobarbital sodium (100 mg/kg, intraperitoneally), into the external oblique muscle and externalized behind the head. Rats were allowed 1 wk recovery from surgery. CRD was performed as described previously with 20 s of distention followed by 2-min rest between distentions of 20, 40, 60 and 80 mmHg. Wires were connected to a Bio Amp, which was connected to a power lab (AD Instruments, Australia) used as an EMG acquisition system with Chart 7 software. The area under the curve during the 20-s distention for the EMG signal was calculated by subtracting the area under the curve for the 20 s before distention<sup>[12]</sup>.

### Evaluation of the colon for inflammation/damage

After behavioral testing, 4 cm of colonic tissue proximal to the anus was removed and rinsed briefly with saline. The proximal half of each colon was placed in 4% paraformaldehyde, embedded in paraffin, cut into 4 μm sections, and used for histologic examination. The distal



**Figure 1** Evaluation of visceral sensitivity in neonatal acetic acid-treated rats when adults. **A:** Abdominal withdrawal reflex (AWR) scores were used as an index in response to distension pressure. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group,  $n = 9$ ; **B:** Typical electromyographic (EMG) activity in the external oblique muscle in response to graded colorectal distension; **C:** Area under the curve (AUC) of EMG activity in the external oblique muscle in response to graded colorectal distension. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group,  $n = 9$ .

half was snap-frozen and stored at  $-80^{\circ}\text{C}$  until use. Myeloperoxidase (MPO) activity was determined later by an enzyme-linked immunosorbent assay (ELISA) kit (RB, Minneapolis, MN, United States).

#### Blood sampling and processing

Under mild sedation with ether, approximately 1 mL blood was taken from the orbital canthus vein plexus. The blood was centrifuged, then the serum was separated and stored at  $-80^{\circ}\text{C}$  until assayed. Serum nesfatin-1 levels were measured using the ELISA kit (RD, CA, United States).

#### ICV cannula and external oblique muscle electrode implantation

Model rats were selected and anesthetized with pentobarbital sodium (100 mg/kg *ip*). A chronic guide cannula was implanted into the right lateral ventricle of the brain following coordinates from Bregma: anteroposterior,  $-0.8$  mm;

lateral,  $-1.5$  mm; dorsoventral,  $-3.5$  mm<sup>[13]</sup>. Two stainless steel screws were fixed to the skull, and then the cannula was secured with dental cement. Finally, two electrodes were implanted in the external oblique muscle as described above. After surgery, rats were housed individually and allowed to recuperate for 1 wk.

#### ICV injection

After ICV cannula and external oblique muscle electrode implantation, model rats were randomly divided into 4 groups and administered, by ICV injection, 8  $\mu\text{g}$  of anti-nesfatin-1/NUCB2<sup>[2]</sup> (Bioss, Beijing, China), 50  $\mu\text{g}$  of  $\alpha$ -helical CRF9-41<sup>[14]</sup> (Tocris, Minneapolis, MN, United States), 50  $\mu\text{g}$  of NBI-27914<sup>[13]</sup> (Tocris, Minneapolis, MN, United States) or 5  $\mu\text{L}$  of vehicle<sup>[2]</sup>. After 1 h, the visceral sensitivity of each group to CRD was measured and compared between groups.

#### Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, United States). All data are expressed as mean  $\pm$  SE. For AWR behavioral grades, a Friedman analysis of variance (ANOVA) was used. Median AWR scores at each distension pressure were compared between treatment groups using the Mann-Whitney  $U$  rank sum test. EMG data were analyzed by two-way repeated-measures ANOVA. Other data were analyzed by the Student  $t$  test or one-way ANOVA where appropriate.  $P$  values  $< 0.05$  were considered statistically significant.

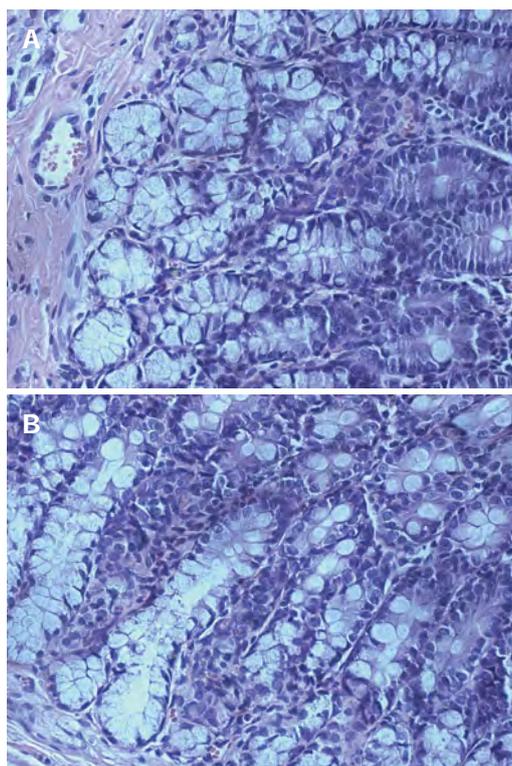
## RESULTS

### Neonatal AA treatment produced persistent visceral hypersensitivity

Visceral sensitivity to CRD was determined at 6 wk of age. Rats treated with AA exhibited higher mean AWR scores at all distension pressures tested than controls (Figure 1A,  $P < 0.05$ ). In a separate experiment, EMG activity, measured in response to graded CRD, was significantly higher in neonatal AA-treated rats than in controls (Figure 1B and C,  $P < 0.05$ ). Taken together, these data showed that rats treated with AA between postnatal days 8 and 21 were more sensitive to CRD than controls, suggesting that neonatal AA treatment produced persistent visceral hypersensitivity when animals became adults.

### Evaluation of adult colons for inflammation

To determine whether persistent visceral hypersensitivity achieved in adults of neonatal AA-treated rats was due to the development of chronic colitis in adults, hematoxylin and eosin-stained sections and MPO activity of the colons of adult rats were examined for histopathologic signs of inflammation. On examination, no significant inflammation or abnormalities in structure were noted in either saline- or AA-treated groups and no inflammatory infiltrates were observed (Figure 2). Likewise, there was



**Figure 2** Photomicrographs of hematoxylin and eosin-stained sections from colons of control adult rats (A) and neonatal acetic acid-treated adult rats (B). No significant inflammation or abnormalities in structure were observed in neonatal acetic acid-treated rats.

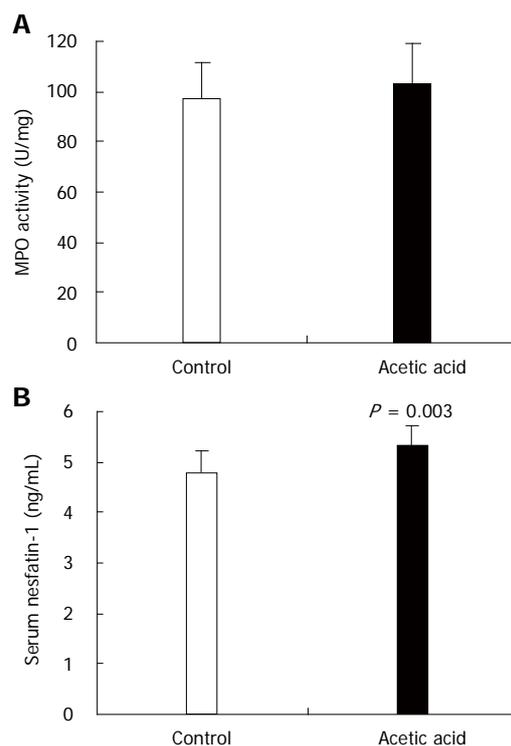
no significant difference in the level of MPO between control and neonatal AA-treated rats (Figure 3A,  $P > 0.05$ ). Thus, these data showed that inflammation/abnormalities were absent in our model, ruling out their involvement in persistent hypersensitivity. Therefore, we have a rat model of persistent visceral hypersensitivity caused by neonatal exposure to a mild acid in the absence of ongoing inflammation.

#### Serum nesfatin-1 levels in rats

The mean serum nesfatin-1 level in the neonatal AA-treated group ( $5.34 \pm 0.37$  ng/mL) was significantly higher than in the control rats ( $4.81 \pm 0.42$  ng/mL; Figure 3B,  $P = 0.003$ ).

#### Effect of ICV administration of antibody on visceral sensitivity

To determine the effect of nesfatin-1 on visceral sensitivity, adult model rats were given ICV injection of anti-nesfatin-1/NUCB2 or vehicle, and visceral sensitivity was measured 1 h later. The AWR scores of nesfatin-1 antibody-treated rats were significantly lower than those of vehicle-treated rats at 20, 40, 60 and 80 mmHg (Figure 4A). Similarly, in model rats, anti-nesfatin-1/NUCB2 treatment caused a significant decline in the EMG activity to graded CRD when compared with vehicle injection. To examine whether brain CRF/CRF1 signaling pathways were involved in visceral hypersensitivity in model rats,



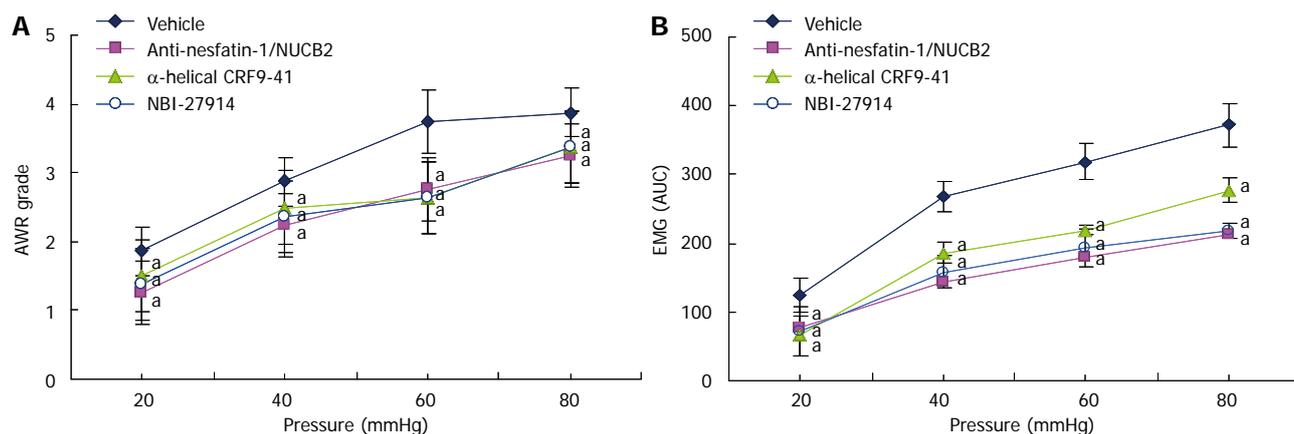
**Figure 3** Myeloperoxidase activity in rat colon (A) and nesfatin-1 levels in rat serum (B). A: There was no difference in myeloperoxidase (MPO) activity in neonatal acetic acid-treated rats compared with control rats; B: The average serum nesfatin-1 level was statistically higher in neonatal acetic acid-treated rats than in controls ( $n = 12$ ).

animals were injected intracerebroventricularly with non-selective CRF receptor antagonists ( $\alpha$ -helical CRF9-41), selective CRF1 receptor antagonist (NBI-27914) or vehicle 1 h before graded CRD. In comparison with model rats receiving a vehicle injection, model rats that received  $\alpha$ -helical CRF9-41 and NBI-27914 showed decreased mean AWR scores and EMG activity at all distension pressures (Figure 4B).

## DISCUSSION

IBS is one of the most common functional gastrointestinal disorders worldwide. The mechanism of this disease is not clear, but an important role for visceral hypersensitivity in the development of symptoms compatible with IBS has become evident. It has been confirmed that a lower pain threshold to colonic distention was observed in patients with IBS compared with healthy subjects<sup>[1]</sup>, and visceral hypersensitivity is a biological marker for IBS<sup>[15]</sup>.

Visceral hypersensitivity may be associated with intestinal irritation (pain or inflammation) in the neonatal period. Nociceptive neuronal circuits are formed during the embryonic and postnatal period when painful stimuli are normally absent or limited. Pain and inflammation during this critical period, particularly before the maturation of the descending inhibitory systems, can lead to prolonged structural and functional alterations in pain



**Figure 4** Effect of anti-nesfatin-1/nucleobindin-2,  $\alpha$ -helical corticotropin releasing factor 9-41 and NBI-27914 treatment on visceral sensitivity in model rats. **A:** Abdominal withdrawal reflex (AWR) scores were used as an index in response to distension pressure. Model rats receiving intracerebroventricular injection of anti-nesfatin-1/nucleobindin-2 (NUCB2),  $\alpha$ -helical corticotropin releasing factor (CRF) 9-41 and NBI-27914 showed decreased mean AWR scores compared with model rats receiving vehicle injection, at 20, 40, 60 and 80 mmHg,  $^aP < 0.05$  vs vehicle group,  $n = 8$ ; **B:** Electromyographic (EMG) activity in the external oblique muscle in response to graded colorectal distension. Compared with vehicle administration, EMG activity in model rats administered intracerebroventricularly with anti-nesfatin-1/NUCB2,  $\alpha$ -helical CRF9-41 or NBI-27914 was significantly decreased at 20, 40, 60 and 80 mmHg compared with model rats receiving vehicle injection,  $^aP < 0.05$  vs vehicle group,  $n = 8$ .

pathways that can last into adult life. This visceral hypersensitivity is associated with central neural sensitization, as well as central sensitization<sup>[16]</sup>.

In this study, a rat model of visceral hypersensitivity associated with IBS was established by intracolonic instillation of dilute AA between P8 and P21. In our model, dilute AA treatment of pups had no effect on the growth of rats. As adults, these rats showed no identifiable peripheral pathology, which was in line with the characteristics of IBS. These rats exhibited higher mean AWR scores and EMG activity at all distension pressures compared with controls. These findings suggest that neonatal AA treatment induced long-lasting visceral hypersensitivity without significant inflammation in the colon, which was in agreement with previous findings<sup>[17]</sup>. Therefore, this model can better reflect the chronic hyperesthetic state of IBS and is applicable to the study of visceral hypersensitivity.

Nesfatin-1 is a recently discovered 82-amino-acid satiety peptide and has a predicted molecular mass of 9.7 kDa. Nesfatin-1, injected intracerebroventricularly or peripherally in rats, reduced food intake in a dose-dependent manner<sup>[2,18]</sup>. As yet, however, the nesfatin-1 receptor has not been identified. Shimizu *et al*<sup>[18]</sup> reported that NUCB2 can be potentially processed into an active derivative, nesfatin-1. However, this mature peptide has not been detected in protein extracts from rat brain<sup>[2]</sup>. Likewise, the western blots of all tissues and cells studied here failed to show a band at 9.7 kDa<sup>[19,20]</sup>. It has been confirmed that nesfatin-1 exists in the blood of rodents and humans using a sandwich-type ELISA, but normal values have not yet been established for nesfatin-1<sup>[21]</sup>. Nesfatin-1 can cross the blood-brain barrier without saturation<sup>[22,23]</sup>, therefore the measurement of nesfatin-1 in the blood may partly reflect its level in the brain. The present study showed that the average serum nesfatin-1 level was significantly higher in neonatal AA-treated rats than in con-

trol rats, suggesting nesfatin-1 may be associated with a visceral hypersensitivity state in model rats.

Furthermore, we found that model rats receiving ICV injection of anti-nesfatin-1/NUCB2 showed decreased mean AWR scores and EMG activity at 20, 40, 60 and 80 mmHg compared with model rats receiving vehicle injection. Results suggest that ICV injection of nesfatin-1 antibody may neutralize endogenous nesfatin-1 and therefore dramatically attenuate visceral hypersensitivity in model rats.

Taken together, these results strongly suggest that nesfatin-1 may be involved in visceral hypersensitivity in model rats. To date, the evidence for the role of a brain CRF/CRF1 signaling system in the modulation of visceral sensitivity in rodents has been based on the study of visceral hypersensitivity in acute models of stress<sup>[24]</sup>. In this model, our results also showed that ICV administration of  $\alpha$ -helical CRF9-41 and NBI-27914 caused a significant decrease in mean AWR scores and EMG activity at 20, 40, 60 and 80 mmHg in comparison with ICV administration of vehicle, which was consistent with the previous studies<sup>[25,26]</sup>. These data demonstrate that brain CRF/CRF1 signaling pathways may be involved in visceral hypersensitivity in the present study.

Previous studies have shown that nesfatin-1 was colocalized with CRF in the PVN<sup>[5]</sup>. ICV administration of nesfatin-1 increased the incidence of c-Fos expression in CRF neurons, and nesfatin-1 increased cytosolic  $Ca^{2+}$  concentration in the CRF-immunoreactive neurons isolated from the PVN<sup>[27]</sup>. Taken together with our present study, these results suggested that nesfatin-1 may be implicated in brain CRF/CRF1 signaling pathways, which then contribute to visceral hypersensitivity in model rats.

In conclusion, nesfatin-1 may be associated with the visceral hypersensitivity state of IBS, and this may be mediated at least in part by brain CRF/CRF1 signaling pathways.

## COMMENTS

## Background

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder in clinical practice, but the pathophysiology of IBS has not been completely elucidated. Visceral hypersensitivity is common in IBS patients and has recently been considered as a biological marker for IBS. Nesfatin-1 is a recently discovered 82-amino-acid satiety peptide. Nesfatin-1 is co-localized with corticotropin releasing factor (CRF) in the hypothalamic paraventricular nucleus (PVN). Furthermore, intracerebroventricular administration of nesfatin-1 induced c-Fos expression in CRF neurons, and nesfatin-1 increased cytosolic Ca<sup>2+</sup> concentrations in single CRF neurons in the PVN. It is now well established that the brain CRF/CRF1 signaling system modulates pain responses. These observations suggest that nesfatin-1 may be involved in the autonomic regulation of visceral sensation.

## Research frontiers

Visceral hypersensitivity is a topic of intense research in gastrointestinal disorders. The research hotspot in terms of the visceral hypersensitivity mechanism is the signaling system and the exact sites mediating modulation.

## Innovations and breakthroughs

The authors found that the average serum nesfatin-1 level was significantly higher in neonatal acetic acid-treated rats than in control rats, suggesting nesfatin-1 may be associated with a visceral hypersensitivity state in IBS-like model rats. Currently, no reports have been published discussing the role of nesfatin-1 in IBS. The purpose of the present study, therefore, was to investigate the effect of nesfatin-1 on visceral sensitivity in IBS and the possible underlying mechanisms of this action.

## Applications

The study results suggest that nesfatin-1 may be associated with the visceral hypersensitivity state of IBS, and this may be mediated, at least in part, by brain CRF/CRF1 signaling pathways. This may provide new targets for the treatment of IBS.

## Peer review

The authors investigated serum nesfatin-1 in a rat model of visceral hypersensitivity associated with IBS to explore the role of nesfatin-1 in the pathogenesis of IBS-like visceral hypersensitivity. The study found that nesfatin-1 may be associated with visceral hypersensitivity in model rats and may be implicated in brain CRF/CRF1 signaling pathways. Overall, the study has been well designed and the results are of great scientific significance.

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**P- Reviewers** Liu S, Zheng HC **S- Editor** Huang XZ  
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## Psychometrics of chronic liver disease questionnaire in Chinese chronic hepatitis B patients

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Supported by The National TS Major Project of China, No. 2008ZX10002-001 and No. 2012ZX10002001

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Received: January 08, 2013 Revised: March 25, 2013

Accepted: March 28, 2013

Published online: June 14, 2013

### Abstract

**AIM:** To evaluate psychometrics of the Chinese (mainland) chronic liver disease questionnaire (CLDQ) in patients with chronic hepatitis B (CHB).

**METHODS:** A cross-sectional sample of 460 Chinese patients with CHB was selected from the Outpatient Department of the Eighth Hospital of Xi'an, including CHB (CHB without cirrhosis) ( $n = 323$ ) and CHB-related cirrhosis ( $n = 137$ ). The psychometrics includes reliability, validity and sensitivity. Internal consistency reliability was measured using Cronbach's  $\alpha$ . Convergent and discriminant validity was evaluated by item-scale correlation. Factorial validity was explored by principal component analysis with varimax rotation. Sensitivity was assessed using Cohen's effect size (ES), and independent sample  $t$  test between CHB and CHB-related cirrhosis groups and between alanine aminotransferase (ALT) normal and abnormal groups after stratifying the disease (CHB and CHB-related cirrhosis).

**RESULTS:** Internal consistency reliability of the CLDQ was 0.83 (range: 0.65-0.90). Most of the hypothesized item-scale correlations were 0.40 or over, and all of such hypothesized correlations were higher than the alternative ones, indicating satisfactory convergent and discriminant validity. Six factors were extracted after varimax rotation from the 29 items of CLDQ. The eligible Cohen's ES with statistically significant independent sample  $t$  test was found in the overall CLDQ and abdominal, systematic, activity scales (CHB vs CHB-related cirrhosis), and in the overall CLDQ and abdominal scale in the stratification of patients with CHB (ALT normal vs abnormal).

**CONCLUSION:** The CLDQ has acceptable reliability, validity and sensitivity in Chinese (mainland) patients with CHB.

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**Key words:** Chronic hepatitis B; Chronic liver disease questionnaire; Reliability; Validity; Sensitivity

**Core tip:** Chronic hepatitis B (CHB) is a common chronic liver disease in mainland China, and its adverse prognosis might impair the patients' health-related quality of life (HRQoL). The chronic liver disease questionnaire (CLDQ) is the first liver specific HRQoL instrument, however, few studies have examined psychometrics of the Chinese (mainland) CLDQ in CHB patients. This study tested psychometrics of the Chinese (mainland) CLDQ in patients with CHB (including CHB without cirrhosis and CHB-related cirrhosis). The findings will help find suitable disease-specific questionnaire in the management of CHB in mainland China and provide evidence for expanding the use of the CLDQ.

Zhou KN, Zhang M, Wu Q, Ji ZH, Zhang XM, Zhuang GH. Psychometrics of chronic liver disease questionnaire in Chinese chronic hepatitis B patients. *World J Gastroenterol* 2013; 19(22): 3494-3501 Available from: URL: <http://www.wjgnet.com>

## INTRODUCTION

Chronic hepatitis B (CHB), caused by persistent infection with hepatitis B virus (HBV), is a common chronic liver disease in mainland China. According to the latest national hepatitis B seroepidemiological survey<sup>[1]</sup>, the estimated current HBV carriers in mainland China run up to 93 million, including 20-30 million patients with CHB<sup>[2]</sup>. CHB patients suffer recurrent symptoms in the long disease natural history and are at a high risk of developing fatal complications of cirrhosis and hepatocellular carcinoma<sup>[3,4]</sup>. Therefore, CHB may result in a heavy disease burden not only in premature death but also in health impairment<sup>[5-8]</sup>.

Health-related quality of life (HRQoL) is defined as how the individual rates his/her life in terms of physical, psychological and social aspects<sup>[9]</sup>. In comparison with clinical parameters, HRQoL is a more holistic assessment of health status considering the individual's functional health and well-being, especially in chronic disease in which mortality is not an immediate concern<sup>[3]</sup>. Due to the complex natural history and phases of CHB<sup>[10]</sup>, it is particularly important to use HRQoL as the primary endpoint for evaluating health and treatment effects in patients with such disease.

Generally, HRQoL can be measured by generic and disease-specific instruments. Generic instruments are used to compare the HRQoL between groups of patients, but disease-specific questionnaires distinguish the impairment of a specific illness and are more sensitive to the change<sup>[11,12]</sup>. The 36-item short-form health survey version 2 (SF-36v2) performs well as a generic instrument in patients with CHB<sup>[13]</sup>. However, not every scale or summary component of the SF-36v2 has the required or eligible sensitivity<sup>[14]</sup>. Therefore, a disease-specific HRQoL instrument is recommended as a complement for clinical studies in CHB.

The chronic liver disease questionnaire (CLDQ) is the first liver specific instrument developed by Younossi *et al.*<sup>[15]</sup>. It has been translated into different languages for cross-cultural adaptation<sup>[16-23]</sup> and proved as a valid tool for HRQoL measurement and evaluation in patients with chronic liver disease<sup>[24,25]</sup>. The Chinese (Hong Kong, China) CLDQ has been validated in patients with CHB infection<sup>[14]</sup>. However, a dearth of study assessed psychometrics of the Chinese (mainland) CLDQ in mainland Chinese patients with CHB<sup>[26]</sup>.

The purpose of the study was to evaluate psychometrics including reliability, validity and sensitivity of the Chinese (mainland) CLDQ in CHB patients. The findings of this study will help find suitable disease-specific questionnaire in the management of CHB in mainland China and add to the body of evidence for expanding the use of the CLDQ.

## MATERIALS AND METHODS

### Patients and data collection

Participants were CHB outpatients from the Eighth Hospital of Xi'an, which is the only local infectious disease hospital in Xi'an, Shaanxi Province, China. Inclusion criteria were aged 18 years or over, Chinese-speaking, with diagnosis of CHB (CHB without cirrhosis) or CHB-related cirrhosis. The diagnosis was made following the standards in the Guideline of Prevention and Treatment for Chronic Hepatitis B (2005 version) issued by the Chinese Society of Hepatology and the Chinese Society of Infectious Diseases<sup>[27]</sup>. If the patients had other chronic diseases (including hypertension, diabetes, chronic obstructive pneumonia disease, cardiovascular disease, mental illness, arthritis, tuberculosis, or gallstone), non-hepatitis B related liver disease, malignancies of liver or other organs, liver transplantation, cognitive disorders, or co-infected with human immunodeficiency virus or other types of hepatitis virus (*e.g.*, hepatitis A virus, hepatitis C virus, hepatitis D virus or hepatitis E virus), or refused to give written informed consent, they would be excluded.

Data were collected from April to June 2010. An individual face-to-face interview was administered by the trained interviewers in a quiet and well-lit room. The patients answered questions of sociodemographics and the Chinese (mainland) CLDQ. In addition to this, a free alanine aminotransferase (ALT) test was provided for the patients immediately after the questionnaire survey. The test was conducted in the laboratory of the Eighth Hospital of Xi'an.

### Chinese (mainland) CLDQ

The Chinese (mainland) CLDQ was provided by the developers of the original CLDQ<sup>[15]</sup>. It consists of 29 items measuring six scales on abdominal symptoms (AB), fatigue (FA), systemic symptoms (SY), activity (AC), emotional function (EM) and worry (WO). Each item is rated on a 7-point Likert scale (1 = all of the time to 7 = none of the time). The six scale scores are calculated by the summated averages of corresponding endorsed item scores. The total score is calculated by the mean of all scale scores. Each scale and the total score ranged from 1 to 7, with a higher score indicating better HRQoL.

### Reliability and floor/ceiling effects

Cronbach's  $\alpha$  coefficient was used to assess the internal consistency reliability, with the value greater than 0.70 representing acceptable reliability<sup>[28]</sup>. Floor and ceiling effects were calculated as the number and percentage of CHB patients at the lowest and highest possible scores for each scale and the overall CLDQ. It should be less than 20% regarding both the floor and ceiling effect to ensure that the scales are capturing the full range of potential responses in the population and that changes over time can be detected<sup>[29]</sup>.

**Table 1 Social-demographic characteristics *n* (%)**

| Items                                     | Total<br>( <i>n</i> = 460) | CHB<br>( <i>n</i> = 323) | CHB-related<br>cirrhosis<br>( <i>n</i> = 137) |
|---|----------------------------|--------------------------|---|
| Age (yr)                                  |                            |                          |   |
| 18-35                                     | 240 (52.2)                 | 216 (90.0)               | 24 (10.0)                                     |
| 36-50                                     | 152 (33.0)                 | 87 (57.2)                | 65 (42.8)                                     |
| 51-65                                     | 64 (13.9)                  | 20 (31.3)                | 44 (68.8)                                     |
| 66-76                                     | 4 (0.9)                    | 0 (0.0)                  | 4 (100.0)                                     |
| mean ± SD                                 | 35.75 ± 12.82              | 31.53 ± 10.94            | 45.71 ± 11.36                                 |
| Gender                                    |                            |                          |   |
| Male                                      | 305 (66.3)                 | 212 (69.5)               | 93 (30.5)                                     |
| Female                                    | 155 (33.7)                 | 111 (71.6)               | 44 (28.4)                                     |
| Marital status                            |                            |                          |   |
| Single                                    | 125 (27.2)                 | 119 (95.2)               | 6 (4.8)                                       |
| Married                                   | 328 (71.3)                 | 200 (61.0)               | 128 (39.0)                                    |
| Others                                    | 7 (1.5)                    | 4 (57.1)                 | 3 (42.9)                                      |
| Education level                           |                            |                          |   |
| No schooling and primary                  | 63 (13.7)                  | 31 (49.2)                | 32 (50.8)                                     |
| Secondary                                 | 311 (67.6)                 | 217 (69.8)               | 94 (30.2)                                     |
| Tertiary                                  | 86 (18.7)                  | 75 (87.2)                | 11 (12.8)                                     |
| Occupation                                |                            |                          |   |
| Peasants                                  | 153 (33.3)                 | 73 (47.7)                | 80 (52.3)                                     |
| Non-peasants                              | 307 (66.7)                 | 250 (81.4)               | 57 (18.6)                                     |
| Annual per capita household incomes (RMB) |                            |                          |   |
| < 5000                                    | 236 (51.3)                 | 147 (62.3)               | 89 (37.7)                                     |
| 5000-9999                                 | 111 (24.1)                 | 88 (79.3)                | 23 (20.7)                                     |
| ≥ 10000                                   | 113 (24.6)                 | 88 (77.9)                | 25 (22.1)                                     |
| Received ALT test                         |                            |                          |   |
| Yes                                       | 375 (81.5)                 | 311 (82.9)               | 64 (17.1)                                     |
| No  | 85 (18.5)                  | 12 (14.1)                | 73 (85.9)                                     |

CHB: Chronic hepatitis B; ALT: Alanine aminotransferase.

### Validity

The hypothesized item-scale correlation ( $\gamma$  coefficient)  $\geq 0.40$  was considered as satisfactory convergent validity<sup>[30]</sup>. Discriminant validity was supported whenever the hypothesized item-scale correlation was significantly higher than the correlation of the item with other scales<sup>[31]</sup>. Factorial validity of the CLDQ was explored using the principal component analysis with varimax rotation. The predictive items with factor loading coefficient (FLC)  $\geq 0.45$ <sup>[32]</sup> and the extracted factors with an eigenvalue  $\geq 1.0$ <sup>[33]</sup> were considered to be relevant.

### Sensitivity

Sensitivity was evaluated by Cohen's effect size (ES), and independent sample *t* test between CHB and CHB-related cirrhosis groups and between ALT normal and abnormal groups after stratifying the disease (CHB and CHB-related cirrhosis). The ES value was calculated as the difference between group mean scores divided by overall standard deviation. According to Cohen, the ES of 0.2-0.5 is small, of 0.5-0.8 moderate, and those of 0.8 or above large<sup>[31]</sup>. Besides, multiple linear regression analysis was used to further prove sensitivity of the CLDQ under the influence of ALT (normal *vs* abnormal). The six scales and overall CLDQ scores were dependent variables, respectively, while the controlled independent variables were age, gender, marital status, education level,

**Table 2 Internal consistency reliability, floor and ceiling effects of the chronic liver disease questionnaire (*n* = 460) *n* (%)**

| Items              | Cronbach's $\alpha$ | Floor and ceiling effects <sup>1</sup> |                 |
|--------------------|---------------------|--|-----------------|
|                    |                     | Floor effects                          | Ceiling effects |
| Abdominal symptoms | 0.80                | 1 (0.2)                                | 50 (10.9)       |
| Fatigue            | 0.82                | 1 (0.2)                                | 1 (0.2)         |
| Systemic symptoms  | 0.65                | 2 (0.4)                                | 16 (3.5)        |
| Activity           | 0.66                | 1 (0.2)                                | 36 (7.8)        |
| Emotional function | 0.90                | 1 (0.2)                                | 5 (1.1)         |
| Worry              | 0.85                | 1 (0.2)                                | 17 (3.7)        |
| Overall            | 0.83                | 1 (0.2)                                | 1 (0.2)         |

<sup>1</sup>Floor and ceiling effect: The number and percentage of the chronic hepatitis B patients at the lowest and highest possible scores.

occupation, annual per capita household incomes and the disease (CHB and CHB-related cirrhosis).

### Ethics statement

The study protocol was reviewed and approved by the Human Research Ethics Committee of Xi'an Jiaotong University. The written informed consent was obtained from each recruited patient before the questionnaire survey.

### Statistical analysis

A database was built using the software EpiData 3.1 and the data were double-entered by two different persons to capture data entry errors. All analyses were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, United States).

## RESULTS

### Sociodemographics

A total of 460 patients were recruited. In the face-to-face interview, they understood the questions of the CLDQ well, and finished the questionnaire completely. The patients aged 35.75 ± 12.82 years (range: 18-76 years), with 305 (66.3%) males and 155 (33.7%) females. Three hundred and twenty-eight (71.3%) patients were married. Education levels of the patients were no schooling and primary (*n* = 63, 13.7%), secondary (*n* = 311, 67.6%) and tertiary (*n* = 86, 18.7%); 153 (33.3%) were peasants and the others were non-peasants. Annual per capita household incomes (RMB) of the patients were < 5000 (*n* = 236, 51.3%), 5000-9999 (*n* = 111, 24.1%) or  $\geq 10000$  (*n* = 113, 24.6%). With respect to the disease, the CHB and CHB-related cirrhosis patients accounted for 70.2% (*n* = 323) and 29.8% (*n* = 137) respectively. Three hundred and seventy five (81.5%) patients received ALT test immediately after the questionnaire survey, 311 (82.9%) with CHB and 64 (17.1%) with CHB-related cirrhosis. The detailed information of patients with CHB or CHB-related cirrhosis is shown in Table 1.

### Reliability and floor/ceiling effects

Cronbach's  $\alpha$  of the total CLDQ was 0.83, with the

**Table 3** Convergent validity and discriminant validity of the chronic liver disease questionnaire (*n* = 460)

| Items   | Item-scale correlation (Spearman $\gamma$ ) |                   |                   |                   |                   |                   |
|---|---|-------------------|-------------------|-------------------|-------------------|-------------------|
|   | AB  | FA                | SY                | AC                | EM                | WO                |
| Abdominal symptoms  |   |                   |                   |                   |                   |                   |
| 1. Abdominal bloating                                     | 0.82 <sup>1</sup>                           | 0.40              | 0.42              | 0.38              | 0.33              | 0.22              |
| 5. Abdominal pain   | 0.79 <sup>1</sup>                           | 0.41              | 0.56              | 0.39              | 0.35              | 0.30              |
| 17. Abdominal discomfort                                  | 0.91 <sup>1</sup>                           | 0.52              | 0.57              | 0.47              | 0.46              | 0.33              |
| Fatigue   |   |                   |                   |                   |                   |                   |
| 2. Tiredness or fatigue                                   | 0.46  | 0.78 <sup>1</sup> | 0.48              | 0.42              | 0.53              | 0.37              |
| 4. Feel sleepy during the day                             | 0.23  | 0.61 <sup>1</sup> | 0.30              | 0.27              | 0.30              | 0.23              |
| 8. Decreased strength                                     | 0.45  | 0.79 <sup>1</sup> | 0.50              | 0.64              | 0.52              | 0.41              |
| 11. Decreased energy                                      | 0.46  | 0.79 <sup>1</sup> | 0.50              | 0.62              | 0.65              | 0.42              |
| 13. Drowsiness  | 0.37  | 0.76 <sup>1</sup> | 0.43              | 0.42              | 0.50              | 0.31              |
| Systemic symptoms   |   |                   |                   |                   |                   |                   |
| 3. Bodily pain  | 0.61  | 0.47              | 0.71 <sup>1</sup> | 0.40              | 0.46              | 0.33              |
| 6. Shortness of breath                                    | 0.46  | 0.50              | 0.66 <sup>1</sup> | 0.49              | 0.42              | 0.30              |
| 21. Muscle cramps   | 0.35  | 0.31              | 0.59 <sup>1</sup> | 0.36              | 0.28              | 0.21              |
| 23. Dry mouth   | 0.32  | 0.35              | 0.64 <sup>1</sup> | 0.26              | 0.38              | 0.31              |
| 27. Itching   | 0.35  | 0.31              | 0.65 <sup>1</sup> | 0.29              | 0.28              | 0.23              |
| Activity  |   |                   |                   |                   |                   |                   |
| 7. Not able to eat as much as you would like              | 0.37  | 0.49              | 0.35              | 0.78 <sup>1</sup> | 0.41              | 0.28              |
| 9. Trouble in lifting or carrying heavy objects           | 0.41  | 0.54              | 0.49              | 0.84 <sup>1</sup> | 0.38              | 0.28              |
| 14. Bothered by a limitation of the diet                  | 0.45  | 0.53              | 0.43              | 0.73 <sup>1</sup> | 0.44              | 0.28              |
| Emotional function  |   |                   |                   |                   |                   |                   |
| 10. Anxiety   | 0.36  | 0.57              | 0.46              | 0.43              | 0.81 <sup>1</sup> | 0.53              |
| 12. Unhappiness   | 0.34  | 0.50              | 0.36              | 0.39              | 0.80 <sup>1</sup> | 0.44              |
| 15. Irritability  | 0.33  | 0.53              | 0.42              | 0.41              | 0.78 <sup>1</sup> | 0.43              |
| 16. Difficulty in sleeping at night                       | 0.30  | 0.44              | 0.39              | 0.32              | 0.70 <sup>1</sup> | 0.33              |
| 19. Mood swings   | 0.36  | 0.51              | 0.42              | 0.35              | 0.77 <sup>1</sup> | 0.54              |
| 20. Difficulty in falling asleep at night                 | 0.33  | 0.38              | 0.40              | 0.35              | 0.67 <sup>1</sup> | 0.35              |
| 24. Depression  | 0.39  | 0.58              | 0.48              | 0.43              | 0.80 <sup>1</sup> | 0.58              |
| 26. Problems with concentration                           | 0.36  | 0.54              | 0.43              | 0.36              | 0.73 <sup>1</sup> | 0.48              |
| Worry   |   |                   |                   |                   |                   |                   |
| 18. Worries about the impact of the liver disease         | 0.19  | 0.32              | 0.30              | 0.24              | 0.47              | 0.78 <sup>1</sup> |
| 22. Worries that symptoms will develop into major problem | 0.33  | 0.45              | 0.38              | 0.31              | 0.53              | 0.89 <sup>1</sup> |
| 25. Worries that the condition is getting worse           | 0.33  | 0.41              | 0.38              | 0.31              | 0.53              | 0.88 <sup>1</sup> |
| 28. Worries about never feeling any better                | 0.31  | 0.39              | 0.34              | 0.30              | 0.50              | 0.83 <sup>1</sup> |
| 29. Availability of a liver for transplant                | 0.19  | 0.15              | 0.24              | 0.18              | 0.22              | 0.35 <sup>2</sup> |

Convergent validity: <sup>1</sup>The hypothesized item-scale correlations  $\geq 0.40$ ; <sup>2</sup>correlations  $< 0.40$ . Discriminant validity: The hypothesized item-scale correlations are higher than the alternative ones. AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function; WO: Worry.

range from 0.65 to 0.90 in the six scales. Both floor and ceiling effect percentages of the six scales and total score of the CLDQ were less than 20% (Table 2).

### Validity

The hypothesized item-scale correlation of the six scales (range) were AB (0.79-0.91), FA (0.61-0.79), SY (0.59-0.71), AC (0.73-0.84), EM (0.67-0.81) and WO (0.35-0.89), all were higher than the range of the corresponding items with other scales, indicating better convergent and discriminant validity (Table 3).

Six factors were extracted from the 29 items of CLDQ, which explained 61.69% of the total variance. Factor loadings of the 29 items were not entirely consistent with the six scales. The items of EM scale loaded on factor 1, except for “difficulty in sleeping at night” (item 16) and “difficulty in falling asleep at night” (item 20), which loaded on factor 6. Factor 2 covered WO scale. The items of FA and AC scales loaded on factor 3, except for “tiredness or fatigue” (item 2), “feel sleepy during the day” (item 4) and “drowsiness” (item 13) loaded on fac-

tor 5. Both of AB scale and item 3 (bodily pain) loaded on factor 4 (Table 4).

### Sensitivity

Regarding the between-group comparison in CHB and CHB-related cirrhosis patients, the eligible ES was found in AC (0.71), SY (0.52), AB (0.33) scales and the overall CLDQ (0.37). After controlling the influences of the disease (CHB and CHB-related cirrhosis), the ES in AB (0.30) scale and the overall CLDQ (0.23) were satisfactory by comparison between the normal and abnormal ALT groups in the stratification of patients with CHB. Meanwhile, the corresponding between-group independent sample *t* test was statistically significant ( $P < 0.05$ ) (Tables 5 and 6). Other eligible ES was also found in patients with normal or abnormal ALT after stratification, including SY (0.22) scale (CHB), and AB (0.27), FA (0.23), AC (0.23) scales and the overall CLDQ (0.21) (CHB-related cirrhosis). However, the corresponding between-group *t* test was not statistically significant ( $P > 0.05$ ) (Table 6).

Based on controlling the influences of sociodemo-

**Table 4 Factor analysis of the chronic liver disease questionnaire (n = 460)**

| Items   | F 1 (EM) | F 2 (WO) | F 3 (AC + FA) | F 4 (AB + SY) | F 5 (FA) | F 6 (sleep) |
|---|----------|----------|---------------|---------------|----------|-------------|
| Abdominal symptoms  |          |          |               |               |          |             |
| 1. Abdominal bloating                                     |          |          |               | 0.68          |          |             |
| 5. Abdominal pain   |          |          |               | 0.82          |          |             |
| 17. Abdominal discomfort                                  |          |          |               | 0.75          |          |             |
| Fatigue   |          |          |               |               |          |             |
| 2. Tiredness or fatigue                                   |          |          |               |               | 0.52     |             |
| 4. Feel sleepy during the day                             |          |          |               |               | 0.75     |             |
| 8. Decreased strength                                     |          |          | 0.66          |               |          |             |
| 11. Decreased energy                                      |          |          | 0.63          |               |          |             |
| 13. Drowsiness  |          |          |               |               | 0.72     |             |
| Systemic symptoms   |          |          |               |               |          |             |
| 3. Bodily pain  |          |          |               | 0.68          |          |             |
| 6. Shortness of breath                                    |          |          |               | (0.32)        |          |             |
| 21. Muscle cramps   |          |          |               | (0.35)        |          |             |
| 23. Dry mouth   |          |          |               | (0.32)        |          |             |
| 27. Itching   |          |          |               | (0.28)        |          |             |
| Activity  |          |          |               |               |          |             |
| 7. Not able to eat as much as you would like              |          |          | 0.76          |               |          |             |
| 9. Trouble in lifting or carrying heavy objects           |          |          | 0.64          |               |          |             |
| 14. Bothered by a limitation of the diet                  |          |          | 0.65          |               |          |             |
| Emotional function  |          |          |               |               |          |             |
| 10. Anxiety   | 0.73     |          |               |               |          |             |
| 12. Unhappiness   | 0.78     |          |               |               |          |             |
| 15. Irritability  | 0.77     |          |               |               |          |             |
| 16. Difficulty in sleeping at night                       |          |          |               |               |          | 0.79        |
| 19. Mood swings   | 0.66     |          |               |               |          |             |
| 20. Difficulty in falling asleep at night                 |          |          |               |               |          | 0.75        |
| 24. Depression  | 0.65     |          |               |               |          |             |
| 26. Problems with concentration                           | 0.55     |          |               |               |          |             |
| Worry   |          |          |               |               |          |             |
| 18. Worries about the impact of the liver disease         |          | 0.69     |               |               |          |             |
| 22. Worries that symptoms will develop into major problem |          | 0.82     |               |               |          |             |
| 25. Worries that the condition is getting worse           |          | 0.81     |               |               |          |             |
| 28. Worries about never feeling any better                |          | 0.75     |               |               |          |             |
| 29. Availability of a liver for transplantation           |          | 0.51     |               |               |          |             |
| Eigenvalue  | 36.14%   | 7.94%    | 5.44%         | 4.57%         | 4.01%    | 3.59%       |
| Cumulative  | 36.14%   | 44.08%   | 49.52%        | 54.09%        | 58.10%   | 61.69%      |

Kaiser-Meyer-Olkin measure of sampling adequacy: 0.929. Bartlett’s test of sphericity:  $P < 0.001$ . The items with factor (F) loading coefficient less than 0.45 are written in bracket. EM: Emotional function; WO: Worry; AC: Activity; FA: Fatigue; AB: Abdominal symptoms; SY: Systemic symptoms.

**Table 5 Sensitivity of the chronic liver disease questionnaire: scores (mean ± SD) and effect size**

| Scales  | Total<br>(n = 460) | CHB<br>(n = 323) | CHB-related<br>cirrhosis<br>(n = 137) | ES                  |
|---------|--------------------|------------------|---------------------------------------|---------------------|
| AB      | 5.48 ± 1.11        | 5.59 ± 1.07      | 5.22 ± 1.16                           | 0.33 <sup>1,b</sup> |
| FA      | 4.76 ± 1.11        | 4.82 ± 1.08      | 4.61 ± 1.17                           | 0.19                |
| SY      | 5.41 ± 0.86        | 5.54 ± 0.82      | 5.09 ± 0.87                           | 0.52 <sup>1,b</sup> |
| AC      | 5.45 ± 1.13        | 5.69 ± 1.03      | 4.89 ± 1.15                           | 0.71 <sup>1,b</sup> |
| EM      | 4.85 ± 1.10        | 4.87 ± 1.13      | 4.80 ± 1.01                           | 0.06                |
| WO      | 5.06 ± 1.19        | 5.03 ± 1.23      | 5.11 ± 1.08                           | -0.07               |
| Overall | 5.17 ± 0.83        | 5.26 ± 0.83      | 4.95 ± 0.78                           | 0.37 <sup>1,b</sup> |

<sup>1</sup>The effect size (ES)  $\geq 0.20$ . Effect size is calculated as the difference between chronic hepatitis B (CHB) and CHB-related cirrhosis groups mean score divided by the overall standard deviation. Significant difference between CHB and CHB-related cirrhosis groups by independent sample *t* test: <sup>b</sup> $P < 0.01$ . AB: Abdominal symptoms; FA: Fatigue; SY: Systemic symptoms; AC: Activity; EM: Emotional function; WO: Worry.

graphics and the disease (CHB and CHB-related cirrhosis), multiple linear regression analysis detected differenc-

es (unstandardized coefficient, 95%CI) in AB scale [-0.39, (-0.63, -0.15),  $P = 0.001$ ], SY scale [-0.23, (-0.41, -0.06),  $P = 0.009$ ] and the overall CLDQ [-0.22, (-0.40, -0.04),  $P = 0.018$ ] with statistical significance under the influence of ALT (normal *vs* abnormal) (Table 7).

## DISCUSSION

The CLDQ is a non-generic, disease-specific instrument for assessing HRQoL in patients with chronic liver disease. We used the Chinese (mainland) CLDQ in patients with CHB and proved that this instrument has acceptable reliability, validity and sensitivity.

Internal consistency reliability of AB, FA, EM, WO scales and the overall CLDQ were satisfactory, with Cronbach’s  $\alpha$  coefficient greater than 0.70. It was consistent with the reports from similar studies using the original and other different language versions of CLDQ<sup>[15,16,19,34]</sup>. However, Cronbach’s  $\alpha$  of SY (0.65) and AC (0.66) scales were less than the recommended value of 0.70. This is probably due to heterogeneous manifestations of system-

**Table 6** Sensitivity of the chronic liver disease questionnaire in different alanine aminotransferase results after stratifying the disease

| Scales             | CHB with ALT test ( <i>n</i> = 311) |                             |                               |                     | CHB-related cirrhosis with ALT test ( <i>n</i> = 64) |                            |                              |                   |
|--------------------|-------------------------------------|-----------------------------|-------------------------------|---------------------|--|----------------------------|------------------------------|-------------------|
|                    | Total score                         | Normal<br>( <i>n</i> = 170) | Abnormal<br>( <i>n</i> = 141) | ES                  | Total score  | Normal<br>( <i>n</i> = 29) | Abnormal<br>( <i>n</i> = 35) | ES                |
| Abdominal symptoms | 5.59 ± 1.08                         | 5.73 ± 1.04                 | 5.41 ± 1.11                   | 0.30 <sup>1,b</sup> | 5.34 ± 1.16  | 5.51 ± 1.17                | 5.20 ± 1.14                  | 0.27 <sup>1</sup> |
| Fatigue            | 4.81 ± 1.08                         | 4.88 ± 1.09                 | 4.72 ± 1.08                   | 0.15                | 4.61 ± 1.13  | 4.75 ± 1.21                | 4.49 ± 1.06                  | 0.23 <sup>1</sup> |
| Systemic symptoms  | 5.54 ± 0.83                         | 5.62 ± 0.82                 | 5.44 ± 0.84                   | 0.22 <sup>1</sup>   | 5.17 ± 0.83  | 5.24 ± 0.83                | 5.11 ± 0.84                  | 0.16              |
| Activity           | 5.70 ± 1.03                         | 5.76 ± 1.00                 | 5.62 ± 1.06                   | 0.14                | 4.94 ± 1.21  | 5.09 ± 1.30                | 4.81 ± 1.14                  | 0.23 <sup>1</sup> |
| Emotional function | 4.85 ± 1.13                         | 4.94 ± 1.16                 | 4.75 ± 1.09                   | 0.17                | 4.77 ± 1.06  | 4.85 ± 1.14                | 4.71 ± 1.00                  | 0.13              |
| Worry              | 5.02 ± 1.23                         | 5.10 ± 1.23                 | 4.93 ± 1.23                   | 0.14                | 4.98 ± 1.20  | 4.94 ± 1.31                | 5.01 ± 1.12                  | -0.06             |
| Overall            | 5.25 ± 0.84                         | 5.34 ± 0.83                 | 5.15 ± 0.84                   | 0.23 <sup>1,a</sup> | 4.97 ± 0.81  | 5.06 ± 0.88                | 4.89 ± 0.74                  | 0.21 <sup>1</sup> |

<sup>1</sup>The effect size (ES) ≥ 0.20. Effect size was calculated as the difference between alanine aminotransferase (ALT) normal and abnormal group mean scores divided by standard deviation of the total score. Significant difference by independent sample *t*-test between ALT normal and abnormal groups: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01.

**Table 7** Multiple linear regression analysis (*n* = 460)

| Dependent variable | <i>B</i> | <i>SE</i> | <i>t</i> | <i>P</i> | 95%CI        |
|--------------------|----------|-----------|----------|----------|--------------|
| Abdominal symptoms | -0.39    | 0.12      | -3.22    | 0.001    | -0.63, -0.15 |
| Systemic symptoms  | -0.23    | 0.09      | -2.63    | 0.009    | -0.41, -0.06 |
| Overall CLDQ       | -0.22    | 0.09      | -2.37    | 0.018    | -0.40, -0.04 |

Independent variable: alanine aminotransferase (normal *vs* abnormal). The other controlled independent variables were age, gender, marital status, education level, occupation, annual per capita household incomes and the disease [chronic hepatitis B (CHB) and CHB-related cirrhosis]. CLDQ: Chronic liver disease questionnaire.

atic symptoms severity and activity limitations in patients with CHB or CHB-related cirrhosis and, consequently, influences the corresponding scoring. Percentages of the floor and ceiling effects regarding the six scales and total score of the CLDQ were less than 20%, indicating that this instrument can capture the full range of potential responses and detect small changes in CHB patients during treatment process.

The convergent and discriminant validity of the CLDQ was satisfactory, proving that the items of each scale can reflect the major characteristics consistently. Lam *et al*<sup>[14]</sup> and Mahmoudi *et al*<sup>[35]</sup> reported similar results in their studies. However, “availability of a liver for transplant” (item 29) in the present study did not better correlate to WO scale ( $\gamma = 0.35$ ), possibly due to few patients confronted with the problem of liver transplantation. Therefore, this item was less correlated to WO scale than the other four items within the same dimension.

The factor structure of the Chinese (mainland) CLDQ conform to the six scale structure of the original US version, with a new scale of “sleep” (factor 6) covering the item “difficulty in sleeping at night” (item 16) and “difficulty in falling asleep at night” (item 20) being the only major difference. Such new factor was also found in the Spanish, Italian and German population<sup>[19,20,34]</sup>. It was reasonable to believe that sleeping habits vary among cultures (napping habits and bedtimes), so it may not be surprising that these items clustered differently. For patients with CHB or CHB-related cirrhosis, they may have sleep difficulties due to various reasons other than emotional

problems, including pain or other disease-related factors. Except for the two items of “sleeping difficulty”, all other items of the EM scale loaded on factor 1 consistently.

The items of WO, AC and AB scales loaded on factor 2, 3 and 4. However, AC and AB scales also combined with other items. The finding showed that “decreased strength” (item 8) and “decreased energy” (item 11) of FA scale loaded on factor 3 (AC scale), whereas “bodily pain” (item 3) of SY scale loaded on factor 4 (AB scale), which demonstrated that decreased strength and energy highly correlate to activity<sup>[14]</sup>, and bodily pain may be better related to AB, especially abdominal pain in CHB patients. “Tiredness or fatigue” (item 2), “feel sleepy during the day” (item 4) and “drowsiness” (item 13) were found loading on factor 5 as measuring fatigue symptoms. However, the FLC of “shortness of breath” (item 6) (0.32), “muscle cramps” (item 21) (0.35), “dry mouth” (item 23) (0.32) and “itching” (item 27) (0.28) were less than the recommended value of 0.45. It was probably because that most of the patients were in CHB stage and did not have distinct clinical manifestations as the items reflected.

By comparison between CHB and CHB-related cirrhosis groups, the CLDQ was sensitive in detecting differences in the overall and AB, SY and AC scale scores. It suggests that, based on measuring the physical and psychological health, the CLDQ could further detect the difference of hepatitis-related physical symptoms in CHB patients with or without cirrhosis. After controlling for influences of the disease (CHB and CHB-related cirrhosis), the CLDQ was very good in measuring differences in the overall and AB and SY scale scores between normal and abnormal ALT groups in CHB patients without cirrhosis. Multiple linear regression analysis also confirmed this result through further controlling other confounding factors such as sociodemographics.

However, due to close scores, FA, EM and WO scales did not show significant difference in CHB patients with or without considering ALT test results and controlling for influences of the disease (CHB and CHB-related cirrhosis). Specifically, the difference of between-group comparison in AC scale changed from significant to not significant after stratification, indicating the confounding role of the disease (CHB and CHB-related cirrhosis).

Unlike such finding, Lam *et al*<sup>14</sup> used the Chinese (Hong Kong, China) CLDQ in patients with CHB infection and reported significant differences in FA, AC, EM and WO scales between uncomplicated and complicated CHB patients. Such discrepancy might be the result that CHB-related complications have impact on the patients' health regardless of the disease (CHB and CHB-related cirrhosis) and, subsequently, influence the scoring of the corresponding scales. Therefore, the sensitivity of FA, AC, EM and WO scales of the CLDQ in patients with CHB or CHB-related cirrhosis need further examination.

In patients with CHB-related cirrhosis, the ES values of AB (0.27), FA (0.23), AC (0.23) scales and the overall CLDQ (0.21) were greater than 0.20, confirming the major impact of ALT on the hepatitis-related physical health. Other scales including SY (0.16), EM (0.13) and WO (-0.06) did not have the required ES value, indicating the similar conditions of hepatitis-related symptoms and mental health under the influence of ALT (normal *vs* abnormal) in cirrhotic patients. However, no statistical significance of independent sample *t* test was found in any scales and the overall CLDQ in the present study. The probable explanation for this might be the small sample size ( $n = 64$ ). Different from such result, Lam *et al*<sup>13</sup> found significant differences in SY scale and the overall CLDQ between impaired liver function and cirrhosis groups in CHB patients. Accordingly, more attention should be paid to proving the sensitivity of the CLDQ in CHB-related cirrhosis patients under the influence of normal and abnormal ALT.

There were some limitations in the present study. First, the Chinese (mainland) CLDQ was administered using a face-to-face interview, the performance of the instrument by self-completion will need to be confirmed by future work. Second, the responsiveness of the Chinese (mainland) CLDQ in detecting changes with disease progression or ALT status will also need to be determined. Third, this study was conducted in the single-site of the Eighth Hospital of Xi'an. Therefore, it was limited to generalize the results to all of Chinese mainland CHB patients.

The Chinese (mainland) CLDQ has been proved as a valid tool for assessing HRQoL in patients with CHB. Both reliability and validity were demonstrated to be strongly satisfactory, and better sensitivity was confirmed in detecting the difference of AB, SY and AC scales and the overall CLDQ, especially the differences in AB scale and the overall CLDQ under the influence of ALT status. The Chinese (mainland) CLDQ can be a suitable disease-specific questionnaire for evaluating the change of HRQoL and treatment effects of CHB in clinical practice. Future work in a larger cohort of patients is needed to further prove the responsiveness of the Chinese (mainland) CLDQ.

The patients are at high risk of developing fatal complications of cirrhosis and hepatocellular carcinoma, which consequently lead to the impairments of the patients' health-related quality of life (HRQoL).

### Research frontiers

HRQoL should be used as the primary endpoint in the evaluation of treatment effectiveness for patients with CHB, since it is a more holistic assessment of health status considering the individual's functional health and well-being.

### Innovations and breakthroughs

The chronic liver disease questionnaire (CLDQ) has been translated into many different languages for cross-cultural adaptation and proved as a valid tool for HRQoL measurement and evaluation in patients with chronic liver disease. However, a dearth of study assessed psychometrics of the Chinese (mainland) CLDQ in mainland Chinese patients with CHB. This study determined that the Chinese (mainland) CLDQ has acceptable reliability, validity and sensitivity in patients with CHB. It can be applied to mainland Chinese CHB patients to evaluate their HRQoL.

### Applications

The Chinese (mainland) CLDQ can be used as a suitable disease-specific questionnaire for evaluating the change of HRQoL and treatment effects of CHB in clinical practice. The CLDQ can be used as a cross-cultural HRQoL measure in international studies that include mainland Chinese.

### Terminology

Reliability concerns the random variability associated with measurements. Validity refers to the extent to which a test measures what it is intended to measure. Sensitivity is the ability of measurements to detect differences between patients or groups of patients.

### Peer review

The authors tested psychometrics of the Chinese (mainland) CLDQ and proved that this questionnaire was reliable, valid and sensitive for Chinese mainland patients with CHB. The study was well done and used appropriate methodology to validate and test the questionnaire.

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## COMMENTS

### Background

Chronic hepatitis B (CHB) is a common chronic liver disease in mainland China.

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P- Reviewer Tiniakos DG S- Editor Huang XZ  
L- Editor Ma JY E- Editor Xiong L



## Nodular regenerative hyperplasia related portal hypertension in a patient with hypogammaglobulinaemia

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Author contributions: Lal BK designed and wrote the case report; Stanley A chose the case and gave valuable ideas throughout the case report writing.

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Received: November 20, 2012 Revised: January 30, 2013

Accepted: February 5, 2013

Published online: June 14, 2013

### Abstract

Nodular regenerative hyperplasia (NRH) of liver is a relatively rare liver disorder, but a frequent cause of noncirrhotic portal hypertension. We present a lady with common variable immune deficiency who presented with upper gastrointestinal bleeding and deranged liver function tests but preserved synthetic function. Upper gastrointestinal endoscope showed bleeding gastric varices and non-bleeding oesophageal varices. Although her oesophageal varices were eradicated by repeated endoscopic band ligation, the gastric varices failed to resolve after repeated endoscopic histocryl injection and she eventually needed transjugular intrahepatic portosystemic shunt placement. Liver biopsy showed NRH. We review the association of hypogammaglobulinaemia and NRH and discuss the appropriate management of portal hypertension in NRH.

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**Key words:** Nodular regenerative hyperplasia; Liver; Portal hypertension; Hypogammaglobulinaemia; Gastro-oesophageal varices

**Core tip:** Nodular regenerative hyperplasia (NRH) is still an evolving concept. Although a rarely identified liver

disorder, it is a frequent cause of noncirrhotic portal hypertension. Liver involvement in primary hypogammaglobulinemia mainly consists of NRH leading to chronic cholestasis and portal hypertension. Optimal management of gastric variceal bleed remains unclear. Histoacryl injection is the endoscopic method of choice for gastric variceal bleed but one should keep a lower threshold for transjugular intrahepatic portosystemic shunt procedure for recurrent gastric variceal bleed.

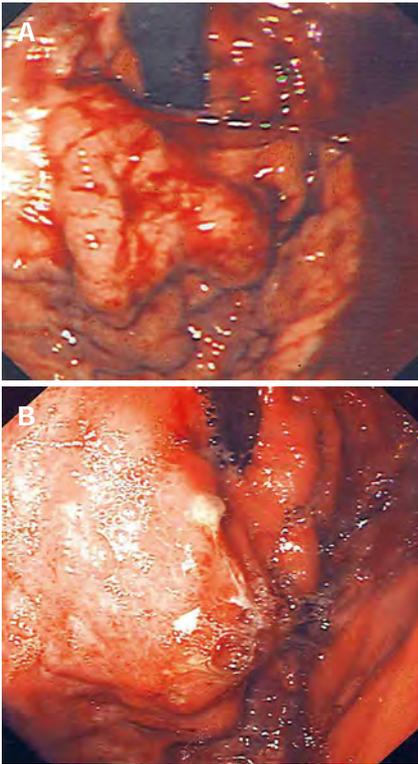
Lal BK, Stanley A. Nodular regenerative hyperplasia related portal hypertension in a patient with hypogammaglobulinaemia. *World J Gastroenterol* 2013; 19(22): 3502-3504 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3502.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3502>

### INTRODUCTION

Nodular regenerative hyperplasia (NRH) is the main cause of non-cirrhotic portal hypertension in the western world being responsible for 14%-27% of these cases<sup>[1-3]</sup>. It is characterized by diffuse regenerative hepatocytic nodules without fibrosis. Common variable immune disorder (CVID) is a heterogeneous group of primary immunodeficiency conditions involving formation of antibody production and various cellular immune system defects. NRH is the main liver pathology found in patients with CVID with abnormal liver function test (LFT). It is associated with intrasinusoidal T-cell infiltration, portal vein endothelitis, autoimmune disease and peripheral lymphocytic abnormalities which suggest an autoimmune mechanism.

### CASE REPORT

A 50-year-old lady presented with upper gastrointestinal bleeding. She had a history of bronchiectasis and idiopathic thrombocytopenic purpura and had previously



**Figure 1** Gastric varices. A: Gastric varices pre glue injection; B: Gastric varices post glue.

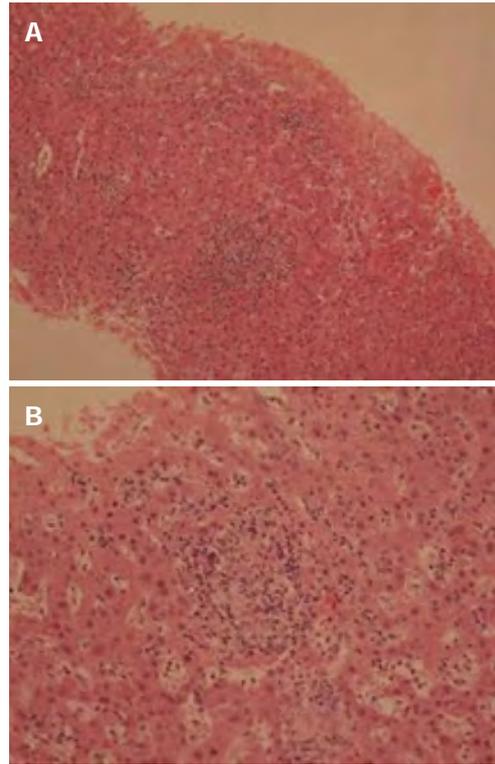
been diagnosed with CVID. She was taking regular intravenous immunoglobulin infusion.

She was haemodynamically stable on presentation with pulse 90 per minute and blood pressure 131/65 mmHg. She had further episodes of haematemesis after admission and emergency endoscopy revealed large gastric fundal varices with evidence of active bleeding (Figure 1) and non-bleeding oesophageal varices. Haemostasis was achieved by applying glue (Histoacryl) to the gastric varices and her esophageal varices were banded. She was given five days of antibiotics and started on oral propranolol for prevention of variceal rebleeding.

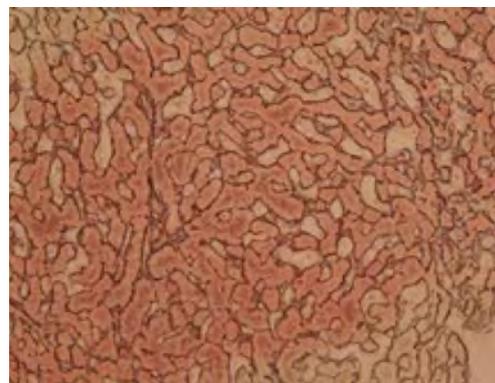
She had mildly elevated liver enzymes (bilirubin 22 mmol/L, aspartate aminotransferase 55 U/L, alanine aminotransferase 35 U/L, gamma-glutamyl transpeptidase 45 U/L) with preserved synthetic function (albumin 41 g/L and prothrombin time 16 s). Ultrasonography of the abdomen revealed a coarse liver echo texture and normal flow in all hepatic veins. There was some bidirectional flow noted in the portal vein suggestive of portal hypertension but no ascites was seen.

To investigate her mildly elevated liver enzymes and portal hypertension percutaneous liver biopsy was undertaken. This showed features suggestive of NRH (Figures 2 and 3). Inflammatory cell infiltrate with granulomas were seen with a reactive appearance and immunophenotype suggestive that the NRH was related to CVID.

Subsequently, her oesophageal varices were eradicated by repeated band ligation but her gastric varices failed to resolve despite of repeated histoacryl injection (Figure 1).



**Figure 2** Nodule formation and granuloma on liver biopsy. A: Nodule; B: Granuloma.



**Figure 3** Thick liver cell plates on reticulin, but no fibrosis on liver biopsy.

Eventually, she had transjugular intrahepatic portosystemic shunt (TIPS) procedure with a covered graft which was placed after 6 mo of initial presentation resulting into satisfactory reduction in the portosystemic gradient with reversal of flow in the varices. She had complete resolution of her gastric varices post procedure and has remained on 6-mo TIPS checks by portography since.

She remains under regular follow up jointly in the liver, immunology, respiratory and haematology clinics. After nine years follow-up she remains well with normal LFTs.

## DISCUSSION

NRH is usually associated with malignant, prothrom-

botic or rheumatologic conditions. Although liver disease and abnormal LFTs are found in approximately 10% of CVID patients<sup>[4,5]</sup> liver lesions associated with primary hypogammaglobulinaemia has been poorly described<sup>[6]</sup>. Two recent studies have identified NRH as the main histological correlate in patients with hypogammaglobulinemia<sup>[2-6]</sup>. Subsequent portal hypertension was frequently observed in only one patient cohort<sup>[6]</sup>. There has also been a case report which shows the association of hypogammaglobulinemia and major gastrointestinal bleeding from gastric varices as a result of cirrhosis of unknown cause (on biopsy)<sup>[7]</sup>.

Management of patients with NRH is aimed at the treatment of the underlying systemic disorder and any complications related to the portal hypertension. A fundamental concept is that the synthetic function of the liver is generally preserved in NRH, despite the potential for development of significant portal hypertension. Liver transplantation is therefore rarely needed for NRH<sup>[8]</sup>.

The immediate approach to variceal bleeding and ascites in patient with NRH does not differ from that of any other patients with the same condition. In the management of gastric varices or intractable or recurrent oesophageal variceal bleeding, TIPS should be considered<sup>[9]</sup>. Gastric variceal bleeding can be particularly challenging to the clinician. Histoacryl injection is the endoscopic method of choice for gastric variceal bleeding with immediate haemostasis figures of over 90% reported<sup>[9,10]</sup>. TIPS is used at a lower threshold for gastric compared with oesophageal variceal bleeding with uncontrolled studies demonstrating initial haemostasis obtained in over 90%, and rebleeding rates of 15%-30%<sup>[11]</sup>. As hepatic encephalopathy is rare in NRH because of preserved hepatic synthetic function, Porto systemic shunt surgery or TIPS is more suitable to treat and prevent refractory gastric variceal bleed in patients with NRH<sup>[11]</sup>. Balloon occluded retrograde transvenous obliteration is a technique for patients with gastric varices and gastrosplenic shunts, although it is rarely used outside Asia<sup>[12]</sup>. Non-cardioselective beta-blockers are an alternative to TIPS for secondary prophylaxis, although the evidence is limited<sup>[11]</sup>.

In conclusion, NRH is still an evolving concept. Although a rarely identified liver disorder, it is a frequent cause of noncirrhotic portal hypertension. Liver involvement in primary hypogammaglobulinaemia mainly consists of NRH leading to chronic cholestasis and portal hypertension. Optimal management of gastric variceal bleed remains unclear. Histoacryl injection is the endoscopic method of choice for gastric variceal bleed but one should keep a lower threshold for TIPS procedure for recurrent gastric variceal bleed.

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**P- Reviewer** Bayrahtar Y **S- Editor** Gou SX  
**L- Editor** A **E- Editor** Xiong L



## Mini-loop ligation of a bleeding duodenal Dieulafoy's lesion

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Received: January 15, 2013 Revised: March 4, 2013  
Accepted: March 15, 2013  
Published online: June 14, 2013

### Abstract

Two percent of gastrointestinal hemorrhages are caused by Dieulafoy's lesions, which are located in duodenum in only 15% of cases. There are no recommendations regarding the prime endoscopic treatment technique for this condition. A 61-year-old woman presented with melena without signs of hemodynamic instability. During an urgent upper endoscopy, blood oozing from the normal mucosa of the duodenum was seen and this was classified as a Dieulafoy's lesion. A mini-loop was opened at the rim of a transparent ligation chamber, at the end of the endoscope, and after aspiration of the lesion, closed and detached. Complete hemostasis was achieved without early or postponed complications. In every day clinical practice, mini-loop ligation is rarely used because of possible complications, such as site ulceration, organ perforation, re-bleeding and possible inexperience of the operator. To the best of our knowledge this is the first case of successful treatment of bleeding duodenal Dieulafoy's lesion by mini-loop ligation.

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**Key words:** Dieulafoy's lesion; Duodenum; Endoscopy; Mini-loop; Hemostasis

**Core tip:** This is a case of 61-year-old woman who presented with upper gastrointestinal hemorrhage caused by duodenal Dieulafoy's lesion that presented with blood oozing from the normal mucosa on upper endoscopy. Complete hemostasis was achieved using a mini-loop ligation without a mucosal lesion, such as ulceration, on two month follow-up endoscopy. Thus, mini-loop ligation is an effective, easy to use and safe method for the treatment of Dieulafoy's lesion. However, case reports with longer follow-up are needed for a definitive statement because of the substantial risk of re-bleeding from a residual aberrant artery.

Gomerčić Palčić M, Ljubičić N. Mini-loop ligation of a bleeding duodenal Dieulafoy's lesion. *World J Gastroenterol* 2013; 19(22): 3505-3507 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3505.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3505>

### INTRODUCTION

Dieulafoy's lesion (DL), a condition first reported in 1884, and named in 1898, refers to dilated submucosal arterial malformation (width of 1-3 mm) that protrudes through overlying epithelium erosion accompanied with normal surrounding mucosa<sup>[1]</sup>. It can be found in any segment of the gastrointestinal (GI) tract with a preference for the lesser curvature of the stomach, 6 cm distally from the esophagogastric junction<sup>[2]</sup>. The incidence of DL is unknown because it is usually an asymptomatic condition that is often undiagnosed; however, its most common complication, GI hemorrhage, accounts for 2%-3% of all GI hemorrhage. A combination of abundant arterial bleeding with an inapproachable site of the lesion implies urgent and reliable treatment; the available endoscopic hemostatic methods are thermal, mechanical and injection<sup>[3,4]</sup>. To the best of our knowledge mini-loop ligation has never been reported as a treatment option in a case of DL.

## CASE REPORT

A 61-year-old woman was admitted to our emergency unit because of melanic stool without abdominal pain, nausea or vomiting. Her previous history indicated that she did not have any serious health problems, just arterial hypertension and hyperlipoproteinemia, which were adequately regulated with chronic therapy. There was no history of alcohol abuse or excess intake of non-steroidal anti-inflammatory drugs (NSAIDs). She was anemic, but showed no signs of hemodynamic instability. Although a nasogastric tube aspirate was clear after insertion, a rectal examination showed evidence of melena. Laboratory tests revealed low levels of erythrocytes  $2.8 \times 10^9/L$  and hemoglobin 79 g/L with normal coagulation parameters. After signing an informed consent, the patient underwent an urgent upper endoscopy (GIF Q160, Olympus Optical Co., Japan). Blood oozing from a pin-point defect with normal surrounding mucosa of the duodenum was visualized and that was suggestive of DL (Figure 1A). A detachable nylon ring was opened at the rim of a transparent ligation chamber attached to the tip of the endoscope (Figure 1B). The lesion was aspirated into the chamber and subsequently the mini-loop was closed and detached, achieving complete hemostasis (Figure 1C). A blood transfusion was administered later, together with pantoprazole in the infusion over a 48 h period. A repeated endoscopy showed that the mini-loop was located at the site of the lesion with no visible sign of acute hemorrhage. The patient was discharged from the hospital on the fourth day with a satisfactory complete blood count (erythrocytes  $3.9 \times 10^9/L$  and hemoglobin 98 g/L). On follow up examination two months later, no pathology of a duodenal mucosa was observed, such as ulceration on the site, and values of her complete blood count were normal.

## DISCUSSION

DL of the duodenum is an uncommon cause of upper GI bleeding, currently experiencing an increase of its frequency because operators' increased awareness of this condition. The duodenum is the second commonest site of DLs, accounting for 15% of patients, second only to the lesser curvature of the stomach, which accounts for around 70%<sup>[3,5]</sup>. Although the incidence of DL is unknown, it accounts for 2%-3% of all GI hemorrhages. Clinical presentations of this condition are extremely wide-ranging: from symptom free, and therefore, frequently undiagnosed cases, to signs of severe and often fatal GI hemorrhage. These lesions usually appear among the elderly, predominantly in men, and are associated with multiple co-morbidities such as chronic renal and heart failure. Other risk factors include the usage of NSAIDs, and antiplatelet and anticoagulant therapy. The patient we treated was an elderly woman with a history of arterial hypertension and hyperlipoproteinemia.

Upper endoscopy, the gold standard, is diagnostic in 90% of the cases; other available diagnostic techniques are angiography and endoscopic ultrasound (EUS). Selective catheter-directed embolization, especially in patients

at high risk for surgery, is usually a salvage method in cases of unsuccessful endoscopic treatment, particularly if the lesion cannot be visualized because of excessive hemorrhage<sup>[6]</sup>. EUS is used to help detect the site of the submucosal vessel, especially in cases of spouting bleeding and to confirm ablation of a DL after therapy.

The endoscopic appearance of a DL is typically arterial spurting or oozing streaming from a minimal defect of the normal surrounding mucosa, or, less commonly, a protruding vessel without signs of active bleeding or with an adherent clot. In this case, blood oozing out of the normal mucosa was visualized without the possibility to distinguishing the underlying vessel. Once the diagnosis is established, several endoscopic techniques can be used, depending on the site of the lesion, severity of symptoms and previous operator experience. Available endoscopic procedures are electrocoagulation, heat probe coagulation, argon plasma coagulation, local epinephrine injection, sclerotherapy, banding and hemoclip. There is no consensus as to which is the most appropriate method. Based on the available data, endoscopic mechanical haemostatic methods seem to be superior to thermal or injection treatment methods<sup>[7,8]</sup>. However, the most commonly used methods are injection therapy and mechanical endoscopic therapy with hemoclips. In our case, a mechanical hemostatic method was used; the bleeding vessel with the surrounding mucosa was aspirated into the transparent ligation chamber and a preloaded nylon ring, called a mini-loop, was closed and detached with consequent complete hemostasis. A search of the published literature using PubMed with the phrase "Dieulafoy's lesion" resulted in 211 articles, of which the first was dated to early 1978 reporting two cases of DL of the jejunum<sup>[9]</sup>. First report of a duodenal DL was published in 1993<sup>[10]</sup> and to the best of our knowledge, there have been no reports of mini-loop ligation as a choice of endoscopic treatment for DL. Despite the fact that the first choice therapy for the bleeding duodenal DLs among most endoscopists includes injection of epinephrine and electrocoagulation<sup>[11]</sup>, mechanical techniques are used more frequently and endoscopic band ligation (EBL) has been demonstrated to be an effective treatment for bleeding esophageal, stomach, duodenal and rectal DLs<sup>[12,13]</sup>. Chung *et al.*<sup>[14]</sup> and Yanar *et al.*<sup>[15]</sup> demonstrated higher efficacy of mechanical hemostatic therapy in initial hemostasis and lowering re-bleeding rate with hemoclip and band ligation compared to injection therapy in patients with DL. On the other hand, EBL has been shown to be as effective as injection therapy with epinephrine, with or without thermal therapy, with a significantly longer length of hospitalization among patients treated with injection therapy or combined therapy with epinephrine and thermal therapy<sup>[16]</sup>. In a study that compared two mechanical methods, EBL and endoscopic hemoclip placement (EHP), no difference was detected in efficacy or safety in the management of bleeding gastric DLs and only one patient from each group experienced re-bleeding<sup>[17]</sup>. A larger study (66 patients) showed a lower recurrent bleeding rate in patients treated with EBL compared with EHP (3.1% *vs* 14.7%)<sup>[18]</sup>. A combined therapy, such as

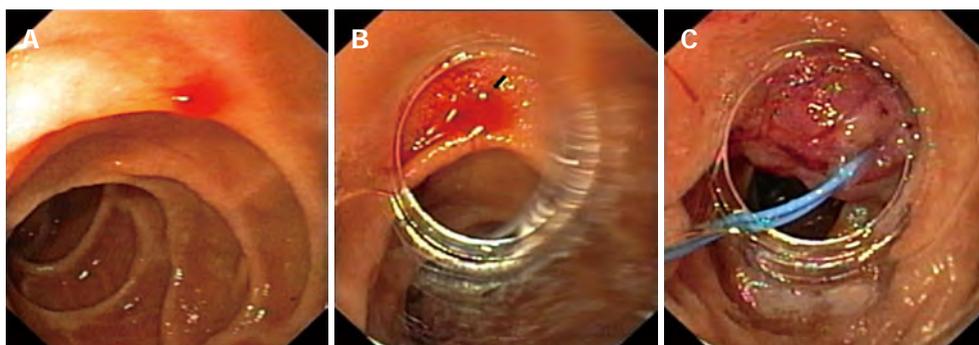


Figure 1 Sixty-one-year-old woman was admitted to our emergency unit because of melanic stool without abdominal pain, nausea or vomiting. A: Bleeding duodenal Dieulafoy's lesion; B: Visible bleeding Dieulafoy's lesion through the rim of a transparent ligation chamber attached to the tip of the endoscope; C: The mini-loop is closed and detached, achieving complete hemostasis.

EBL with EUS, promises a new era in diagnostic efficacy in terms of establishing accurate diagnosis, guiding EBL and confirming success of the treatment<sup>[19]</sup>.

Therapeutic endoscopy is the first line of treatment of DL. However, the choice of technique is not specified. In our case, endoscopic mini-loop ligation proved to be an effective, easy to use and safe method for the treatment of DL. Mini-loop ligation may become a method of choice in resolving bleeding DL; however, case reports with longer follow-up periods are needed for a definitive statement because of the substantial risk of re-bleeding from a residual aberrant artery.

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**P- Reviewers** Chamberlain SM, Kouraklis G, Loffroy R, Wilcox CM  
**S- Editor** Gou SX **L- Editor** Stewart G **E- Editor** Xiong L



## Colonic mucormycosis presented with ischemic colitis in a liver transplant recipient

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Received: January 17, 2013 Revised: April 3, 2013

Accepted: April 28, 2013

Published online: June 14, 2013

### Abstract

Mucormycosis is an uncommon opportunistic fungal infection with high mortality in liver transplant recipients. Mucormycosis of the gastrointestinal tract can manifest with features similar to ischemic colitis. Typically signs and symptoms of non-gangrenous ischemic colitis resolve spontaneously within 24-48 h. On the other hand, the clinical course of the mucormycosis is commonly fulminant. We encountered a case of invasive fungal colitis presenting with abdominal pain and hematochezia in a liver transplant recipient. Endoscopic examination showed multiple shallow ulcerations and edema with mucosal friabilities on the sigmoid and distal descending colon, which was consistent with ischemic colitis. However, the histological examination obtained from endoscopic biopsies showed fungal hyphae with

surrounding inflammatory cells and mucosal necrosis. The patient was successfully managed with antifungal agent without surgical treatment. Thus, early diagnosis and treatment is essential for improving the prognosis of invasive fungal infection after liver transplantation.

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**Key words:** Mucormycosis; Liver transplantation; Colon; Ischemia

Do GW, Jung SW, Jun JB, Seo JH, Nah YW. Colonic mucormycosis presented with ischemic colitis in a liver transplant recipient. *World J Gastroenterol* 2013; 19(22): 3508-3511 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3508.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3508>

### INTRODUCTION

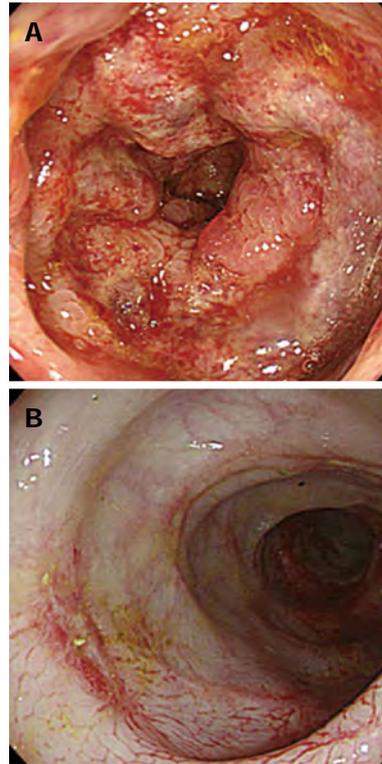
Mucormycosis is a rare but highly invasive opportunistic fungal infection in liver transplant recipients. Clinical manifestations can be rhino-sino-orbital, rhinocerebral, pulmonary, gastrointestinal, genitourinary, cutaneous and disseminated<sup>[1]</sup>. Mucormycosis of the gastrointestinal tract manifests with features similar to ischemic colitis<sup>[2]</sup>. *Mucor*, one of fungi that cause mucormycosis, has a special affinity for blood vessels<sup>[3]</sup>, which may explain the clinical presentation of colonic infection as an ischemic colitis pattern. Ischemic colitis is associated with inadequate blood flow in the colon leading to colonic inflammation, one of the most common vascular disorders of the intestinal tract. Most cases of ischemic colitis are transient and resolve spontaneously without complication<sup>[4]</sup>. However, a deteriorating course of ischemic colitis should warrant further investigation. Herein we present a case of colonic mucormycosis presenting with ischemic colitis in a liver transplant recipient with diabetes mellitus who had been on immunosuppressive agents.



**Figure 1** Abdominopelvic computed tomography scan. Mild to moderate symmetrical wall thickening with decreased mural attenuation was observed from the hepatic flexure to distal sigmoid colon.

## CASE REPORT

A 42-year-old man with chronic hepatitis B underwent deceased donor liver transplantation (DDLT) due to hepatocellular carcinoma in a background of liver cirrhosis. He suffered from diabetes mellitus for 26 mo and was treated with insulin. He had an extremely high blood glucose level after he stopped insulin therapy in the last few months on his own initiative. His general condition was good after transplantation and there were no immediate complications such as acute rejection or infection. Post-operative immunosuppressive agents with methylprednisolone, mycophenolate mofetil, and tacrolimus were used. On the fifth day after DDLT, the patient complained of abdominal pain and small amount of hematochezia. On physical examination, his vital signs were normal except slightly elevated pulse rate (101 beats/min). His abdomen was soft but diffusely tender without peritoneal irritation signs. Laboratory analysis revealed a leukocyte count of  $27800/\text{mm}^3$  with 92% segment form, hemoglobin of 12.1 g/dL, a platelet count of  $65000/\text{mm}^3$ , and C-reactive protein of 3.48 mg/dL. A computed tomography scan of the abdomen revealed mild to moderate symmetrical wall thickening with decreased mural attenuation was observed from the hepatic flexure to distal sigmoid colon (Figure 1). Because of suspected ischemic colitis, the patient was treated with bowel rest, intravenous fluid replacement, and antibiotics. However, the patient's symptoms continued to deteriorate. On the seventh day after DDLT, the decision was made to perform an endoscopic evaluation to elucidate the exact cause of abdominal pain and rectal bleeding. The sigmoidoscopic examination revealed multiple shallow ulcerations, edema with mucosal friabilities, and loss of the normal vascular appearance on the sigmoid and distal descending colon (Figure 2A). Microscopic examination showed several elongated fungal hyphae that stained light blue with haematoxylin and eosin (Figure 3A), were periodic acid Schiff positive and stained dark brown to black with the Grocott-Gomori methenamine silver method (Figure 3B). The fungal hyphae consisted of non-septated filaments, irregularly wide (6-9  $\mu\text{m}$  diameter) hyphae with frequent right angle

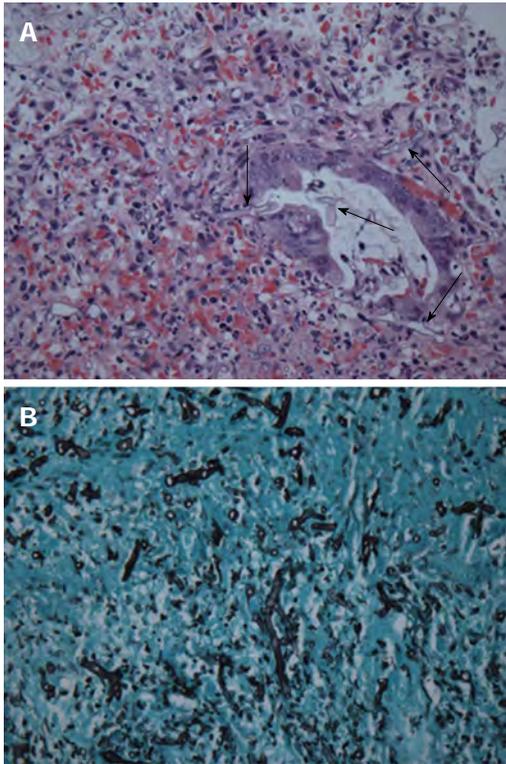


**Figure 2** Sigmoidoscopic findings. A: The mucosa shows multiple shallow ulcerations, edema with friability of the mucosa, and loss of the normal vascular appearance; B: Two weeks after anti-fungal therapy, the mucosal edema and ulcerative lesions are markedly improved and mucosal scar changes were seen on the sigmoid colon.

branching. These morphological features suggested infection by *Mucoraceae*. Based on the clinical findings and the morphological features of the fungal organisms, the diagnosis of mucormycosis was made. The patient was initially treated with intravenous high dose (1 mg/kg per day) amphotericin B, later replaced with liposomal amphotericin B due to azotemia. A follow-up sigmoidoscopy showed significant reduction in mucosal edema and inflammation compared to previous endoscopic findings (Figure 2B). The patient's symptoms disappeared and he made a good recovery.

## DISCUSSION

Mucormycosis is an uncommon fungal infection caused by fungi of the class *Zygomycetes*, order *Mucorales* and *Entomorphothorales*<sup>[5]</sup>. The following risk factors are associated with mucormycosis: neutropenia, history of diabetes mellitus, hyperglycemia, acidosis, preexisting renal impairment, deferoxamine therapy, immunosuppressive agents and steroid use<sup>[1]</sup>. Our patient was diagnosed with diabetes mellitus before this diagnosis and received postoperative immunosuppressive agents after liver transplantation. Mucormycosis manifests a variety of different syndromes in humans, particularly in solid organ transplant recipients<sup>[1,6]</sup>. Although unusual, mucormycosis of the gastrointestinal tract may occur as a result of ingesting fungal spores. A review of the 116 solid organ



**Figure 3 Histologic findings.** A: Microscopic examination of the biopsy specimen obtained by endoscopy shows several fungal hyphae (arrows) (hematoxylin and eosin stain, magnification  $\times 400$ ); B: Right-angle branching, non-septate hyphae of *Mucoraceae* are shown (Grocott's methenamine silver stain, magnification  $\times 400$ ).

transplant recipients with mucormycosis found that the site of involvement of gastrointestinal mucormycosis ( $n = 13$ , 11.2%), included stomach in 9 (69.2%), colon 1 (7.6%), esophagus 1 (7.6%) and liver 1 (7.6%)<sup>[1]</sup>. Non-specific abdominal pain and distention associated with nausea and vomiting are the most common symptoms. Fever and gastrointestinal bleeding may also occur<sup>[7]</sup>. In our case, the patient complained of abdominal pain and hematochezia. We performed a sigmoidoscopy to determine the exact cause of those symptoms. One previous study showed that endoscopic examination is a valuable diagnostic tool to establish an accurate diagnosis of transplant recipients with lower gastrointestinal complaints<sup>[8]</sup>. These symptoms occur typically in ischemic colitis<sup>[2]</sup>. In a review of 85 patients, endoscopic appearance of transient ischemic colitis included edematous and fragile mucosa, segmental erythema, scattered erosion, longitudinal ulcerations, petechial hemorrhages interspersed with pale areas, and purple hemorrhagic nodules<sup>[9]</sup>. The endoscopic findings for our patient resembled ischemic colitis. Successful current treatment for mucormycosis involves a combined approach; rapidity of diagnosis, the control of the underlying predisposing illness, appropriate surgical debridement of infected tissue and prompt initiation of antifungal therapy<sup>[5]</sup>. Until recently, only the polyene class of drugs, including amphotericin B deoxycholate and its lipid derivatives, demonstrated activity against the agents of mucormycosis. The lipid formulations of amphoteri-

cin are significantly less nephrotoxic than amphotericin B deoxycholate and can be safely administered at higher doses for a longer period of time<sup>[7]</sup>. Surgery is essential in decreasing the fungal burden by removing infarcted tissues, where amphotericin B cannot distribute. In a recent study, a patient who was precluded from extensive surgical intervention did not respond to amphotericin B but was treated successfully with a combination of posaconazole and liposomal amphotericin B without surgical treatment<sup>[10]</sup>. Mucormycosis is generally a severe infection with an overall survival rate of patients of approximately 50%<sup>[7]</sup>. Appropriate treatment must be instituted early if the clinical suspicion is high, even before the results of culture and/or histopathological studies have been obtained, and currently, the administration of systemic amphotericin B and/or surgical debridement of the lesions are considered standard therapy<sup>[7]</sup>.

In this case, the patient with diabetes mellitus had received immunosuppressive agents after liver transplantation and presumptive diagnosis of ischemic colitis was initially suspected. The patient was treated with supportive care including bowel rest, intravenous fluid replacement, and antibiotics. However, the patient's symptoms showed a deteriorating condition. Non-improving ischemic colitis warrants further inquiry into causes in an immunosuppressed patient, especially liver transplant recipients with risk factors of mucormycosis. He was finally diagnosed by endoscopic biopsies and successfully treated with strict control of blood sugar level and antifungal agent. Thus, early diagnosis and treatment are essential for improving the prognosis of invasive fungal infection such as colonic mucormycosis after liver transplantation.

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**P- Reviewers** Nielsen OH, Rodrigo L **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Zhang DN



## Transarterial embolization of metastatic mediastinal hepatocellular carcinoma

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**Author contributions:** Chen CC designed the research and drafted the article; Yeh HZ performed endosonographic biopsy to prove the diagnosis of metastatic mediastinal hepatocellular carcinoma and revised the manuscript; Chang CS was the attending physician for this patient, discussed the treatment plan with the patient, and revised the manuscript; Hung SW performed the transarterial embolization for the metastatic mediastinal hepatocellular carcinoma and wrote the technical aspect of the procedure; Ko CW, Lien HC and Wu CY gave intellectual advice and revised the manuscript.

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Received: January 12, 2013 Revised: April 14, 2013

Accepted: April 18, 2013

Published online: June 14, 2013

### Abstract

This paper introduces an innovative treatment for extrahepatic metastasis of hepatocellular carcinoma. A 71-year-old patient had a stable liver condition following treatment for hepatocellular carcinoma, but later developed symptomatic mediastinal metastasis. This rapidly growing mediastinal mass induced symptoms including cough and hoarseness. Serial sessions of transarterial embolization (TAE) successfully controlled this mediastinal mass with limited side effects. The patient's survival time since the initial diagnosis of the mediastinal hepatocellular carcinoma was 32 mo, significantly longer than the 12 mo mean survival period of patients with similar diagnoses: metastatic hepatocellular carcinoma and a liver condition with a Child-Pugh class A score. Currently, oral sorafenib is the treatment of choice for metastatic hepatocellular carcinoma. Recent

studies indicate that locoregional treatment of extrahepatic metastasis of hepatocellular carcinomas might also significantly improve the prognosis in patients with their primary hepatic lesions under control. Many effective locoregional therapies for extrahepatic metastasis, including radiation and surgical resection, may provide palliative effects for hepatocellular carcinoma-associated mediastinal metastasis. This case report demonstrates that TAE of metastatic mediastinal hepatocellular carcinoma provided this patient with tumor control and increased survival time. This finding is important as it can potentially provide an alternative treatment option for patients with similar symptoms and diagnoses.

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**Key words:** Embolization; Hepatocellular carcinoma; Lymphatic metastasis; Endosonography; Mediastinal neoplasm

**Core tip:** This report presents a patient with metastatic hepatocellular carcinoma localized to his mediastinum. The metastatic tumor grew rapidly, with  $\alpha$ -fetoprotein (AFP) elevation rates correlating with the size of the tumor and tumor growth. We used several sessions of transarterial embolization to control this metastatic tumor. After every successful intervention, the tumor had been stabilized and AFP levels were decreased significantly, resulting in a survival time of the patient that is much longer when compared to the mean survival times of others with similar diagnoses. Transarterial embolization could be an alternative treatment choice for patients with mediastinal metastasis of hepatocellular carcinoma.

Chen CC, Yeh HZ, Chang CS, Ko CW, Lien HC, Wu CY, Hung SW. Transarterial embolization of metastatic mediastinal hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(22): 3512-3516 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3512.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3512>

## INTRODUCTION

Liver cancer in men is the fifth most frequently diagnosed cancer and the second most frequent cause of cancer death worldwide. In women, it is the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death<sup>[1]</sup>. Although advances in treatment of hepatocellular carcinoma (HCC) have resulted in prolonged survival of HCC patients, the incidence of extrahepatic metastasis associated with HCC has also risen<sup>[2]</sup>. Patients with extrahepatic spread have a poor prognosis<sup>[3]</sup>. Current managements for these patients are oral sorafenib and possibly transarterial chemoembolization (TACE) for the hepatic tumors if the patient has enough remaining liver function<sup>[4]</sup>. There are few studies examining the efficacy of locoregional management for extrahepatic metastasis. Furthermore, there are even less reports of lymph node-directed therapy in distant lymph node metastasis<sup>[5]</sup>. In this report, a 71-year-old patient with symptomatic isolated progression of mediastinal lymphadenopathy of HCC was treated with transarterial embolization (TAE). The symptoms were controlled and survival time was longer than expected.

## CASE REPORT

A 71-year-old man visited the Taichung Veterans General Hospital for the management of his HCC. His HCC tumor was 3 cm, localized to the right lobe, and diagnosed by elevated  $\alpha$ -fetoprotein (AFP) levels and hypervascular appearance on abdominal computer tomography (CT). The patient was a hepatitis B carrier with a class A Child-Pugh score and had early stage HCC as assessed by the Barcelona Clinic Liver Cancer staging classification (stage A). However, this patient did not want to receive curative surgery due to old age and personal preference.

He received TACE treatment twice during the first year following his HCC diagnosis. His serum AFP level decreased from 1171 to 14.92 ng/mL following this TACE therapy. One year later, his serum AFP level increased again, but an abdominal CT scan and angiography of the liver did not reveal definite hepatic hypervascular tumors. Two years after the diagnosis of HCC, he started to experience hoarseness due to left vocal cord paralysis. A chest CT scan revealed a 5 cm tumor over the aortopulmonary window of the mediastinum (Figure 1). Metastatic HCC was identified pathologically with endosonography-guided fine needle aspiration. After discussions with the surgeon, radiation oncologist and interventional radiologist, he refused surgery and radiation.

Transarterial embolization of mediastinal tumor was adopted as the treatment modality. Although available now, molecular targeting agents for HCC were not yet a standard care option for patients at that time. The TAE procedure was performed as follows: the first branch of the right superior bronchial artery was identified as the main artery supplying the left hilar metastatic lesion during angiography. Lipiodol was administered *via* this artery as the embolizer, and a chest CT scan verified the

metastatic lymph nodes were mostly occupied by lipiodol (Figure 2). His AFP level decreased from 17089 to 1061 ng/mL and this TAE session was considered successful. There were no adverse effects like cough, chest pain, fever or dyspnea. Thereafter, he received another four sessions of TAE *via* several supplying arteries for viable or recurrent components. AFP levels decreased following each successful TAE, but these levels would rebound quickly due to recurrence of the tumor. During the 16-mo follow-up period, his AFP level was approximately 1000-4000 ng/mL. In addition, he began to have night coughs. On the sixth session of TAE, embolization was performed *via* a small branch of the left branch of internal mammary artery identified by high-resolution chest CT (Figure 3). AFP levels decreased significantly to 16.64 ng/mL and this session was considered successful and the patient remained in stable condition for seven months with no TAE-related complications. However, several months later, the patient began to experience progressive night cough and elevation of AFP levels. A chest CT scan showed progression of mediastinal tumors (Figure 4). A seventh TAE for recurrent mediastinal lymphadenopathy was performed for controlling of tumor. However, his lung condition deteriorated progressively eventually causing him to expire due to pneumonia-related respiratory failure. The patient's survival time since the initial diagnosis of mediastinal metastasis was 32 mo. During the follow up period, his hepatic tumor remained well embolized and his liver function remained stable without evidence of decompensation.

## DISCUSSION

The natural course of HCC is unique. Unlike cancers of the colon, breast, lung, and others, whose causes of death are typically due to systemic processes with development of disseminated diseases, HCCs usually present as local lesions and subsequent development of systemic diseases are relatively uncommon<sup>[6]</sup>. The causes of death from HCC may not only be due to progressive cancer, but may also be due to liver failure.

The prognosis of patients with extrahepatic metastases is usually very poor. The one year and three year survival rates of patients who underwent surgical resection of extrahepatic metastases of HCC was 24% and 7%, respectively. For patients who did not undergo resection, these rates dropped to 8% and 0%, respectively<sup>[7]</sup>. Poor prognostic factors of extrahepatic metastasis of HCC are poor performance status, a high Child-Pugh score, a large number and size of intrahepatic lesions, the presence of macroscopic vascular invasion, a symptomatic extrahepatic metastasis, and high AFP levels<sup>[8]</sup>. In this report, the patient exhibited two of these prognostic factors: high AFP level and symptomatic extrahepatic metastasis. He survived for 32 mo following the initial diagnosis of extrahepatic metastasis despite these poor prognostic factors and his decision not to have surgery. The mean survival time for HCC patients with a Child-Pugh class A score and who had developed extrahepatic

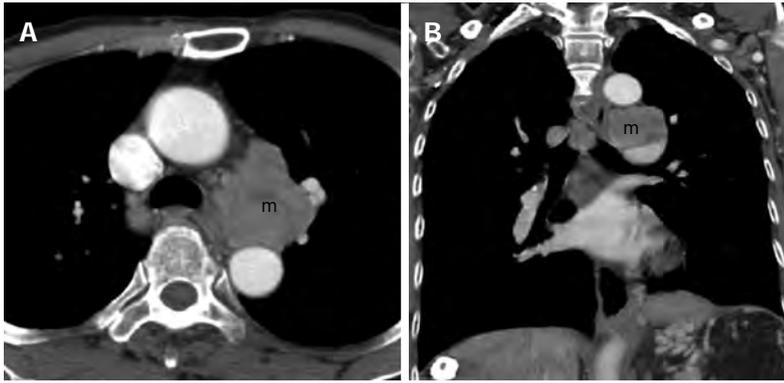


Figure 1 A chest computer tomography scan of the aortopulmonary window of the mediastinum revealed a 5 cm metastatic lymphadenopathy (m). A: Transverse view; B: Sagittal view.



Figure 2 First session of transarterial embolization for mediastinal tumor. A: An angiogram of the right superior bronchial arterial branch showed a metastatic hepatocellular carcinoma (M) in the left hilum supplying by the first branch of right superior bronchial artery. Embolization was performed by lipiodol only; B: Chest computer tomography (CT) scan: Lipiodol retention in posterior portion of the mass (T) over aortopulmonary window region and decreased in size, but the anterior portion of the mass (M) over aortopulmonary window region increased in size as compared with previous CT.

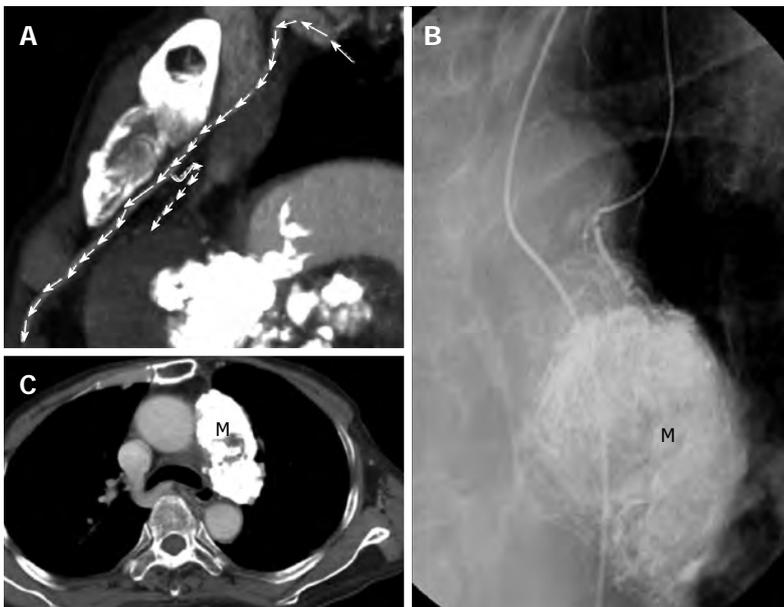


Figure 3 Successful control of mediastinal metastasis via selection of the supplying vessel. A: Multi-detector row computed tomography showed a supplying artery from small branch of left internal mammary artery to residual tumor (arrows); B: Tumor supplying artery of left internal thoracic (mammary) artery was selected via a 3 Fr microcatheter. Transarterial embolization for tumor (M) was done by using 6 mL of lipiodol; C: Follow-up chest computed tomography showed the metastatic tumor (M) in left anterior mediastinum was well embolized.

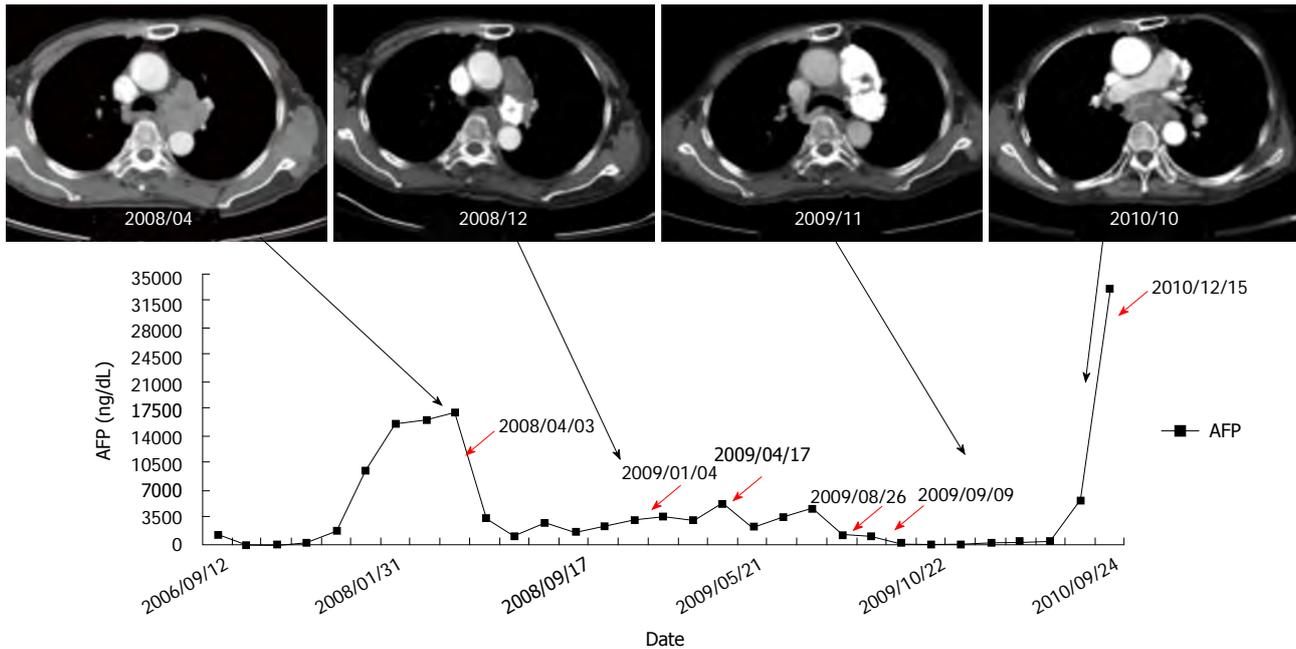
metastasis was recently reported to be 12.0 mo<sup>[6]</sup>. Therefore, this patient far exceeded the expected survival time for someone with his diagnosis.

Current management of metastatic HCC is TACE for primary tumor control and possible chemotherapy, radiation or molecular targeting with sorafenib for extrahepatic lesions<sup>[4]</sup>. Some evidence suggests that treatment of extra-hepatic metastasis of HCC can significantly improve the prognosis of patients whose hepatic lesions are under control<sup>[7,8]</sup>.

Previous studies indicate that surgery for HCC-

associated pulmonary<sup>[9]</sup>, adrenal<sup>[10]</sup> and local lymph node metastasis<sup>[11]</sup> may be beneficial when added to these traditional treatments. Many effective locoregional therapies for extrahepatic metastasis have also been reported, including irradiation for bone<sup>[12]</sup>, lymph node<sup>[13]</sup>, brain<sup>[14]</sup>, and adrenal<sup>[15]</sup> metastases, and percutaneous ablation for adrenal<sup>[16]</sup> and bone metastases<sup>[17]</sup>. Further, TAE has been used as a therapy for adrenal metastasis<sup>[18]</sup> while this is the first report of TAE for mediastinal metastasis.

The most common sites of lymph node metastasis of HCC are locoregional lymph nodes including paraaortic,



**Figure 4** Alpha-fetoprotein levels in time course of treatment. Every session of transarterial embolization is shown with a red arrow. The computed tomography (CT) images represented the progression of mediastinal tumor and treatment response of transarterial embolization. The CT image on the far right was taken about three months before death of the patient. The tumor compressed the left bronchus, which may explain the cause of the patient's pneumonia.

portohepatic, periceliac and peripancreatic nodes. The most common site of HCC-associated distant metastatic lymphadenopathy is the mediastinal lymph nodes<sup>[19]</sup>. Because lymph node metastasis or dissemination itself is not the usual cause of death<sup>[5]</sup>, there are relatively few studies examining the management of metastatic lymph nodes. Lymph node metastases that cause agonizing pain, or rarely life-threatening conditions like pericardial or esophageal invasion<sup>[20]</sup>, may be indications for locoregional treatment such as radiotherapy or surgical resection.

Excellent radiotherapy responsiveness was reported in patients whose intrahepatic HCC was concurrently under control<sup>[13]</sup>. However, radiation at the mediastinum is associated with cardiovascular toxicity, which may present with acute pericarditis, restrict cardiomyopathy, ischemic heart disease, congestive heart failure and valvular heart disease<sup>[21]</sup>. Moreover, radiation therapy at the mediastinum is also associated with a 5%-10% risk of symptomatic radiation pneumonitis<sup>[22]</sup>. Subtle lung function impairment induced by radiation injury may further reduce the capacity of a patient to deal with future cardiopulmonary stress. Surgical resection may be an option if the patient has good liver function and isolated lymph node metastasis<sup>[23]</sup>. However, the existence of possible occult metastasis and the variable degree of hepatic decompensation in HCC patients greatly reduce the benefits of surgery.

Currently, the mainstay treatment for metastatic HCC is sorafenib<sup>[4]</sup>, while options including heavy ion or proton radiotherapy also remain. TAE for mediastinal metastatic HCC may be an alternative option for certain patients with localized metastatic lymph nodes. Furthermore, TAE does not cost more than the other treatment mo-

dalities. In this report, the patient did not present significant side effects during several sessions of TAE and had satisfactory control of his tumor. However, several challenges associated with performing TAE in the mediastinum remain including: the considerable challenge of locating the supplying vessels, the close proximity to many critical arteries in this part of the body such as the spinal cord artery, and the risk of reflux of embolizer to other vital areas. Above all, the key challenge to providing a safe and successful TAE is selection of the supplying artery. Thus, it remains unclear whether this technique can be widely used.

In conclusion, this report showed that the use of TAE for treatment of a metastatic mediastinal HCC provided temporal tumor control and increased survival time. In the right environment, TAE should be an alternative option for well-selected patients with mediastinal metastasis of HCC.

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**P- Reviewers** Enjoji M, Ferraioli G, Ramia JM, Tsuchiya A  
**S- Editor** Huang XZ **L- Editor** A **E- Editor** Xiong L



## A long adult intussusception secondary to transverse colon cancer

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Author contributions: Xu XQ and Li BL designed the report; Xu XQ, Hong T, Liu W and He XD were attending doctors for the patient; Xu XQ, Li BL and Zheng CJ performed surgical operation; Xu XQ and Hong T organized the report; Xu XQ wrote paper.

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Received: February 28, 2013 Revised: April 2, 2013

Accepted: April 10, 2013

Published online: June 14, 2013

### Abstract

The occurrence of adult intussusception arising from colorectal cancer is quite rare. We present the case of a 76-year-old man with sudden abdominal pain and vomiting. Clinical symptoms included severe abdominal distension and tenderness. Computed tomography scan of the abdomen revealed left-sided colocolic intussusception with a lead point. The patient underwent a left hemicolectomy with right transverse colostomy. Pathologic evaluation revealed moderately differentiated adenocarcinoma invading the muscularis propria; the regional lymph nodes were negative for cancer cells. The postoperative course was uneventful.

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**Key words:** Adult intussusception; Colon cancer; Surgery; Hemicolectomy

**Core tip:** Intussusception is a common cause of bowel obstruction in pediatric patients, but it is rare in adults

and it is often difficult to diagnose. We present the case of a 76-year-old man with sudden abdominal pain and vomiting. The patient underwent a left hemicolectomy with right transverse colostomy. This article reports the complete diagnosis and management of the patient.

Xu XQ, Hong T, Liu W, Zheng CJ, He XD, Li BL. A long adult intussusception secondary to transverse colon cancer. *World J Gastroenterol* 2013; 19(22): 3517-3519 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3517.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3517>

### INTRODUCTION

Intussusception is most often encountered in infants and children, and only 5% of cases occur in adults. It accounts for about 1% of all cases of adult bowel obstruction. Adult intussusception of the colon is rare and related to malignant lesions<sup>[1]</sup>. We describe a case of adult intussusception of the transverse colon caused by a malignant tumor in an elderly man preoperatively diagnosed by X-ray, barium enema and computed tomography (CT) scan.

### CASE REPORT

A 76-year-old male with no significant medical history was admitted as a surgical emergency with sudden abdominal pain and vomiting and 2 episodes of bright red bloody stool. Physical examination revealed severe abdominal distension and tenderness.

The abdominal X-ray showed distension of the ascending and right half transverse colon (Figure 1). A barium enema showed the meniscus sign in the contrast material-filled distal sigmoid (Figure 2). CT showed a giant mass at the transverse colon. CT also showed "bowel within bowel" consistent with colocolic component of the intussusception with distension of the ascending



Figure 1 Abdominal X-ray showing distension of the ascending and right half transverse colon.



Figure 2 Barium enema examination showing the meniscus sign in the contrast material-filled distal sigmoid.

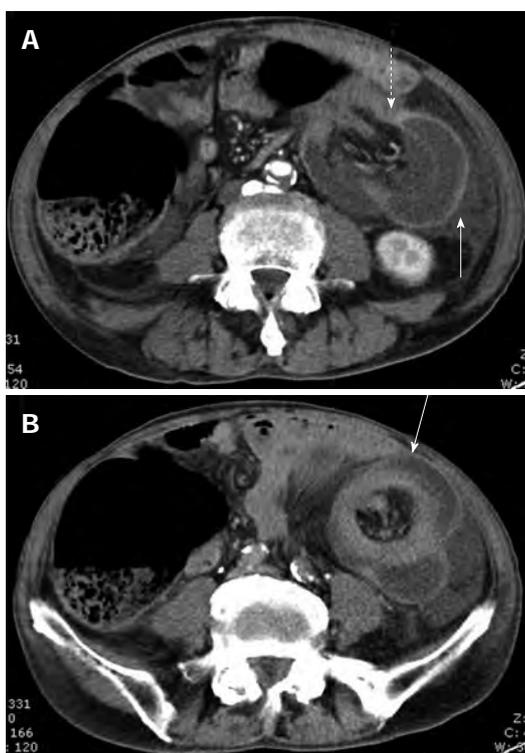


Figure 3 Abdominal computed tomography showing colocolic component of the intussusception (A, dashed arrow), thick-walled descending colon invaginated by transverse colon with mesenteric fat and vessels (A, solid arrow), and the typical "target sign" of colon within colon (B, solid arrow).

colon and invaginated left transverse colon into the descending colon with mesenteric fat and vessels (Figure 3).

The diagnosis of colocolic intussusception at the transverse colon was made. The patient was admitted and underwent emergency laparotomy, and radiologic findings were confirmed during the operation. A giant mass was palpable through the splenic flexure of colon which extended till the sigmoid colon. The mass comprised an intussusception of both the left part of the transverse colon and the great omentum (Figure 4). An extended left hemicolectomy with right transverse colostomy was performed. The patient had an uneventful recovery and was discharged 10 d after the surgery.



Figure 4 Abdominal computed tomography showing enlargement of the descending colon wall with the intussusception (arrow), suggesting the existence of a tumor in the head.

Gross examination of the resected specimen revealed a 25-cm colocolic intussusception which contained a 3.0 cm × 3.0 cm × 2.5 cm protuberant tumor originating from the splenic flexure of colon and the whole great omentum in the descending colon (Figure 5). The final pathological report showed moderately differentiated adenocarcinoma invading the muscularis propria; the regional lymph nodes were negative for cancer cells.

## DISCUSSION

Colo-colonic intussusception is rare in adults (< 5%), but it is the most common cause of intestinal obstruction in infants aged 6-18 mo<sup>[1]</sup>. The characteristic pediatric presentation triad of abdominal pain, palpable abdominal mass and bloody stool is rarely seen in adult cases. Most patients present with subacute (24.4%) or chronic (51.2%) symptoms of abdominal pain, nausea, vomiting and constipation; this is the main reason why preoperative diagnosis is difficult<sup>[2]</sup>. The best preoperative diagnosis method of intussusception is CT scan. Mostly, adult intussusception is related to bowel pathology and 38%-45% cases occur in the colon, while 52%-55% occur in the small intestine<sup>[3]</sup>. It has been reported that 33%-77% of adult colonic intussusception cases are associated with malignant lesions. Adult intus-



**Figure 5** Gross examination of the resected specimen reveals a 25-cm colocolic intussusception. A: Perioperative view after mobilization of the left colon with forceps inside the intussusception invaginated transverse colon with great omentum; B: Operative specimen showing about 25 cm overlapped colon in the intussusceptions; C: The specimen contained a 3.0 cm × 3.0 cm × 2.5 cm protuberant tumor, thought to be the lead point of the intussusception (arrow).

susception of the colon mostly occurs in the flexible regions such as the sigmoid and transverse colon and the cecum<sup>[4]</sup>. For the management of adult intussusception, most authors think that laparotomy is mandatory due to the high incidence of underlying malignancy in colonic intussusceptions and the inability to differentiate non-operatively benign from malignant causes in enteric intussusceptions<sup>[5]</sup>.

In this case, we report a relatively complete process for the diagnosis of adult intussusceptions with detailed medical pictures. Adult intussusception must be considered in the differential diagnosis of patients with abdominal pain and vomiting. The work-up must include X-ray, ultrasound and CT scan of the abdomen, and even a barium enema. Emergent surgical interventions are required once the diagnosis of intussusception is made, due to the high risk of malignancy.

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**P- Reviewers** Petronella P, Zaniboni A **S- Editor** Gou SX  
**L- Editor** Logan S **E- Editor** Xiong L



## Hemolymphangioma: A rare differential diagnosis of cystic-solid or cystic tumors of the pancreas

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Supported by The Public Technology Research and Social Development Project of Science and Technology Department of Zhejiang Province, China, Grant No.2010C33142

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Received: January 18, 2013 Revised: March 2, 2013

Accepted: April 9, 2013

Published online: June 14, 2013

### Abstract

We report a case of pancreatic hemolymphangioma. Hemolymphangioma is a malformation of both lymphatic vessels and blood vessels. The incidence of this disease in the pancreas is extremely rare. To the best of our knowledge, only seven cases have been reported worldwide (PubMed). A 39-year-old woman with a one-day history of abdominal pain was admitted to our hospital. There was no obvious precipitating factor. The preoperative examination, including ultrasonography and computed tomography, showed a cystic-solid tumor in the pancreas, and it was considered to be a mucinous cystadenoma or cystadenocarcinoma. Pancreatic

body-tail resection combined with splenectomy was performed. After the operation, the tumor was pathologically demonstrated to be a pancreatic hemolymphangioma. Although pancreatic hemolymphangioma is rare, we believe that it should be considered in the differential diagnosis of cystic-solid tumors of the pancreas, particularly when there is no sufficient evidence for diagnosing cystadenoma, cystadenocarcinoma or some other relatively common disease of the pancreas.

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**Key words:** Pancreatic neoplasm; Hemolymphangioma; Differential diagnosis; Computed tomography; Ultrasonography

**Core tip:** This article reports an extremely rare case of pancreatic hemolymphangioma, which is a cystic-solid tumor. The imaging findings are different from those reported in the literature. Although it is rare and the definitive diagnosis depends on histological evidence, it should be considered preoperatively in the differential diagnosis of cystic-solid or cystic tumors of the pancreas, particularly when there is no sufficient evidence for diagnosing cystadenoma, cystadenocarcinoma or some other relatively common disease of the pancreas.

Dong F, Zheng Y, Wu JJ, Fu YB, Jin K, Chao M. Hemolymphangioma: A rare differential diagnosis of cystic-solid or cystic tumors of the pancreas. *World J Gastroenterol* 2013; 19(22): 3520-3523 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3520.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3520>

### INTRODUCTION

Hemolymphangioma is a malformation of both lymphatic vessels and blood vessels. The incidence of pancreatic

hemolympangioma is extremely rare. To the best of our knowledge, only seven cases have been reported worldwide (PubMed)<sup>[1-7]</sup>. Macroscopically, it is a multilocular cystic mass with endothelium covering the connective septa<sup>[2,3]</sup>. The tumor typically presents as a mass with heterogeneous enhancement on computed tomography (CT)<sup>[1,6,7]</sup>. Here, we report a case of pancreatic hemolympangioma with different imaging findings.

## CASE REPORT

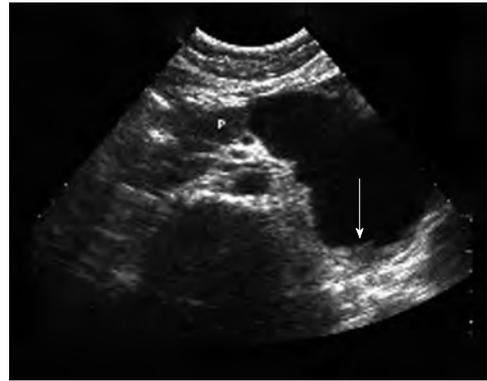
A 39-year-old woman was admitted to our hospital with a complaint of abdominal pain for one day. There was no obvious precipitating factor, no weight loss and no family history of cancer. A physical examination showed mild tenderness in the left hypochondrium. Laboratory tests, including serum tumor markers, were normal.

The ultrasonography and CT findings of the tumor were as follows: (1) Location: the tumor was located at the body and tail of the pancreas; (2) Shape: it was irregular, approximately 10 cm × 7 cm in size, and had a clear border (Figures 1 and 2); (3) Composition: it was a cystic-solid tumor. The cystic area, with a mean CT attenuation value of 30 Hounsfield units (HU), comprised the main part. There was a cord-like septum in the center of the cystic area (Figure 2A, arrow). The solid part was located at the back of the tumor, with a mean CT attenuation value of 68 HU (Figure 2E, arrow); (4) Enhancement: it showed almost no change on contrast-enhanced CT images (Figure 2B-D, Figure 2F-H) compared with pre-contrast images, as assessed with the CT attenuation value; and (5) Neighborhood: the adjacent organs were compressed, and no enlarged lymph nodes were found. Based on these findings, mucinous cystadenoma or cystadenocarcinoma was initially considered.

The patient underwent an exploratory laparotomy. During the operation, the mass was identified as a large dark red mass located at the body and tail of the pancreas, slightly adhering to the gastric wall, posterior peritoneum and left kidney, with bloody fluid inside. Pancreatic body-tail resection combined with splenectomy was performed. Microscopically, some dysplastic lymphatic vessels and blood vessels were observed in the tumor (Figure 3). The pathological diagnosis was hemolympangioma. Her postoperative course was uneventful. She is currently enjoying a normal life without signs of recurrence.

## DISCUSSION

Hemolympangioma is a congenital malformation of the vascular system. The formation of this tumor may be explained by obstruction of the venolymphatic communication between dysembryoplastic vascular tissue and the systemic circulation<sup>[1-3]</sup>. Only seven cases have been reported previously<sup>[1-7]</sup>. Most of these cases were women (6 of 7), and most of the tumors were located at the head of the pancreas (6 of 7). A patient with pancre-



**Figure 1 Ultrasonography.** The tumor is located at the body and tail of the pancreas and is irregularly shaped. The cystic part has a low-intensity echo level, and the solid part (arrow) displays an isointense echo.

atic hemolympangioma can be asymptomatic for a long time. Abdominal pain and awareness of an abdominal mass are the most common symptoms<sup>[7]</sup>.

The imaging findings of pancreatic hemolympangioma typically show a mass with heterogeneous enhancement<sup>[1,6,7]</sup>. In our case, the tumor showed different imaging findings. It presented as a cystic-solid tumor (a single large cyst and a small solid part), with no enhancement in the three-phase contrast scan. We believe that the cystic part might be due to the rupture and fusion of the vascular cavity and that the solid part represented the residual and compressed vascular tissue. The lack of enhancement in the three-phase contrast scan may be related to the relatively small number of blood vessels in the tumor and slow-moving blood flow in the dysplastic blood vessels.

The differential imaging diagnosis for this case includes other cystic-solid or cystic lesions of the pancreas. A mucinous cystadenoma typically shows a single large cyst with a smooth contour, fine septa, even wall, and potentially, small nodules. A thick wall and large nodules might suggest the diagnosis of cystadenocarcinoma. Both mucinous cystadenomas and cystadenocarcinomas can present septa, walls, and nodule enhancement. A solid pseudopapillary tumor of the pancreas is typically found in young women presenting a cystic-solid mass with enhancement of the solid part. A pancreatic pseudocyst typically shows a unilocular lesion with a history of pancreatitis. However, the definitive diagnosis of this tumor was made using histological evidence<sup>[1,3]</sup>.

An operation is required to treat this tumor. Two operative methods are available: tumor enucleation and partial pancreatectomy<sup>[2]</sup>. Because the diagnosis is made postoperatively, pancreatoduodenectomy is typically used for the suspicion of malignancy or invasion<sup>[1,6]</sup>.

Although rare, pancreatic hemolympangioma should be considered in the differential diagnosis when a cystic-solid tumor of the pancreas is found, particularly when there is no sufficient evidence for diagnosing cystadenoma, cystadenocarcinoma or some other relatively common disease.

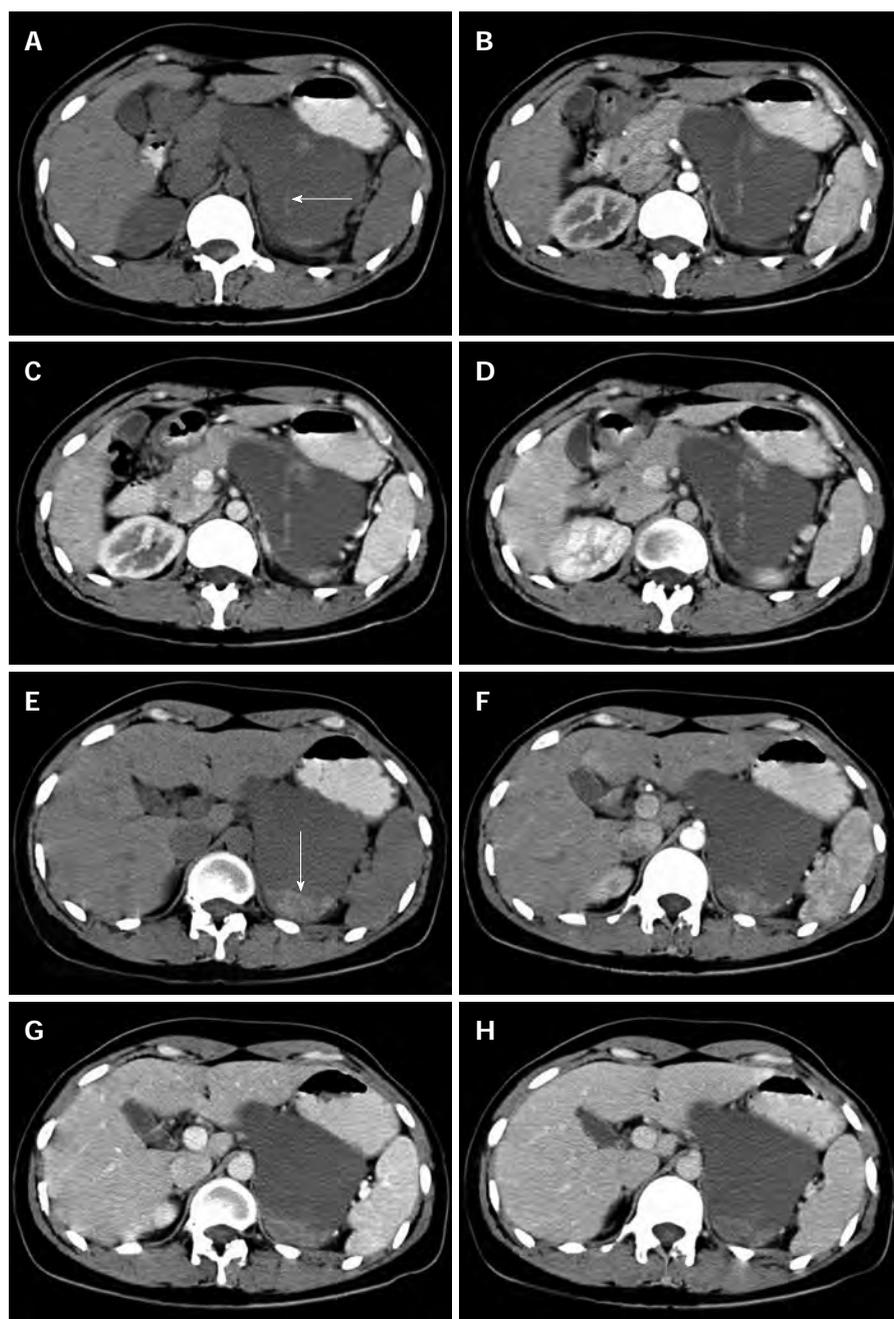


Figure 2 Computed tomography images. A-D: There is a septum (arrow) in the center of the cystic part. Neither the cystic part nor the septum shows enhancement in the arterial phase (20 s after contrast injection) (B), pancreatic phase (45 s after contrast injection) (C), or portal phase (80 s after contrast injection) (D) compared with the pre-enhanced phase (A); E-H: The solid part (arrow) shows no enhancement in the arterial phase (20 s after contrast injection) (F), pancreatic phase (45 s after contrast injection) (G), or portal phase (80 s after contrast injection) (H) compared with the pre-enhanced phase (E).

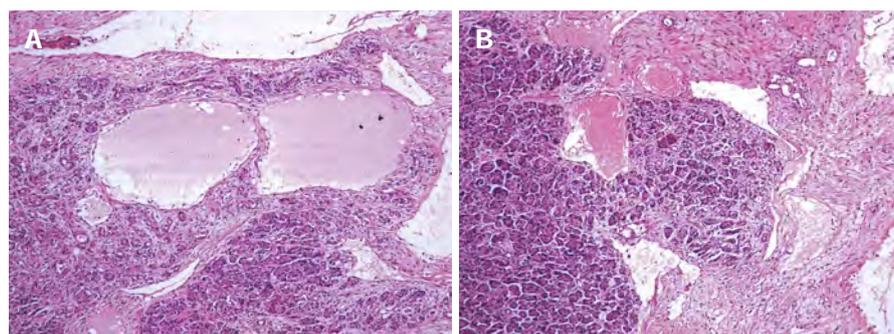


Figure 3 Pathological sections. Some dysplastic lymphatic vessels (A) and blood vessels (B) can be observed in the tumor. Hematoxylin and eosin stain,  $\times 100$ .

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## ACKNOWLEDGMENTS

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We thank Min-Ming Zhang, a responsible Professor and the Director of our department, who provided us with support during the work.

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**P- Reviewers** Vila JJ, Zhang XP **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Xiong L



## Hepatoid adenocarcinoma of the extrahepatic duct

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Author contributions: All authors contributed equally to this work; Han GP performed the pathological diagnosis; Wang Y and Liu YY summarized the clinical materials and generated the photographs; all authors wrote the manuscript.

Supported by The Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry of China, No. 2009-1001

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Received: January 10, 2013 Revised: April 29, 2013

Accepted: May 22, 2013

Published online: June 14, 2013

### Abstract

Hepatoid carcinoma is a unique type of extrahepatic tumor associated with hepatic differentiation and displays the morphological and functional features of hepatocellular carcinoma. Hepatoid carcinoma of the extrahepatic duct has rarely been reported in the literature. We report a 62-year-old man who presented with epigastric discomfort, xanthochromia, dull pain of the right shoulder, nausea and pruitus. Microscopic examination of the extrahepatic duct indicated that the tumor was primarily composed of "hepatoid cells", which were characterized by an eosinophilic cytoplasm, enlarged nucleus and prominent nucleoli. The cells were arranged in nests or proliferated in a trabecular pattern. Immunohistochemistry indicated that the tumor cells were positive for hepatocyte paraffin 1 and cytokeratins 8 and 18. Based on these findings, this case was diagnosed as hepatoid carcinoma of the extrahepatic duct.

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**Key words:** Extrahepatic bile ducts; Hepatocellular;

Adenocarcinoma; Immunohistochemistry; Surgical pathology; Differential diagnosis

**Core tip:** Hepatoid adenocarcinoma (HAC) is a rare type of extrahepatic adenocarcinoma that resembles hepatocellular carcinoma. Although the stomach is the most common location for this tumor, the lung, pancreas, esophagus, papilla of Vater, colon, urinary bladder, renal pelvis, ovaries, uterus and cervix have also been reported as primary locations. To the best of our knowledge, HAC arising primarily from the extrahepatic duct has not previously been reported in the literature. This report presents a rare case of HAC of the hepatic duct and performs a differential diagnosis based on immunochemical results, detailed clinical history and endoscopic findings.

Wang Y, Liu YY, Han GP. Hepatoid adenocarcinoma of the extrahepatic duct. *World J Gastroenterol* 2013; 19(22): 3524-3527 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3524.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3524>

### INTRODUCTION

Hepatoid adenocarcinoma (HAC) of the stomach was first described by Ishikura *et al* in 1985. Carcinomas with hepatoid differentiation have since been described in a variety of locations, including the lung, kidney, female reproductive tract, pancreas, gallbladder and stomach (the most prevalent site)<sup>[1-6]</sup>. An adenocarcinoma of the papilla of Vater showing hepatoid differentiation has previously been reported<sup>[2]</sup>. This tumor was proposed to be a specific type of carcinoma of the Vater. HAC is characterized by hepatic differentiation, which is determined based on both morphological and functional features<sup>[7]</sup>.

To the best of our knowledge, cases of HAC of the extrahepatic duct have not previously been reported. Herein, we present a rare case of HAC of the hepatic duct, which may be proposed as a new site of this carcinoma.

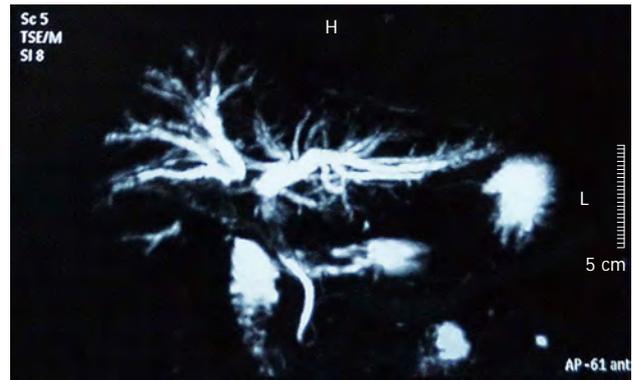
## CASE REPORT

A 62-year-old man presented with epigastric discomfort, xanthochromia, dull pain of the right shoulder, nausea and pruritus. A physical examination identified edema in both lower extremities and his feet. Epigastric tenderness to deep palpation was present but rebound tenderness was undetected. Muscular tension was negative. The liver and spleen were unreachable under the ribs. The hepatojugular reflux was negative. The patient denied any history of infection. Caput medusae, spider angioma and palmar erythema were not observed. Bowel sounds were detected twice per minute. Hepatitis B surface antigen (HbsAg) and hepatitis C-antibody were negative. Cholecystectomy, laparoscopic common bile duct exploration and cholangio-enterostomy were performed.

Abdominal ultrasonography revealed hepatic adipose infiltration, intrahepatic cholangiectasis, and choledochectasia. The above description included the possibility of upper hepatic duct obstruction. Magnetic resonance cholangiopancreatography (MRCP) demonstrated that the branches of the intrahepatic bile duct, the ductus hepaticus sinister and the ductus hepaticus dexter flowed normally. Intrahepatic cholangiectasis and aclasis of the ductus hepaticus sinister and the ductus hepaticus dexter were observed (Figure 1). The common hepatic duct, gall bladder and upper segment of the common bile duct could not be visualized. The imaging suggested obstructive jaundice of the upper segment of the bile duct. The obstruction was observed in the proximity of the hepatic portal area, and a lesion occupying the hepatic portal area was considered.

During the exploratory laparotomy, a small degree of abdominal dropsy and intrahepatic cholestasis were observed. The spleen was normal and the gallbladder wall was thickened. Foci of severe inflammation adhered to surrounding tissues were detected and the wall of the common bile duct was thickened and the lumen dilated (diameter of 3 cm). A solid space-occupying lump could be felt within the ampulla of the common bile duct. Therefore, cholecystectomy, laparoscopic exploration of the common bile duct and a cholangioenterostomy were performed. Intraoperative consultation reported a small number of malignant cells in the necrotic tissues, however, the origin of the cells could not be confirmed. A repeated search for the origin of the cells failed to identify a primary lesion in the liver, gastrointestinal tract or pancreas. A choledochojejunostomy was subsequently performed.

A gross examination revealed a purple and red solid mass in the hepatic duct corresponding to the occupying lesion observed by MRCP, measuring  $1.5 \times 1.2 \times 1.0 \text{ cm}^3$ . In addition, one tubular mass in the common hepatic duct (1 cm in diameter and 0.2 cm in depth), one tubular mass in the ductus hepaticus sinister ( $1.0 \times 0.6 \times 0.2 \text{ cm}^3$ ), one gray and yellow solid mass in the ductus hepaticus dexter ( $1.2 \times 1.2 \times 0.5 \text{ cm}^3$ ), a mass of fragmented red and purple contents in the hepatic duct ( $3.5 \times 3.0 \times 0.4 \text{ cm}^3$ ), one gallbladder containing bile (4 cm



**Figure 1** Magnetic resonance cholangiopancreatography. Magnetic resonance cholangiopancreatography revealed an obstruction in the proximity of the hepatic portal area and the absence of hepatic nodules within the liver lobes. L: Left; H: Height.

in diameter and 9 cm in length), and two lymph nodes attached to the mesentery were also observed.

Microscopically, the tumor was primarily composed of “hepatoid cells”, which were characterized by an eosinophilic cytoplasm, enlarged nucleus, and prominent nucleoli. The tumor cells were arranged in nests or proliferated in a trabecular pattern. The tumor consisted of marginal areas of dysplastic glands and well-differentiated intestinal-type adenocarcinomatous tissue, which formed the bulk of the tumor (Figure 2A-C). None of the lymph nodes dissected at surgery showed tumor metastasis. Immunohistochemistry indicated that the tumor cells were positive for hepatocyte paraffin 1 (HepPar-1), cytokeratins 8 and 18 (CK8/18), polyclonal carcino-embryonic antigen (pCEA) and S-100 (Figure 2D-G). Approximately 15% of the tumor cells were positive for Ki-67 (Figure 2H).

Based on the above findings, the case was diagnosed as HAC of the hepatic duct was made.

## DISCUSSION

HAC a rare variant of extrahepatic adenocarcinoma, consists of foci of both adenomatous and hepatocellular differentiation that behave morphologically and functionally similar to hepatocellular carcinoma (HCC)<sup>[8,9]</sup>. As previously mentioned, the primary characteristics of HAC are histopathological features that suggest hepatoid differentiation resembling HCC. The tumor is generally composed of large or polygonal cells with abundant eosinophilic cytoplasm and proliferates in a solid or trabecular pattern, although medullary proliferation is occasionally observed<sup>[10]</sup>. The hepatoid nature of the cells can be proven based on the detection of bile production<sup>[11]</sup>. Primary gastric HAC has been the most frequently analyzed type of HAC. Glandular and hepatocyte differentiation both have been observed and the explanation for this phenomenon is “enteroblastic differentiation”. Because the stomach and liver are derived from the primordial foregut, prosoplasia that occurs during the maturation of the cells may induce the formation described above.

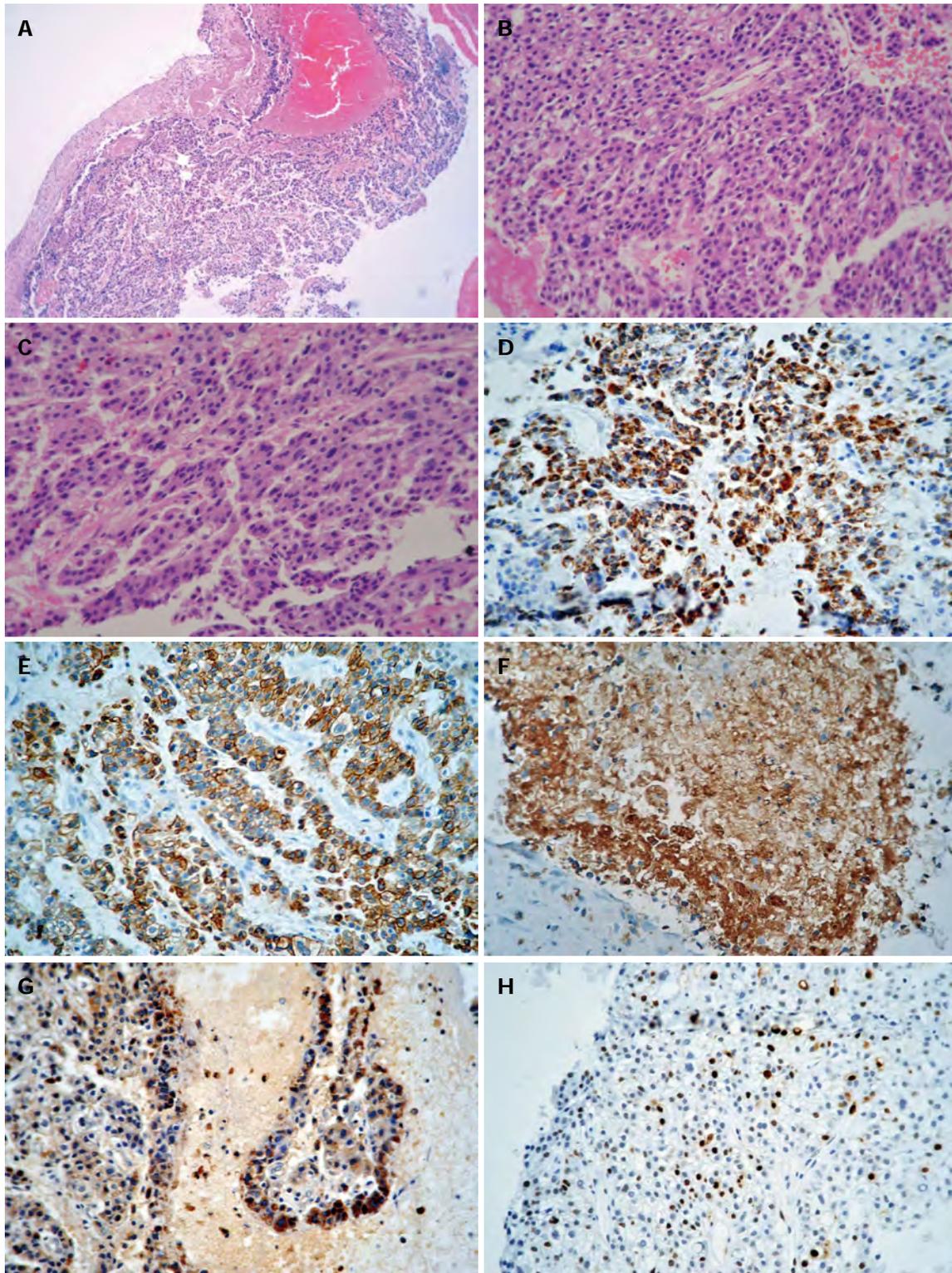


Figure 2 Histological and immunohistochemical features. A: Low-power microphotograph showing hepatoid areas (hematoxylin and eosin; original magnification,  $\times 100$ ); B, C: Hepatoid adenocarcinoma composed of cells with a clear cytoplasm arranged in a nested or trabecular pattern (hematoxylin and eosin; original magnification,  $\times 400$ ); D-H: Intracytoplasmic expression of hepatocyte paraffin 1, cytokeratin 8/18, polyclonal carcinoembryonic antigen and S-100 as well as Ki-67-positive nuclei (original magnification,  $\times 400$ ).

Clear cell carcinomas of the gallbladder with or without hepatoid differentiation are often associated with high serum  $\alpha$ -fetoprotein (AFP) levels<sup>[10]</sup>, however, not all HACs are associated with AFP overproduction<sup>[11]</sup>. In the present case, the preoperative serum AFP level was

6.84  $\mu\text{g/L}$ , and immunohistochemistry suggested that AFP protein was not expressed. However, focal positivity for HepPar-1 suggested the presence of hepatoid differentiation, and pCEA positivity indicated canalicular differentiation and hepatocellular origin. CK8/18 ex-

pression in this case resembled HCC and could also be considered to be as a marker of hepatocellular origin.

HCC generally arises in cirrhotic livers and with hepatitis virus infection, however, the preoperative examination revealed that this patient was negative for HBsAg. In the present case, the rough region of the ductus hepaticus sinister was observed by the operator during laparoscopic exploration of the common bile duct. Combined with the clinical findings, the extrahepatic ductal origin of the HAC was verified by the clinical presentation, its morphological similarity to HCC, the immunohistochemical identification of liver-synthesized proteins, and bile production<sup>[2]</sup>.

The differential diagnosis of HAC and HCC with invasion into the hepatic duct is primarily dependent upon tumor location. No primary lesion was identified in the liver, gastrointestinal tract or pancreas during surgery. In addition, MRCP and computed tomography angiography (not shown) did not reveal lumps in the liver. The literature has shown that HAC is generally found in elderly males, and poor outcomes are observed. The pathological findings in immunohistochemical analyses may also aid in the differential diagnosis of HAC from HCC: an evaluation using a panel of immunohistochemical markers (*e.g.*, cytokeratin 19, palate, lung and nasal epithelium clone, HepPar-1 and carcinoembryonic antigen), combined with detailed clinical history and endoscopic findings is essential for a definitive diagnosis.

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## Reducing risk of transjugular intrahepatic portosystemic shunt using ultrasound guided single needle pass

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Author contributions: All the authors contributed to the writing and the revision of this letter.

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Received: February 26, 2013 Revised: April 11, 2013

Accepted: May 7, 2013

Published online: June 14, 2013

### Abstract

Delayed liver laceration following transjugular intrahepatic portosystemic shunt (TIPS) is a serious and likely underdiagnosed complication. It is however an important complication following TIPS, which remains one of the most technically challenging interventional procedures performed. In addition to laceration, a number of complications regarding bleeding and perforation are well described following TIPS procedures. We feel the adoption of techniques such as ours and that of other authors described in the literature using an ultrasound-guided percutaneous transhepatic approach with a small caliber needle provides a safer and less traumatic procedure and should reduce complications of bleeding and almost completely eliminate the risk of liver laceration. Our procedure was successfully performed under conscious sedation rather than general anaesthesia further reducing the overall procedural risk to the patient.

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**Key words:** Transjugular portal systemic shunt; Ultrasound guided; Haemorrhage; Complication; Reducing; Morbidity; Death; Liver; Laceration

**Core tip:** Transjugular intrahepatic portosystemic shunt (TIPS) for complications of portal hypertension is com-

monly formed by accessing a portal vein branch from a metal cannula wedged in a hepatic vein. A number of serious procedural complications including bleeding and perforation following TIPS have been described. We feel the adoption of techniques such as ours and that of other authors described in the literature using an ultrasound-guided percutaneous transhepatic approach with a small caliber needle provides a safer and less traumatic procedure and should reduce complications of bleeding and almost completely eliminate the risk of liver laceration.

Leong S, Kok HK, Govender P, Torreggiani W. Reducing risk of transjugular intrahepatic portosystemic shunt using ultrasound guided single needle pass. *World J Gastroenterol* 2013; 19(22): 3528-3530 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i22/3528.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i22.3528>

### TO THE EDITOR

We read with interest the excellent article by Liu *et al*<sup>[1]</sup> regarding the delayed liver laceration following transjugular intrahepatic portosystemic shunt (TIPS) for portal hypertension. This is a serious and likely underdiagnosed complication. It is however an important complication following TIPS, which remains one of the most technically challenging interventional procedures performed. In addition to laceration, a number of complications regarding bleeding and perforation are well described following TIPS procedures.

In their paper, Liu *et al*<sup>[1]</sup> correctly established a number of factors which contribute to bleeding and liver injury following TIPS: (1) liver cirrhosis with coagulopathy; (2) liver parenchyma and vascular injury during TIPS; and (3) early stent anticoagulation with low molecular weight heparin. While we fully agree with these postulations, we feel that another important factor is the

needle size used for percutaneous access and tract creation between the portal vein and the hepatic veins during TIPS. In our department, we have recently adopted a new technique utilising a 22-gauge microneedle and percutaneous liver puncture under direct ultrasound visualisation that dramatically decreases the risk of laceration.

This modification may help refine and improve the outcome of patients undergoing TIPS placement. The creation of the shunt between the portal and hepatic veins is traditionally performed under fluoroscopic guidance, and is considered the most difficult step in the establishment of a TIPS<sup>[2]</sup>, and is usually successful only after several punctures<sup>[3]</sup>. This “blind” fluoroscopic procedure can be refined with fluoroscopic methods including wedged hepatic venography<sup>[4]</sup> and superior mesenteric artery arterial portography<sup>[5]</sup>. Ultrasound assisted TIPS which facilitates the creation of the portosystemic shunt have also been described including placement of metallic overlying skin markers<sup>[6]</sup>, placement of metallic coils adjacent to or microwire placement within the portal vein<sup>[7]</sup>. These methods attempt to reduce the time spent achieving the portosystemic shunt and thus, the risk of bleeding complications.

More interestingly, the removal of the step requiring blind puncture of the portal vein has been described in the recent literature. Raza *et al*<sup>[8]</sup> described a single-pass technique to access the right portal and right hepatic veins under ultrasound guidance using an 18-gauge, 20-cm Chiba needle (Cook) with a technical success rate of 73% and no evidence of post-procedure puncture site haemorrhage. Liang *et al*<sup>[2]</sup> also achieved 100% technical success and no evidence of post-TIPS internal hemorrhage with a similar technique in patients with severely distorted liver parenchyma to obtain a left portal vein and inferior vena cava (IVC) shunt, using an 18-gauge, 20-cm needle. Whilst small in patient numbers, these studies highlight the feasibility of ultrasound guidance to reduce the number of punctures required to achieve a portosystemic shunt, which thus reduces the risk of bleeding.

To further reduce the risk of bleeding in these patients who are often coagulopathic, it is suggested to use a further modification of Raza *et al*<sup>[8]</sup> and Liang *et al*<sup>[2]</sup> technique using initial access with a 22-gauge, 20-cm Chiba needle (Cook) in an attempt to reduce the bleeding risk from the most challenging step of the TIPS procedure, especially in high risk patients. This method has also been described in a small series by Gazzera *et al*<sup>[9]</sup> in 8 patients with 100% technical success. This method requires pre-procedural drainage of ascites, if present, under ultrasound guidance with a 7-8 Fr pigtail catheter. Embolisation of the transhepatic needle tract can also be considered<sup>[8]</sup> especially in cases where ascites is present<sup>[2]</sup>. Where available, intravascular ultrasound may be useful to guide shunt creation especially in cases with portal vein thrombosis, distorted anatomy, Budd-Chiari syndrome or hepatic tumors<sup>[10]</sup>.

We recently adopted a modified transhepatic single-needle pass for TIPS in our institution. It involved a 53-year-old male with cirrhosis undergoing TIPS for refractory ascites. His pre-procedure work up revealed

a Child-Pugh B and Model for End-stage Liver Disease score of 14. Prior to the modified TIPS procedure, the ascites was drained under ultrasound guidance. Following this, both the neck and abdomen were prepared for a combined transjugular and transhepatic approach followed by administration of conscious sedation with intravenous midazolam and fentanyl. Using real time ultrasound guidance, the right portal vein (Figure 1) was punctured with a 22-gauge, 20-cm Chiba needle (Cook) close to the bifurcation of the main portal vein (MPV), with entry confirmed by aspiration of blood. Following this, the needle was advanced into the right hepatic vein under real-time ultrasound guidance, with confirmation of entry by blood aspiration. A 0.018-inch Nitinol guidewire was then advanced into the hepatic vein, IVC and right atrium. The tract was upsized with a 7-Fr co-axial introducer system. The introducer and stiffener was then removed allowing passage of a 0.035-inch Amplatz Ultrastiff guidewire (Cook) into the right atrium. The right internal jugular vein was the accessed under ultrasound guidance using a micropuncture set (Cook) comprising of a 21-gauge needle, 0.018-inch guidewire and 4-Fr co-axial catheter. This was then upsized to a 6-Fr long vascular sheath into the right atrium. At this point, a 25-mm diameter Amplatz Gooseneck Snare (ev3 Inc) was used to snare the transhepatic wire, achieving through and through access (Figure 2). The sheath was then advanced into the hepatic vein over the transhepatic-transjugular wire until resistance was met. A 6 mm × 20 mm and 8 mm × 20 mm angioplasty balloon catheter (Powerflex Pro, Cordis) was advanced over the wire and the hepatic and portal vein tract was dilated. The sheath was advanced into the dilated tract, and contrast was injected through the sheath to confirm intraportal position. A 0.035-inch, 260-cm long hydrophilic wire (Glide-wire Advantage, Terumo, Japan) and 5-Fr Berenstein catheter was then introduced through the transjugular sheath and manipulated into the MPV and then into the superior mesenteric vein (Figure 3). The transhepatic-transjugular wire was removed at this stage, and the 6-Fr sheath was replaced with a 10-Fr sheath. The procedure was completed as a conventional TIPS with deployment of a 10 mm × 70 mm (Viatorr, WL Gore and Associates) TIPS endoprosthesis stent (Figure 4). The patient remained stable with an uncomplicated post-procedural course and was discharged after 3 d with a satisfactory baseline post-TIPS ultrasound.

We feel the adoption of techniques such as ours and that of other authors described in the literature using an ultrasound-guided percutaneous transhepatic approach with a small caliber needle provides a safer and less traumatic procedure and should reduce complications of bleeding and almost completely eliminate the risk of liver laceration. Finally, our procedure was successfully performed under conscious sedation rather than general anaesthesia further reducing the overall procedural risk to the patient.

In conclusion, the authors should be commended for publication of this important complication of liver laceration following TIPS, which remains one of the most technically challenging interventional procedures but we hope that adoption of a microneedle controlled approach will potentially eliminate such complications.



Figure 1 The right portal vein was punctured with a 22-gauge, 20-cm Chiba needle (Cook) close to the bifurcation of the main portal vein, with entry confirmed by aspiration of blood.



Figure 3 A 0.035-inch, 260-cm long hydrophilic guidewire (Glidewire, Boston Scientific) and 5 Fr Berenstein catheter was then introduced through the transjugular sheath and manipulated into the main portal vein and then into the superior mesenteric vein.



Figure 2 A 25-mm diameter Amplatz Gooseneck Snare (ev3 Inc) was used to snare the transhepatic wire, achieving through and through access.



Figure 4 The procedure was completed as conventional transjugular intrahepatic portosystemic shunt with deployment of a 10 mm x 70 mm (Viatorr, WL Gore and Associates) transjugular intrahepatic portosystemic shunt endoprosthesis stent.

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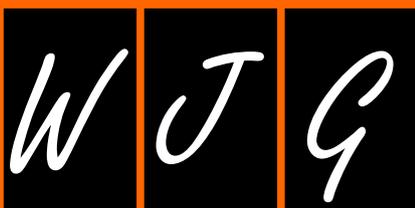
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# World Journal of *Gastroenterology*

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|                         |             |  |
|-------------------------|-------------|--|
| <b>EDITORIAL</b>        | <b>3531</b> | Microscopic colitis: A therapeutic challenge<br><i>Guslandi M</i>  |
| <b>REVIEW</b>           | <b>3534</b> | Liver-spleen axis: Intersection between immunity, infections and metabolism<br><i>Tarantino G, Scalera A, Finelli G</i>  |
|                         | <b>3543</b> | Evaluation of hepatic cystic lesions<br><i>Lantinga MA, Gevers TJG, Drenth JPH</i>   |
| <b>MINIREVIEWS</b>      | <b>3555</b> | Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review<br><i>Liao YF, Zhu W, Li DP, Zhu X</i>  |
| <b>ORIGINAL ARTICLE</b> | <b>3562</b> | Virulence factors of <i>Enterococcus</i> strains isolated from patients with inflammatory bowel disease<br><i>Golińska E, Tomusiak A, Gosiewski T, Więcek G, Machul A, Mikolajczyk D, Bulanda M, Heczko PB, Strus M</i>  |
|                         | <b>3573</b> | Aberrant glycosylation of the anti-Thomsen-Friedenreich glycotope immunoglobulin G in gastric cancer patients<br><i>Kodar K, Izotova J, Klaamas K, Sergeyev B, Järvekülg L, Kurtenkov O</i>  |
|                         | <b>3583</b> | Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption <i>via</i> a TNF- $\alpha$ -regulated mechanism<br><i>Shen ZY, Zhang J, Song HL, Zheng WP</i>                              |
| <b>BRIEF ARTICLE</b>    | <b>3596</b> | Risk factors for colonoscopic perforation: A population-based study of 80118 cases<br><i>Hamdani U, Naeem R, Haider F, Bansal P, Komar M, Diehl DL, Kirchner HL</i>  |
|                         | <b>3602</b> | Evaluation of enterochromaffin cells and melatonin secretion exponents in ulcerative colitis<br><i>Chojnacki C, Wiśniewska-Jarosińska M, Kulig G, Majsterek I, Reiter RJ, Chojnacki J</i>  |
|                         | <b>3608</b> | Diagnostic value of endothelial markers and HHV-8 staining in gastrointestinal Kaposi sarcoma and its difference in endoscopic tumor staging<br><i>Nagata N, Igari T, Shimbo T, Sekine K, Akiyama J, Hamada Y, Yazaki H, Ohmagari N, Teruya K, Oka S, Uemura N</i> |

- 3615** Gastric precancerous lesions are associated with gene variants in *Helicobacter pylori*-susceptible ethnic Malays  
*Maran S, Lee YY, Xu S, Rajab NS, Hasan N, Syed Abdul Aziz SH, Majid NA, Zilfalil BA*
- 3623** *XPC* Lys939Gln polymorphism, smoking and risk of sporadic colorectal cancer among Malaysians  
*Ahmad Aizat AA, Siti Nurfatimah MS, Aminudin MM, Ankathil R*
- 3629** Role of international criteria in the diagnosis of autoimmune hepatitis  
*Abdollahi MR, Somi MH, Faraji E*
- 3634** Focal autoimmune pancreatitis: Radiological characteristics help to distinguish from pancreatic cancer  
*Sun GF, Zuo CJ, Shao CW, Wang JH, Zhang J*
- 3642** Fast-track surgery could improve postoperative recovery in radical total gastrectomy patients  
*Feng F, Ji G, Li JP, Li XH, Shi H, Zhao ZW, Wu GS, Liu XN, Zhao QC*
- 3649** Hepatocellular carcinoma: Clinical study of long-term survival and choice of treatment modalities  
*Wu KT, Wang CC, Lu LG, Zhang WD, Zhang FJ, Shi F, Li CX*
- 3658** Analysis of long non-coding RNA expression profiles in gastric cancer  
*Cao WJ, Wu HL, He BS, Zhang YS, Zhang ZY*
- 3665** Double-balloon enteroscopy in small bowel tumors: A Chinese single-center study  
*Chen WG, Shan GD, Zhang H, Li L, Yue M, Xiang Z, Cheng Y, Wu CJ, Fang Y, Chen LH*
- 3672** Case-matched comparison of laparoscopy-assisted and open distal gastrectomy for gastric cancer  
*Wang W, Chen K, Xu XW, Pan Y, Mou YP*

**META-ANALYSIS**

- 3678** Diagnostic accuracy of endoscopic ultrasound in pancreatic neuroendocrine tumors: A systematic review and meta analysis  
*Puli SR, Kalva N, Bechtold ML, Pamulaparthi SR, Cashman MD, Estes NC, Pearl RH, Volmar FH, Dillon S, Shekleton MF, Forcione D*

**CASE REPORT**

- 3685** Endoscopic transluminal pancreatic necrosectomy using a self-expanding metal stent and high-flow water-jet system  
*Hritz I, Fejes R, Székely A, Székely I, Horváth L, Sárkány Á, Altorjay Á, Madácsy L*

- 3693** Polyarteritis nodosa clinically mimicking nonocclusive mesenteric ischemia  
*Shirai T, Fujii H, Saito S, Ishii T, Yamaya H, Miyagi S, Sekiguchi S, Kawagishi N, Nose M, Harigae H*
- 3699** Esophageal mucosal metastasis from adenocarcinoma of the distal stomach  
*Ki SH, Jeong S, Park IS, Lee DH, Lee JI, Kwon KS, Kim HG, Shin YW*
- 3703** Gastric body diaphragm-like stricture as a rare complication of nonsteroidal anti-inflammatory drugs  
*Wu LL, Yang YS, Cai FC, Wang SF*
- 3707** Ileocecal endometriosis and a diagnosis dilemma: A case report and literature review  
*Tong YL, Chen Y, Zhu SY*

- LETTERS TO THE EDITOR 3711** Response to Abadi and Kusters, *World J Gastroenterol* 19: 429-430  
*Rafiei A, Gilbreath JJ, Merrell DS*

**APPENDIX** I-VI Instructions to authors

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**INDEXING/ABSTRACTING** *World Journal of Gastroenterology* is now indexed in Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®), Journal Citation Reports®, Index Medicus, MEDLINE, PubMed, PubMed Central, Digital Object Identifier, and Directory of Open Access Journals. ISI, Journal Citation Reports®, Gastroenterology and Hepatology, 2011 Impact Factor: 2.471 (32/74); Total Cites: 16951 (7/74); Current Articles: 677 (1/74); and Eigenfactor® Score: 0.06035 (5/74).

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 25/F, Lucky Plaza,  
315-321 Lockhart Road, Wan Chai, Hong Kong, China

Fax: +852-65557188  
Telephone: +852-31779906  
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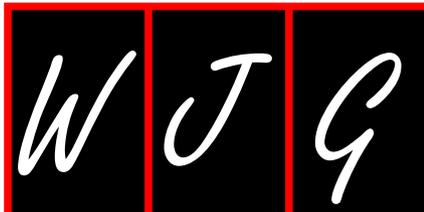
**PUBLICATION DATE**  
June 21, 2013

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## Microscopic colitis: A therapeutic challenge

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Received: January 18, 2013 Revised: April 11, 2013  
Accepted: April 28, 2013  
Published online: June 21, 2013

### Abstract

The treatment of microscopic colitis is mainly based on the use of budesonide, the only drug found effective in controlled clinical trials. After an initial course at a dose of 9 mg daily, however, most patients relapse when the drug is discontinued, hence a maintenance therapy at doses of 6 mg daily or lower is necessary. In order to avoid steroid dependence and drug toxicity different pharmacological agents should be considered as an alternative to indefinite long-term budesonide treatment. Evidence-based guidelines are currently lacking due to the lack of conclusive data concerning the use of either immunosuppressive or anti-tumor necrosis factor agents. For the time being in clinical practice the skilled physician should therefore tailor long term management of microscopic colitis on the single patient.

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**Key words:** Microscopic colitis; Budesonide; Mesalazine; Immunosuppressants

**Core tip:** The efficacy of short-term treatment of microscopic colitis with budesonide is confirmed. Long-term therapy is not advisable because of possible side effects, but the efficacy of alternative drugs such as immunosuppressants or anti-tumor necrosis factor agents remains to be established. For the time being prolonged budesonide treatment in minimal doses, tailored

on the single patient, appears to be the most sensible option.

Guslandi M. Microscopic colitis: A therapeutic challenge. *World J Gastroenterol* 2013; 19(23): 3531-3533 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3531.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3531>

### MICROSCOPIC COLITIS

Microscopic colitis (MC) is an intestinal inflammatory disorder the diagnosis of which relies on specific histopathological findings, namely an increased number of lymphocytes in the colonic epithelium and of subepithelial chronic inflammatory cells (lymphocytic colitis), in some cases with a thickening of the subepithelial layer (collagenous colitis)<sup>[1,2]</sup>.

Radiographic and endoscopic features are constantly normal. The main symptom is chronic watery diarrhea without bleeding, the disease being more common among older individuals, especially of female gender. Although a genetic cause has not been proven, familial occurrence has been reported.

Smoking is a risk factor and bile acid malabsorption is frequent (however, a bile acid binding drug such as cholestyramine may improve symptoms but not histopathology)<sup>[1-3]</sup>.

Medications such as nonsteroidal antiinflammatory drugs, proton pump inhibitor, ticlopidine, sertraline *etc.* can induce MC<sup>[4]</sup>. Hence, accurate information about pharmacological treatment history is mandatory in order to discontinue the supposedly responsible drug.

The only medication found effective in randomized, placebo-controlled trials is budesonide, which, at a dose of 9 mg per day is able to induce clinical remission and histological improvement in about 81% of cases<sup>[5]</sup>. The superior efficacy of budesonide compared with placebo has been shown in four controlled trials involving patients with either collagenous or lymphocytic form of MC<sup>[6-9]</sup>.

By contrast only a small trial comparing prednisolone and placebo for two weeks reported a trend toward clinical response<sup>[10]</sup>. At any rate, budesonide should be preferred to other steroids not only because of fewer side effects, but also because the success rate is higher and the incidence of clinical relapses is lower<sup>[11,12]</sup>.

When budesonide is withdrawn, symptomatic relapse of MC can occur in 60%-80% of cases<sup>[13,14]</sup>. In order to maintain remission budesonide can be successfully administered at a dose of 6 mg daily, up to six months<sup>[14,15]</sup>. After that period there is no published evidence that the drug continues to be effective, but clinical practice shows that budesonide 3-6 mg daily can prevent recurrence, although patients become at risk of becoming steroid dependent and to develop side effects due to long-term steroid therapy. The minimum dose of budesonide should be employed, even 3 mg every other day being occasionally sufficient to maintain clinical remission (Guslandi M, unpublished data), but in order to avoid steroid dependence and drug toxicity other therapeutic options must be considered.

Mesalazine, which is usually well tolerated, would represent an ideal long-term treatment, but evidence of its efficacy in MC is weak, retrospective series reporting benefit in fewer than half the patients as a short term therapy while data for periods exceeding 6 mo are lacking<sup>[16,17]</sup>.

Immunosuppressive agents can be taken into consideration, both in patients with severe symptoms who do not respond to full doses of budesonide or who are experiencing side effects and/or steroid dependence during long-term budesonide treatment. Unfortunately available data with either azathioprine (or 6-mercaptopurine) and methotrexate in MC are extremely limited and inconclusive<sup>[18-20]</sup> despite their not infrequent use in clinical practice by gastroenterologists.

In the attempt to avoid colectomy in severe cases of MC refractory to any other pharmacological treatment, the possible use of biological agents has been tested, with promising results<sup>[21,22]</sup> but more conclusive data are needed.

Thus, long-term management of microscopic colitis remains elusive, especially in patients refractory or intolerant to budesonide, but even in subjects where the drug is effective but continuous intake for an indefinite length of time is not advisable. In those cases physicians must take therapeutic decisions irrespective of evidence-based data, tailoring the treatment on the characteristics and needs of the single patient. Even a recent treatment algorithm proposed by the European Microscopic Colitis Group<sup>[23]</sup> includes drugs such as loperamide, mesalazine, cholestyramine and bismuth, the efficacy of which is questionable and uncertain, pointing out the fact that the use of those medication is empirical. The same applies to immunosuppressants and anti-tumor necrosis factor agents, although the former, in spite of the scarce controlled data, appear to be a sensible and comparatively safe approach. Needless to say, randomized, controlled

studies with azathioprine or methotrexate are eagerly awaited and sorely needed.

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## Liver-spleen axis: Intersection between immunity, infections and metabolism

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Received: February 21, 2013 Revised: May 10, 2013

Accepted: May 18, 2013

Published online: June 21, 2013

### Abstract

Spleen has been considered a neglected organ so far, even though is strictly linked to liver. The spleen plays an important role in the modulation of the immune system and in the maintenance of peripheral tolerance *via* the clearance of circulating apoptotic cells, the differentiation and activation of T and B cells and production of antibodies in the white pulp. Moreover, splenic macrophages are able to remove bacteria from the blood and protect from sepsis during systemic infections. We review the spleen function and its assessment in humans starting from the description of spleen diseases, ranging from the congenital asplenia to secondary hyposplenism. From the literature data it is clear that obesity in humans affects different compartments of immune system, even though there are still few data available on the implicated mechanisms. The intent is to enable clinicians to evaluate the newly recognized role of metabolic and endocrine functions of the spleen with special emphasis to obesity and nonalcoholic fatty liver disease in the context of the available literature.

Moreover, understanding the spleen function could be important to develop appropriate prevention strategies in order to counteract the *pandemia* of obesity. In this direction, we suggest spleen longitudinal diameter at ultrasonography, as simple, cheap and largely available tool, be used as new marker for assessing splenic function, in the context of the so-called liver-spleen axis.

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**Key words:** Spleen size; Obesity; Non-alcoholic fatty liver disease

**Core tip:** From the literature data it is clear that obesity in humans affects different compartments of immune system. The aim of this review is to let clinicians appreciate the new role of metabolic and endocrine functions of the spleen with special emphasis to obesity and nonalcoholic fatty liver disease in the context of the available literature. Moreover, understanding the spleen function could be important to develop appropriate prevention strategies in order to counteract the *pandemia* of obesity. In this direction, we suggest spleen longitudinal diameter at ultrasonography, as simple, cheap and largely available tool, be used as new marker for assessing the liver/spleen axis.

Tarantino G, Scalera A, Finelli G. Liver-spleen axis: Intersection between immunity, infections and metabolism. *World J Gastroenterol* 2013; 19(23): 3534-3542 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3534.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3534>

### INTRODUCTION

In vertebrate evolution, spleen functions were performed by a spleen-like tissue scattered along the digestive tract, as seen in lamprey. Bony fishes and sharks are the first

vertebrates where it appears as individual organ<sup>[1]</sup>.

The spleen is a secondary peripheral lymphoid organ located in the abdominal cavity between the diaphragm and the fundus of the stomach of mammals. Its principal function was preserved during the evolution in all animal classes having that organ, while important differences can be observed histologically. For example, the red pulp is seen only from bony fishes upwards.

It is the largest lymphoid organ in the human body and it has a fundamental role as destruction of red blood cells and as actor in the immune response, filtering the blood from antigenic particles and from abnormal and aged cells. Table 1 summarizes the different functions of the spleen.

The spleen anatomical architecture is extremely sophisticated and little is still known about specific processes that are performed in its differentiation. Mesenchymal, hematopoietic and endothelial cells interact each other thanks to complex, organized and still undiscovered signals leading to the development of its complex micro-architecture<sup>[2-4]</sup>.

## WHAT EVIDENCE HAS SUGGESTED SPLEEN BE CONSIDERED A NEGLECTED ORGAN?

The congenital asplenia may occur with or without other clinically evident abnormalities. In the first case, with asplenia, other defects of organs of the thoracic and abdominal cavities can be found. One example is the heterotaxy syndrome, where there is a failure in the left-right axis specification<sup>[5]</sup>.

If the defect occurs before the ontogenesis of the spleen on the left side, it may not affect splenic development. The second type of congenital asplenia is less common<sup>[6-10]</sup> and includes subjects with no other obvious abnormalities that report recurrent infections from childhood. In those cases the diagnosis of asplenia often remains unravelled, due to the lack of necroscopy.

Studies in mice have highlighted that some genes are crucial for spleen development, such as *Tcf21*, *Bapx1*, *Pbx1*<sup>[11]</sup> and recently also *Tlx1*<sup>[12]</sup>. In this case it can be expected that asplenic animals suffer from additional several anomalies caused by the deficiency of specific genes. However, in the literature are not reported corresponding cases, probably because in humans and mice similar genes do not have overlapping functions, or these subjects die before or soon after birth and/or they were not extensively investigated.

A suitable example may be the Atrx syndrome, where the mutations of this gene result in athalassemia, myelodysplasia and mental retardation<sup>[13]</sup>. Individuals with this syndrome occasionally exhibit asplenia<sup>[14]</sup>, but the inactivation of Atrx similar gene in mice does not end up in asplenia<sup>[15]</sup>.

In individuals with congenital isolated asplenia are reported some mutations<sup>[16]</sup>, but the molecular mecha-

**Table 1 Function of the spleen**

|  |
|--|
| Red pulp   |
| Extramedullary hematopoiesis if required   |
| Facilitating an environment wherein erythrocytes rid themselves of solid waste material                              |
| Blood filter for foreign material and damaged and senescent blood cells  |
| Storage site for iron, erythrocytes, platelets, plasmablasts and plasma cells  |
| Rapid release of antigen-specific antibodies into the circulation produced by red pulp plasma cells                  |
| Defense against bacteria using iron metabolism by its macrophages  |
| White pulp   |
| T cell zone (periarterial lymphatic sheath) and B cell zone (follicles)  |
| Storage site for B and T lymphocytes   |
| Development of B and T lymphocytes upon antigenic challenge  |
| Release of immunoglobulins upon antigenic challenge by B lymphocytes   |
| Production of immune mediators involved in clearance of bacteria such as complement, opsonins, properdin and tuftsin |
| Marginal zone  |
| Phagocytosis of circulating microorganisms and immune complexes by MZ macrophages                                    |
| Development of marginal zone B lymphocytes upon TI-2 antigenic challenge   |
| Blood trafficking of B and T lymphocytes   |
| Release of immunoglobulins upon antigenic challenge by splenic B lymphocytes   |

nisms and the etiology of spleen development are still unknown.

## ANATOMICAL AND HISTOLOGICAL COMPOSITION OF THE SPLEEN (ANIMAL VS HUMAN MODEL)

In the mice the spleen has a characteristic histological organization similar to a sponge, where the fibrous capsule form a reticular network with the trabeculae stemming from its internal side. The splenic artery enters the hilum of the spleen, divides itself into smaller branches and finally gives rise to “central arteriolar” of the white pulp and to the large sinusoids of the red pulp. The central arteriolar are surrounded by a sheath of small T lymphocytes, the so-called PeriArteriolar Lymphoid Sheath (PALS). They communicate with follicles, a highly organized accumulation of T and B lymphocytes. The red pulp, PALS and follicles are also surrounded by the marginal zone, filled with large memory B cells.

The human and mice spleen are not anatomically different. The fact that patients with autoimmune thrombocytopenia purpura and circulating antiplatelet antibodies improve after splenectomy<sup>[17]</sup>, supports the role of the red pulp of the spleen in the displacement of old and damaged platelets, aged erythrocytes and apoptotic cells in humans.

After apoptosis of aged erythrocytes, hemoglobin is digested and iron is released or stored by splenic macrophages. Thus, the spleen is fundamental in the recycling of iron<sup>[18]</sup>.

Interestingly, after abdominal surgery for trauma or neoplasia, the displacement of the spleen is often without immediate consequences. This is one of the reasons for which, until a recent past, the spleen was considered not a vital organ. Consequently, it was believed that the spleen could be removed without major consequences. Recently, Ozban *et al.*<sup>[19]</sup> have disproved this theory, because they have shown that exercise in splenectomized individuals can cause serious problems in form of decreasing splanchnic flow and increasing blood viscosity.

The spleen plays an important role in the modulation of the immune system and in the maintenance of peripheral tolerance *via* the clearance of circulating apoptotic cells, the differentiation and activation of T and B cells and production of antibodies in the white pulp<sup>[20,21]</sup>. Moreover, splenic macrophages are able to remove bacteria from the blood and protect from sepsis during systemic infections.

*Vice versa*, most important differences between mice and humans in the spleen organization and functionality are revealed in the immune response. The marginal sinus in mice and the perifollicular zone in humans are areas of particular activity. B cells in the marginal zone in mouse are highly reactive and specialized against pathogens invasion *via* a T-independent reaction<sup>[20]</sup>, however in humans, the same area contains memory B cells<sup>[22]</sup>. Several chemokines and adhesion molecules regulate the trafficking between the marginal zone and the white pulp.

## ROLE OF SPLEEN IN LIMITING BACTERIAL INFECTION

As seen before, congenital asplenic subjects have an increased risk of developing infective diseases and the lack of the spleen functionality causes more startling effects in the newborn.

Morris *et al.*<sup>[23]</sup> firstly described in 1919 that splenectomized patients are more susceptible to infections, especially caused by *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitidis* (so called encapsulated bacteria)<sup>[23-25]</sup>. The risk of sepsis is 10- to 20-fold higher than non splenectomized population and, especially in young children, death can result<sup>[26]</sup>. Overwhelming post-splenectomy infection can occur some hours after the first signs of deterioration of health and can degenerate to multi-organ failure and death<sup>[27]</sup>. Most asplenic children die of infection during the neonatal period. In fact, among the causes of sudden and unexpected infant death, the congenital asplenia can also be included<sup>[28]</sup>.

Another condition is functional asplenia, when in patients with haematological or metabolic disorders the splenic tissue organization is altered and, for this reason, equally they develop the same type of infections. In patients with functional or anatomical asplenia is quite impossible to quantify the risk of developing infections and sepsis. Therefore, a method to conserve some splenic tissue during abdominal surgery with deracination of the spleen is to transplant small spleen fragments

into the well vascularised greater omentum. Clinical data have shown that this procedure has an important effect in increasing specific antibody responses after pneumococcal vaccination, as well as normalizing IgM levels<sup>[29,30]</sup>, and probably can also reduce the risk of opportunistic infections in immunodeficient subjects<sup>[31]</sup>. The increased susceptibility of hypo or asplenic individuals to encapsulated bacterial infections is mostly due to the lack of IgM memory B cells and to their not adherent reaction to polysaccharide vaccines. The absence of splenic macrophages with the reduced number of B cells in asplenic patients can result in the establishment of a favorable environment to the development of overwhelming bacterial infections.

## SPLEEN AND NATURAL ANTIBODIES

A particular subtype of B-cell population is involved in the immune deficiency and in the reduced response to polysaccharide antigens seen in the asplenic or splenectomized mice. B cells may be divided in two main subpopulations on the basis of life development (fetal or adult), surface markers and functions. Asplenic mice lack B-1a B cells<sup>[32]</sup>, a distinct population from the more conventional B-2 B cells that are involved in the adaptive immunity and collaborating with T cells<sup>[33]</sup>.

Functions of B-1a B cells are mainly three: (1) they can act in an T-cell independent mode during the immune response; (2) they produce natural antibodies and co-operate with the innate immune system to contrast bacterial and viral infections; and (3) in the intestinal mucosa they can differentiate into plasma cells producing IgA<sup>[34]</sup>.

In the specific immune response, produced antibodies have a very high affinity for a particular epitope and they can prevent re-infection from the single pathogen that previously has caused their own production. *Vice versa*, the pentameric IgM isotype produced by B-1a B cells binds various antigens with high avidity and low affinity and is therefore able to neutralize many different antigens. IgM antibodies are the so called “natural antibodies” and in recent years, it has been demonstrated that they may play a role in the protection against malignancy<sup>[35]</sup> and atherosclerosis<sup>[36]</sup>. Asplenic mice not only have a reduced number of B-1a B cells but they have a decreased concentration of serum IgM<sup>[32]</sup>.

B-1a B cells produce IgA immunoglobulins, and in the intestinal mucosa about half of the IgA plasma cells derives from B-1a B cells<sup>[37]</sup>. The homeostasis of the intestine is finely regulated by mucosal IgA. These immunoglobulins interact with antigens presented by the intestinal microbiota and by pathogens, preventing their overgrowth and subsequent invasion.

The precise reason why B-1a B cells are absent in asplenic mice and why their number rapidly declines after splenectomy is not yet defined. B-1a B cells are produced in the fetal liver, contrarily to B-2 B cells that derive from adult bone marrow. It is noteworthy to stress that the

first subtype cannot be replaced after adult bone marrow transplant. Moreover, Ig-positive precursors of B-1a B cells have been detected in the spleen, but it is unknown if these cells persist in the spleen during the adult life and derive from precursors situated in the fetal liver. According to a recent theory the spleen might be central to their generation or survival and therefore splenectomy would lead to the depletion of the B-1a population<sup>[32]</sup>.

## OTHER FUNCTIONS OF THE SPLEEN

An interesting hypothesis relates the spleen to the activity of gut-associated lymphoid tissue (GALT). The dysfunction of GALT is known to predispose to inflammatory bowel diseases (IBD), above all for its role in T cell activation and trafficking in the gut. Moreover, the frequency of IgM memory B cells is decreased in IBD subjects<sup>[38]</sup> establishing a relationship among GALT and spleen in humans.

The spleen also has important hematological functions. The spleen picks up from the circulation platelets that subsequently are stored or can be destroyed by lymphocytes. As storage organ the spleen stores about one third of the human body's platelets.

The thrombocytopaenia is a result of hypersplenism, because of the heightened functions of the spleen in sequestering and break-downing platelets. Conversely, after splenectomy mild thrombocytosis can be observed<sup>[39]</sup> because of the lack of sequestering and destruction of platelets by the spleen, and at the same time it can be observed a slight increase in platelet production in the bone marrow<sup>[40]</sup>. Erythrocytes are stored and removed from the blood circulation in this organ. After splenectomy, the presence in the blood of many substances released from circulating damaged erythrocytes with procoagulant activity can lead to the establishment of a procoagulant state and therefore to the occurrence of thromboembolic events (for example pulmonary embolism, deep vein and portal vein thrombosis).

Beyond haematopoietic stem cells, stem cells of other differentiation lines, such as stromal cells with osteogenic differentiation properties, seem to be present in the spleen, confirmed by *in vitro* studies<sup>[41]</sup>. It would be interesting to explore if in the spleen these osteogenic precursors may represent a monocyte/macrophage lineage common precursor cell population with the ability to differentiate along the osteoclast lineage<sup>[42]</sup>. Animal studies have also shown that splenocytes can differentiate into pancreatic islets and ductal epithelial cells when injected into diabetic non-obese diabetic mice, thereby splenocytes may be useful in the treatment of type 1 diabetes, thanks to their ability of restoring normal glycaemia<sup>[43,44]</sup>. Subsequently, Chong *et al.*<sup>[45]</sup> have questioned the origin of these stem cells.

## ASSESSMENT OF SPLEEN FUNCTION

Over the years, several methods have been developed to

study the activity of the spleen. Because of its ability in purifying the blood from old erythrocytes, the amount of altered red blood cells can be used as index of functionality of the spleen. The detection of Howell-Jolly bodies is one of these, although the sensitivity and specificity are questionable for the hyposplenism<sup>[46,47]</sup>. Other haematological parameters are finding membranes pits, large vacuoles situated near to the plasma membrane, or other cellular changes as acanthocytes, target cells, Heinz bodies (remnants hemoglobin), Pappenheimer bodies (iron granulocytes) and siderocytes<sup>[48]</sup>.

The count of B cells derived from the marginal zone, which have fundamental action in innate immunity, and in particular as defense against the invasion by encapsulated bacteria, is otherwise a possible method for evaluating the immunological activity of spleen<sup>[49]</sup>. B cells derived from the marginal zone and the memory cells producing IgM are in fact reduced in patients with diminished splenic function<sup>[38]</sup>.

All the tests described above could be used with ease in clinical practice, on the other hand they have proved not to be very sensitive and specific, or they are needed to be further studied and validated<sup>[46,50]</sup>.

To date, the radioisotope method is definitely the best way to quantify the filtering activity of the spleen. The (99m) Tc-labeled, heat-altered, autologous erythrocyte scintigraphy with multimodality single photon emission computed tomography (CT) - technology is considered the best approach to gauge all the facets of the splenic function<sup>[51]</sup>. However, this is a method that has the highest cost and it is difficult to perform.

## SPLEEN AS A NEW PLAYER

Nonalcoholic Fatty Liver Disease (NAFLD), the most common cause of liver steatosis, is associated with obesity, mainly visceral type, and insulin resistance. The liver inflammation (nonalcoholic steatohepatitis, the so called NASH) can progress from the simple hepatic steatosis or fatty liver (FL) lasting risk factors, as type 2 diabetes mellitus, major obesity and Metabolic Syndrome (MS). In its natural history, NASH can end up in perisinusoidal fibrosis and cirrhosis. Hepatocytes, during steatosis, are fat-laden and swollen, and in steatohepatitis the hydropic change (ballooning) causes further swelling and also sinusoidal distortion, as visualized by *in vivo* microscopy studies. This evenience leads to the reduction of intrasinusoidal volume and microvascular blood flow, as clearly described by Farrell *et al.*<sup>[52]</sup>. Sinusoidal endothelial cells, Kupffer cells and stellate cells are also involved in the pathological process in conjunction with the activation of the immune system. The microcirculation is skewed by inflammatory cells and platelets recruited in the liver. Animal models confirm these data and evidence that these pathological changes lead to a marked reduction of sinusoidal space (approximately 50% of control), and a decrease in the number of normally perfused sinusoids, according to a review, recently published<sup>[51]</sup>. The micro-

vascular damage is necessary for developing further liver injury and causing disease progression as in NASH. The lipid peroxidation of unsaturated fatty acids by reactive oxygen species is one of the main causes of the sensitivity of hepatic steatosis to ischemia-reperfusion injury. During the whole 24-h-period the most part of time is spent in postprandial state in humans. Therefore, the liver has a fundamental role in maintaining the correct energy state balancing the input, secretion, and oxidation of fatty acids. In abdominally obese men the oxidation of dietary fatty acids, hepatic desaturation and elongation of palmitic acid occur to a greater extent than in non-obese<sup>[53]</sup>.

This means, therefore, that donor's fatty livers are an obstacle to transplantation<sup>[52]</sup>. Between other hepatic cells, the dysfunction of Kupffer cells gives a major contribution to NASH progression. It is noteworthy that the reticular-endothelial system also plays a key role in the spleen and a good method for study Kupffer cell activity is the colloid scintigraphy. Duman *et al*<sup>[54]</sup> have followed 22 patients with biopsy-proven NASH who underwent colloid liver scintigraphy. Liver right/left lobe ratio was altered in all patients after intravenous injection of 185 MBq Tc tin colloid. The shift of colloid to the spleen and a prolonged blood pool clearance time was observed in 55% of patients with NASH.

Previously, Tsushima *et al*<sup>[55]</sup> aimed to determine if there was an association between spleen enlargement and NAFLD, measuring spleen volume at CT. It must be observed that the values were weighted according to the patient's demographic data, the Liver/Spleen (L/S) ratio of CT Hounsfield unit measurements, and liver function tests. L/S ratio was also used to perform the diagnosis. The authors evidenced an increased mean spleen volume ( $P < 0.0001$ ) between NAFLD and controls  $73.0 \pm 24.4 \text{ cm}^3$  (range, 21.1-106.1) in normal subjects and  $141.2 \pm 54.1 \text{ cm}^3$  (range, 44.1-267.3) in NAFLD subjects. Only the L/S ratio ( $P < 0.0001$ ) and age ( $P < 0.01$ ) were significantly correlated to spleen volume at multivariate linear regression analysis and at forward selection stepwise regression.

Basing on the evidence that obesity and insulin resistance are inflammatory chronic diseases and therefore are associated with systemic markers of inflammation, some scholars have attempted to find a non invasive diagnostic method for NASH to help clinicians to decide whether and when to perform liver biopsy.

Patients with histology proven-NAFLD (43 patients with NASH and 40 with FL), compared with healthy subjects, were evaluated with ultrasonographic exams, with particular interest to ultrasonographic spleen longitudinal diameter (SLD) and splenic artery resistive index, and laboratory measurements, as serum interleukin (IL)-6 and vascular endothelial growth factor (VEGF) concentrations. The NASH group demonstrated higher IL-6 blood levels, SLD values, and VEGF concentrations than controls. In this study was estimated that the SLD is more sensitive than IL-6 and VEGF in discriminating NASH from FL, and the optimal cut-off value for SLD is 116 mm (specificity 95% and sensitivity 88%). NASH and FL

subjects have a similar splenic artery resistive index, but it differs when compared with controls. On the other hand, normal values of SLD and IL-6 were associated with FL and normal values of IL-6 could confirm the absence of NASH<sup>[56]</sup>. Further confirmation of these findings comes from another study which highlighted that spleen enlargement may be a distinct feature of NASH, especially early-stage NASH<sup>[57]</sup>. Therefore, we suggest that SLD could be used as new marker for assessing splenic function, independently from its use in distinguishing the simple FL, also called benign, from NASH, the more severe form of NAFLD, benignity not always shared<sup>[58]</sup>.

In this study<sup>[59]</sup>, SLD and blood pressure were significantly correlated with insulin resistance, moreover measures of SLD were well predicted by body mass index values.

To let Authors duplicate this finding, SLD was measured by postero-lateral scanning. It was used the average value obtained by measuring the maximum length and the cranio-caudal diameter. All the indices were measured thrice.

A subsequent study showed that spleen enlargement was found at significant levels (38%) in obese female rats as determined by Cavalieri volume calculation, an unbiased stereological method<sup>[60]</sup>. These recent results clearly indicated that high fat diet caused splenomegaly *via* sinusoidal dilatation and intracellular or intercellular deposits<sup>[61]</sup>. Although these data are encouraging to find a non-invasive method for NAFLD diagnosis, liver biopsy remains the only reliable method to differentiate simple steatosis or FL from NASH in NAFLD subjects<sup>[58]</sup>. On this line, Kikuchi *et al*<sup>[62]</sup> evaluate the efficacy of non-invasive (99m) Tc-phytate scintigraphy in the diagnosis of NASH in humans and in a rat model. In the first study, patients with suspected NAFLD underwent liver biopsy and (99m) Tc-phytate scintigraphy. As region of interest, signal intensities of the liver and spleen were measured. Subsequently, they observed that the L/S uptake ratio at scintigraphy was significantly decreased in NASH subjects when compared to patients with FL. The L/S ratio was an independent predictor in distinguishing NASH from FL. More interestingly, the decrease of L/S ratio was found in all NASH stages, from its earliest stages (stages 1 and 0). In the second study, the authors induced NASH in rats feeding them with a Methionine- and Choline-Deficient (MCD) diet. In this case, the L/S uptake ratio was also significantly decreased after 8 wk of a MCD diet in comparison with control diet-fed rats. From these data, the authors concluded that non-invasive (99m) Tc-phytate scintigraphy is able to discriminate NASH from FL.

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## INFECTIONS TENDENCY IN OBESITY AND THE POSSIBLE LINK WITH THE SPLEEN

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The frequency of ischemic heart disease observed after traumatic splenectomy and the low cholesterol levels

Table 2 Main topics

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| Congenital asplenia in humans  |  |
| There are two types of congenital asplenia: with or without other clinically evident abnormalities   |  |
| Tcf21, Bapx1, Pbx1 and Tlx1 are crucial for spleen development   |  |
| The molecular mechanisms and the etiology of spleen development are still unknown  |  |
| How the anatomical and histological composition of the spleen can guarantee its function?  |  |
| The phagocytosis of old and damaged cells, particles and blood-borne microorganisms from local macrophages takes place in the red pulp                               |  |
| The spleen is fundamental in the recycling of iron   |  |
| Exercise in splenectomized individuals can decrease splanchnic flow and increase blood viscosity   |  |
| Most important differences between mice and humans in the spleen organization and functionality are revealed in the immune response                                  |  |
| Role of spleen in limiting bacterial infection   |  |
| Splenectomized and asplenic patients are more susceptible to infections, especially caused by <i>Haemophilus influenzae</i>  |  |
| Subjects with functional asplenia develop the same type of infections  |  |
| The spleen and natural antibodies  |  |
| B cells may be divided in two main subpopulations on the basis of life development (fetal or adult), superficie markers and functions                                |  |
| Spleen might be central to the generation or survival of the B-1a population and therefore splenectomy would lead to their depletion                                 |  |
| Other functions of the spleen  |  |
| There is a probable relationship among GALT and spleen in humans   |  |
| The spleen also has important hematological functions  |  |
| In the spleen were found stem cells with several differentiation properties: haematological, osteogenic and maybe pancreatic   |  |
| Assessment of spleen function  |  |
| Hematological and immunological parameters should be used in the assessment of spleen function   |  |
| The best approach to gauge all the facets of the splenic function is the radioisotope method   |  |
| Spleen as a new player   |  |
| There is an association between spleen enlargement and NAFLD   |  |
| SLD could be used as new marker for assessing splenic function   |  |
| Initial data have shown that SLD is more sensitive than IL-6 and VEGF in discriminating NASH from FL, and the optimal cut-off value for SLD is 116 mm                |  |
| Infections tendency in obesity and the possible link with the spleen   |  |
| Obese subjects have an increased risk to develop malignancies and infections   |  |
| The pathophysiological mechanisms by which cellular immune functions are affected by obesity are still under investigation but the spleen may have an important role |  |

GALT: Gut-associated lymphoid tissue; NAFLD: Nonalcoholic fatty liver disease; SLD: Spleen longitudinal diameter; VEGF: Vascular endothelial growth factor; IL-6: Interleukin-6; NASH: Nonalcoholic steatohepatitis.

found in patients with hypersplenism are observations that suggest a possible role for the spleen in lipid metabolism and in the etiology of atherosclerosis<sup>[63]</sup>. Previous studies showed that obese subjects, compared to non-obese, have an increased risk to develop cardiovascular disease, hypertension, cerebrovascular disease and type 2 diabetes mellitus. But, it is equally important that they have an impaired immune function, as demonstrated by the higher incidence of malignancies and infections. From the literature data it is clear that obesity in humans affects different compartments of immune system, even though there are still few data available on the implicated mechanisms. Elderly people (> 60 years of age) have

an increased risk of infection, showing their peripheral blood lymphocytes a decreased reactivity to mitogens and an impaired proliferative capacity<sup>[64,65]</sup>. The response of T lymphocytes to concanavalin A and response of B lymphocytes to pokeweed mitogen are decreased in obese subjects<sup>[66]</sup>. In addition to the T lymphocyte population, also natural killer cell activity is suppressed in obese men and women > 60 years of age, as mentioned in a report made by Moriguchi *et al*<sup>[66,67]</sup>. Moreover, the natural killer cells activity and percentage of body fat are negatively correlated in both elderly women<sup>[68]</sup>, and middle-aged men<sup>[69]</sup>. These data suggest that obesity is a risk factor for the progressive deteriorating of cellular immune functions. The pathophysiological mechanisms by which cellular immune functions are affected by obesity are still under investigation but the spleen may have an important role. In the splenic lymphocytes of obese mice, the expression of glucose transporter 1 (GLUT-1), analyzed by Western blot analysis, was lower compared to lean rats. The decreased expression of GLUT-1 in these rats is associated with a defective uptake of glucose into immune cells. It is probable that the decreased proliferation of splenic lymphocytes in obese rats is connected to the decreased expression of GLUT-1 and therefore to an impairment of glucose uptake<sup>[69]</sup>. An interesting report by Miyake *et al*<sup>[70]</sup> evaluates NAsFLD mice fed high-fat and high-calorie diet for 12 wk for assessing the extent of antigen-specific immunity response. NAFLD mice and control mice were immunized with hepatitis B vaccine containing hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) and, subsequently, antibody to HBsAg (anti-HBs) blood levels, HBsAg and HBcAg-specific cellular immune response and functions of whole spleen cells, T lymphocytes, B lymphocytes and spleen dendritic cells (DCs) of NAFLD and control mice were assessed *in vitro*. Interestingly, in NAFLD mice levels of anti-HBs and the proliferation activity of HBsAg and HBcAg-specific lymphocytes were significantly lower compared to controls. Higher levels of inflammatory cytokines were produced and T cells have showed an increased proliferation rate in spleen cells of NAFLD than lean mice. Concurrently, DCs processing and presenting antigen activities were significantly decreased in the spleen of NAFLD mice compared to controls. Moreover, the administration of saturated fatty acids caused impaired antigen processing and presenting capacity of murine DCs. These data emphasize that the modification of antigen-specific immunity in NAFLD mice depends on the action different types of immunocytes, including DCs and lymphocytes, clarifying the role of the spleen in this specific pathological process.

## FUTURE PERSPECTIVES

This paper reports the studies on the use of simpler parameters in assessing the need for medical intervention with respect to healthy and non healthy overweight/obese individuals.

In a not too distant past, the spleen has been considered a neglected and expendable organ. In portal hypertension it was considered an ancillary organ<sup>[71-74]</sup> and it has some importance in infectious disease or as organ localization in lymphoproliferative diseases.

As described before, Table 2, now the spleen is deemed an important component of the immune system, crucial in immune response regulation<sup>[55,75,76]</sup>, and also it has a metabolic asset and it is involved in endocrine function with regard to NAFLD<sup>[51]</sup>.

It is suggested that adoption of a simpler tool to perform measurements could not only reduce the cost of medical care but also provide more reliable identification of patients in need of weight loss<sup>[77-80]</sup>.

Larger and well-implanted studies comprehending better characterized patients should be taken into account to ascertain the validity of this tool.

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**P- Reviewers** Czaja MJ, Maleki I, Mahta MA, Nagarajan P  
**S- Editor** Wen LL **L- Editor** A **E- Editor** Zhang DN



## Evaluation of hepatic cystic lesions

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Received: January 20, 2013 Revised: March 5, 2013

Accepted: March 22, 2013

Published online: June 21, 2013

### Abstract

Hepatic cysts are increasingly found as a mere coincidence on abdominal imaging techniques, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI). These cysts often present a diagnostic challenge. Therefore, we performed a review of the recent literature and developed an evidence-based diagnostic algorithm to guide clinicians in characterising these lesions. Simple cysts are the most common cystic liver disease, and diagnosis is based on typical USG characteristics. Serodiagnostic tests and microbubble contrast-enhanced ultrasound (CEUS) are invaluable in differentiating complicated cysts, echinococcosis and cystadenoma/cystadenocarcinoma when USG, CT and MRI show ambiguous findings. Therefore, serodiagnostic tests and CEUS reduce the need for invasive procedures. Polycystic liver disease (PLD) is arbitrarily defined as the presence of > 20 liver cysts and can present as two distinct genetic disorders: autosomal dominant polycystic kidney disease (ADPKD) and autosomal dominant polycystic liver disease (PCLD). Although genetic testing for ADPKD and PCLD is possible, it is rarely performed because it does not affect the therapeutic management of PLD. USG screening of the liver and both kidneys combined with extensive family history taking are the cornerstone

of diagnostic decision making in PLD. In conclusion, an amalgamation of these recent advances results in a diagnostic algorithm that facilitates evidence-based clinical decision making.

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**Key words:** Coincidental hepatic cystic lesions; Cystic liver disease; Complicated cyst; Polycystic liver disease; Diagnostic algorithm

**Core tip:** We performed a review of the recent literature, and through combining current consensus and recent advances, we developed an evidence-based diagnostic algorithm to guide clinicians in characterising hepatic cystic lesions. Serodiagnostic tests and microbubble contrast-enhanced ultrasound (CEUS) are invaluable in differentiating complicated cysts, echinococcosis and cystadenoma/cystadenocarcinoma when ultrasonography (USG), computed tomography and magnetic resonance imaging show ambiguous findings. As a result, serodiagnostic tests and CEUS reduce the need for invasive procedures. USG screening of the liver and both kidneys combined with extensive family history taking remains the cornerstone of diagnostic decision making in polycystic liver disease.

Lantinga MA, Gevers TJG, Drenth JPH. Evaluation of hepatic cystic lesions. *World J Gastroenterol* 2013; 19(23): 3543-3554 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3543.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3543>

### INTRODUCTION

Hepatic cystic lesions represent a comprehensive heterogeneous cluster with regard to pathogenesis, clinical presentation, diagnostic findings and therapeutic management (Table 1). Hepatic cystic lesions predominantly remain asymptomatic and are found as a mere coinci-

dence on abdominal imaging techniques, such as ultrasonography (USG), computed tomography (CT) and magnetic resonance imaging (MRI)<sup>[1,2]</sup>. The use of these techniques has greatly increased over the last years, and as a corollary, there has been an increase in incidental findings of asymptomatic hepatic cystic lesions<sup>[3]</sup>. In most cases, hepatic cystic lesions will follow a benign course<sup>[4]</sup>. However, it is essential to differentiate benign cysts from potentially harmful cysts, such as echinococcosis, cystadenoma and cystadenocarcinoma, which require specific treatment<sup>[5,6]</sup>. Currently, clinicians must also be aware of changes in the epidemiology of certain hepatic cystic lesions. Echinococcosis has spread to previously non-endemic Western European countries<sup>[7,8]</sup>. For this reason, the early and accurate diagnosis of cysts is crucial. To facilitate the diagnostic process, we provide an overview of the wide spectrum of mono- and polycystic liver diseases based on literature published over the last five years.

## LITERATURE SEARCH

We searched the electronic database PubMed using the following search terms: “liver” and “cyst” and “diagnosis”. We limited our search to articles that were written in English, published between November 2007 and November 2012 and available in full text. A total of 992 articles were identified. For the purpose of this review, we included articles with a main focus on the evaluation of hepatic cystic lesions in humans. Screening the titles and abstracts identified 252 articles meeting these inclusion criteria (Figure 1). Additionally, we searched the reference lists from all eligible reviews for additional leads.

## SIMPLE CYSTS

### Pathogenesis

Simple cysts arise congenitally from aberrant bile duct cells and contain a clear, bile-like fluid<sup>[9]</sup>. Because bile duct epithelium covers the simple cyst inner lining, it is hypothesised that simple cysts arise during embryogenesis when intrahepatic ductules fail to connect with extrahepatic ducts<sup>[4,10]</sup>.

### Clinical features

The prevalence of simple cysts ranges from 2.5% to 18% and increases with age<sup>[11,12]</sup>. More than half of individuals older than 60 years are likely to have one or more simple cysts. Cysts are small in most patients but can grow to over 30 cm in selected cases. In a small fraction of patients, symptoms, such as abdominal pain, early satiety, nausea and vomiting, arise as a result of a mass effect<sup>[3]</sup>. Physical examination may reveal a palpable abdominal mass or hepatomegaly<sup>[1]</sup>. Complications such as haemorrhage, rupture and biliary obstruction are uncommon but are more likely in larger cysts<sup>[13]</sup>. Intracystic haemorrhage is a rare complication of simple cysts and usually presents with severe abdominal pain<sup>[14]</sup>, although asymptomatic presentations are also observed<sup>[15,16]</sup>.

**Table 1 Differential diagnosis of cystic lesions in the liver**

|  |
|--|
| Monocytic disease                            |
| Simple cyst                                  |
| Echinococcosis                               |
| Cystic echinococcosis                        |
| Alveolar echinococcosis                      |
| Cystadenoma                                  |
| Cystadenocarcinoma                           |
| Polycystic disease                           |
| Autosomal dominant polycystic kidney disease |
| Autosomal dominant polycystic liver disease  |

### Laboratory findings

Laboratory findings are predominantly normal, but a minority of patients have raised serum  $\gamma$ -glutamyl-transferase ( $\gamma$ GT)<sup>[17]</sup>. Several studies have shown that serum and cyst fluid levels of carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9) may be elevated<sup>[18]</sup>. CA 19-9 is expressed in the simple cyst inner epithelial lining and leads to elevated cyst fluid and serum CA 19-9 levels<sup>[17]</sup>. CA 19-9 is not helpful in the differential diagnosis of intracystic haemorrhage<sup>[19]</sup>.

### Diagnostic features

Most simple cysts are diagnosed incidentally on USG (Figure 2A), CT (Figure 2B) or MRI. The diagnosis of a simple cyst is based on the following USG criteria: anechoic (*i.e.*, fluid filled cavity), no septations, sharp smooth borders, strong posterior wall echoes (indicating a well-defined fluid/tissue interface), spherical or oval shaped and a relative accentuation of echoes beyond the cyst compared to echoes at a similar depth transmitted through normal adjacent hepatic tissue (Table 2)<sup>[20]</sup>. CT shows a sharply defined homogeneous hypodense lesion (Figure 2B)<sup>[21]</sup>. MRI T1-weighted sequence shows low signal intensity, whereas the T2-weighted sequence shows extremely high signal intensity, which does not enhance after contrast injection<sup>[22]</sup>. USG has a reported sensitivity and specificity of approximately 90% for diagnosing a simple cyst<sup>[23]</sup>, and recent advances in CT and MRI technology might result in even higher sensitivity rates<sup>[12,22,24]</sup>. However, because CT is accompanied with a radiation load and both CT and MRI come at a significantly higher cost, USG remains the most accurate, non-invasive and cost-effective imaging modality for diagnosing simple cysts.

In case of an intracystic haemorrhage (*i.e.*, complicated cyst), USG typically shows a hyperechogenic echo pattern combined with internal echoes that mimic septations or solid portions (Figure 3)<sup>[25]</sup>. In contrast, CT visualises intracystic haemorrhage as a high-density area<sup>[26]</sup>, whereas MRI depicts it as a high signal intensity on T1- and T2-weighted sequences<sup>[27]</sup>. Neither CT nor MRI has additional diagnostic value compared to USG in the diagnosis of cystic bleeding<sup>[15]</sup>. The recent development of microbubble contrast-enhanced ultrasound (CEUS) enables us to visualise vascular flow within septa or solid components of cysts, which is absent in simple cysts with intracystic

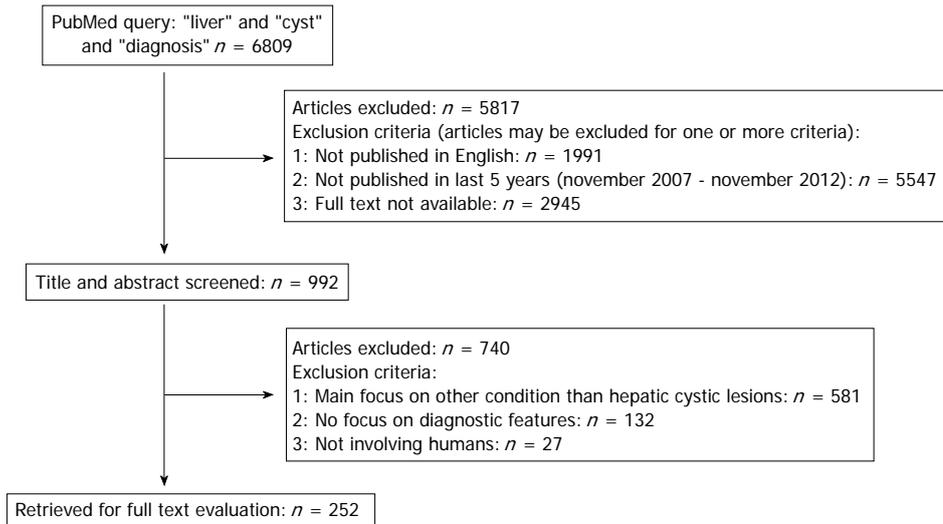


Figure 1 Selection process of retrieved articles.

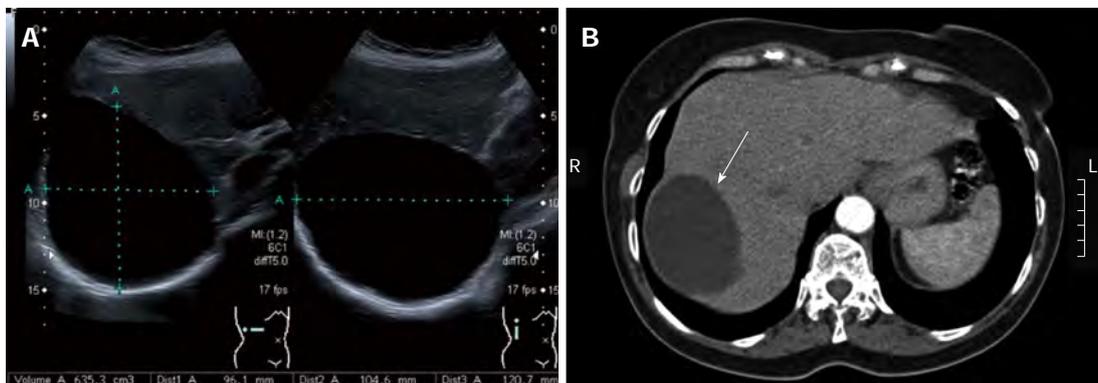


Figure 2 Simple cyst. A: On abdominal ultrasonography. Ultrasonography (USG) demonstrating a large simple cyst occupying the right hepatic lobe. Note the sharp and smooth border, oval shape, and anechoic echo pattern with the absence of septations and strong posterior wall echoes. The cyst size is indicated by the dotted lines; B: On abdominal computed tomography. Computed tomography demonstrating a sharply defined homogeneous hypodense cystic lesion (arrow) occupying the right hepatic lobe, which was diagnosed as a simple cyst.

haemorrhage<sup>[28]</sup>. Therefore, CEUS can accurately characterize these cysts when USG, CT and MRI show ambiguous findings<sup>[29-31]</sup>.

### Therapy

The management of most simple cysts relies on a “wait-and-see” policy, and no further treatment is required in these cases. If there are symptoms, aspiration-sclerotherapy is the preferred treatment<sup>[32,33]</sup>. Laparoscopic or open surgical fenestration techniques are similarly or even more effective in reducing symptoms<sup>[34,35]</sup> but have a significantly higher morbidity and mortality rate<sup>[36]</sup>.

## ECHINOCOCCOSIS

Echinococcosis is a zoonosis caused by larval stages of taeniid cestodes (tapeworms) belonging to the *Echinococcus* species. Two of the six known species cause solitary cystic lesions in humans: (1) *Echinococcus granulosus* (*E. granulosus*), responsible for cystic echinococcosis (CE); and (2) *Echinococcus multilocularis* (*E. multilocularis*), responsible for

alveolar echinococcosis (AE)<sup>[6]</sup>.

Echinococcosis-related deaths are uncommon in developed countries. For example, there were 41 echinococcosis-associated deaths in the United States over an 18-year study period<sup>[37]</sup>. However, echinococcosis is considered to be an emerging disease in Europe<sup>[38,39]</sup>. Thus, CE and AE are diseases with a considerable global disease impact, as indicated by a substantial loss in disability-adjusted life years<sup>[38,40]</sup>.

### Cystic echinococcosis

**Pathogenesis:** Humans become infected by acting as intermediate hosts of *E. granulosus* after ingestion of *Echinococcus* eggs, which are excreted by infected carnivores (dogs and other canids)<sup>[6]</sup>. Infection is typically observed in areas containing large numbers of the intermediate hosts of the parasite (sheep and goats) that are in close contact with the final host (herding dogs)<sup>[41-43]</sup>.

**Clinical features:** Although CE has a worldwide geographic distribution, the highest prevalence of CE is

**Table 2** Ultrasonography features for the diagnosis of monocytic diseases of the liver

|                            | Simple cyst                                  | Cystic echinococcosis             | Alveolar echinococcosis                            | Cystadenoma and cystadenocarcinoma                       |
|----------------------------|--|-----------------------------------|--|--|
| Border                     | Sharp and smooth                             | Laminated                         | Irregular  | Irregular  |
| Shape                      | Spherical or oval                            | Round or oval                     | Irregular  | Round or oval  |
| Echo pattern               | Anechoic <sup>1</sup>                        | Anechoic or atypical <sup>2</sup> | Hyperechogenic outer ring and hypoechogenic centre | Hypoechogenic with hyperechogenic septations             |
| Appearance                 | No septa                                     | Multiseptated                     | Multivesicular                                     | Septated and/or solid structures (papillary projections) |
| Wall                       | Strong posterior wall echoes                 |                                   |  | Wall enhancement   |
| Posterior acoustic feature | Relative <sup>3</sup> accentuation of echoes |                                   | Dorsal shadowing (calcified areas)                 | Dorsal shadowing (calcified areas)                       |

<sup>1</sup>Fluid-filled cavity; <sup>2</sup>Snowflake-like inclusions or floating laminated membranes; <sup>3</sup>Compared to echoes at a similar depth transmitted through normal adjacent hepatic tissue.



**Figure 3** Complicated simple cyst on abdominal ultrasonography. Ultrasonography (USG) demonstrating a cystic lesion with a hyperechogenic echo pattern combined with internal echoes that mimic septations or solid portions (arrow) in a patient presenting with severe abdominal pain with a known history of multiple simple cysts (asterisks). Because of the known history of simple cysts, the lesion was diagnosed as a complicated simple cyst (*i.e.*, intracystic haemorrhage).

found in the temperate zones, including the Mediterranean, Central Asia, Australia and some parts of America<sup>[44]</sup>.

Because cyst growth in the liver is slow (ranging from 1-5 millimetres in diameter per year), CE can remain asymptomatic for a long time. In approximately 90% of cases, the primary presentation is a spherical, fluid-filled vesicle with an inner cellular layer and an outer laminated layer located in the liver, lungs or both<sup>[45]</sup>. Symptoms occur when cysts exert mass effects within the organ or surrounding tissues or rupture, often presenting as a sudden onset of abdominal pain. Secondary cholangitis (rupture into the biliary tree), biliary obstruction and intraperitoneal rupture followed by anaphylaxis are common complications of CE and require hospitalisation<sup>[6]</sup>. Worldwide mortality rate estimates vary between 2.2%-5.0%<sup>[45,46]</sup>, although the exact mortality rate of CE in developed countries remains unknown.

**Diagnostic features:** The diagnosis of CE is based on the following criteria: endemic region history, clinical findings (*e.g.*, abdominal pain, fever, chest pain, and dyspnea), pathognomonic USG features and positive

immuno diagnostic tests<sup>[47]</sup>. USG shows a round or oval-shaped, anechoic or atypical (*i.e.*, snowflake-like inclusions or floating laminated membranes) echo pattern with multiple septa confined by a laminated border (Table 2)<sup>[47]</sup>. USG has a reported specificity of 90% and is used in combination with CT when surgical treatment is considered. MRI has not been proven to be cost-effective and has no added value<sup>[48]</sup>. The currently used serodiagnostic tests to reveal *E. granulosus* antibodies have a sensitivity of 93.5% and specificity of 89.7%<sup>[49]</sup>.

**Therapy:** The treatment of CE, including surgery (open or laparoscopic), percutaneous treatments [*e.g.*, puncture aspiration injection re-aspiration (PAIR) method] and chemotherapy<sup>[50]</sup>, is indicated to reduce symptoms and prevent complications<sup>[51]</sup>. PAIR is the treatment of choice for CE, as a recent review showed that PAIR resulted in parasitological clearance (*i.e.*, negative serodiagnostic tests) in 95.8% of cases<sup>[52]</sup>.

### Alveolar echinococcosis

**Pathogenesis:** AE is endemic in the Northern hemisphere (*e.g.*, North America, Asia, China, Japan and Europe). AE occurs when *E. multilocularis* eggs, found in the excrement of foxes, are ingested. The spread from endemic areas to previously non-endemic Western European countries is most likely due to an increasing fox population and spillover from these wild carnivores to domestic hosts<sup>[7,8]</sup>.

**Clinical features:** The ingested eggs develop into an alveolar structure composed of numerous small vesicles that vary in diameter from smaller than 1 mm to 3 cm. Each vesicle has the same wall structure as CE. These vesicles grow slowly and are able to reach a maximum diameter of 15-20 cm, similar to simple cysts<sup>[53]</sup>. In approximately 99% of cases, the infection is initially confined to a solitary alveolar lesion in the liver<sup>[45]</sup>. After the primary infection, AE usually has an asymptomatic phase of 5-15 years prior to the development of symptoms. Symptoms are related to mass effect or are nonspecific, such as weight loss or fatigue<sup>[54]</sup>. In contrast to the encapsulated growth pattern of CE, AE eventually leads to liver failure

due to an infiltrative neoplastic growth with potential metastasis to adjacent and distant (*e.g.*, lungs, spleen, bone, and brain) organs<sup>[55,56]</sup>.

**Diagnostic features:** Typical USG aspects are observed in 70% of cases and include irregular shape and border, hyperechogenic outer ring and hypoechogenic centre, multivesicular appearance and dorsal shadowing due to calcified areas (Table 2)<sup>[47]</sup>. Atypical USG aspects include small hyperechogenic nodules (amorphous AE), large lesions with massive necrosis (pseudocyst) and small calcified lesions (inert AE)<sup>[57]</sup>. In contrast to CE, MRI is superior to CT in detecting AE lesion margins<sup>[58,59]</sup>. Similar to CE, high diagnostic sensitivity (90%-100%) and specificity (95%-100%) are attained with serodiagnostic tests, and in 80%-95% of cases, AE can be differentiated from CE with the help of serologically obtained purified *Echinococcus* antigens<sup>[60]</sup>.

**Therapy:** The approach to the management of AE resembles that of a hepatic malignancy. The cornerstone of treatment for AE includes radical surgery followed by a 2-year period of chemotherapy<sup>[6]</sup>. A recent study concluded that AE can be cured in 42% of cases by complete surgical removal of the parasitic mass. Early diagnosis could even improve this rate further<sup>[61]</sup>.

## CYSTADENOMA AND CYSTADENOCARCINOMA

### Pathogenesis

Cystadenoma and cystadenocarcinoma are biliary cyst tumours that originate from the biliary epithelium<sup>[62]</sup>. Analogous to simple cysts, cystadenoma is considered to be a congenital disorder<sup>[63]</sup>. The exact mechanism of carcinogenesis in cystadenoma remains unknown. Several studies have suggested that cystadenocarcinoma develops from the ectopic remnants of primitive foregut sequestered within the liver<sup>[63]</sup>. In contrast, the malignant transformation of cystadenoma into cystadenocarcinoma is considered to be an alternative mechanism of carcinogenesis, as some cystadenocarcinomas may co-exist with cystadenoma<sup>[64]</sup>. This hypothesis is supported by the observation that the presence of cystadenoma increases the chance of developing cystadenocarcinoma<sup>[65]</sup>.

### Clinical features

Less than 5% of all cystic lesions of the liver are cystic neoplasms<sup>[2]</sup>. The clinical presentation of cystadenoma and cystadenocarcinoma is asymptomatic or tends to mimic symptoms of simple cysts or echinococcosis<sup>[66,67]</sup>. Studies have reported a predominance in women, with a mean age of onset varying from 40-60 years<sup>[64,65]</sup>. Cystadenomas appear to be slow growing, but exact growth rates are unknown. One case series evaluated 75 patients and recorded a variability in cyst size from 1.5-35 cm<sup>[68]</sup>. One study involving 63 cases diagnosed with cystadenocarcinoma demonstrated infiltrative growth in neighbour-

ing organs in 33 cases (52%) and distant metastases in 15 cases (24%)<sup>[5]</sup>. For that reason, it is necessary to diagnose cystadenocarcinoma in an early stage.

### Laboratory findings

In general, liver function tests are normal. A review of 13 cases found that serum concentrations of  $\gamma$ GT and alkaline phosphatase (AP) were elevated in 3 cases<sup>[69]</sup>. One study reported a rise in serum levels of CEA in 3 of 22 cystadenocarcinoma cases (14%) and a rise in the serum concentration of CA 19-9 in 4 of 11 cases (36%)<sup>[5]</sup>. Similar results have been reported in cases with cystadenoma: one study showed elevated serum concentrations of CEA or CA 19-9 in 2 of 3 cases<sup>[63]</sup>. Consequently, laboratory studies are not helpful in differentiating cystadenoma and cystadenocarcinoma from complicated cysts or echinococcosis.

### Diagnostic features

The USG characteristics of cystic neoplasms for both cystadenoma and cystadenocarcinoma are the following: a round or oval shape, irregular border, hypoechogenic echo pattern with hyperechogenic septations or solid structures (*i.e.*, papillary projections), wall enhancement and dorsal shadowing due to calcified areas (Table 2)<sup>[2]</sup>. Because of these typical cystic neoplastic features, which are absent in simple cysts, USG is a useful technique to easily discriminate between cystic neoplasms and simple cysts<sup>[2]</sup>. Like USG, CT and MRI show markedly similar characteristics for cystadenoma and cystadenocarcinoma: internal septations, thickened and irregular wall, papillary projections, calcifications and wall enhancements<sup>[62]</sup>. Cystadenomas predominantly have thinner septa and more regular walls<sup>[70]</sup>, whereas solid structures, intracystic haemorrhage and vascularised septations on contrast-enhanced CT are more suspicious for cystadenocarcinoma<sup>[62]</sup>. However, in most cases, differentiation between cystadenoma and cystadenocarcinoma is not possible<sup>[1]</sup>. The same problem arises in differentiating echinococcosis and complicated cysts from cystadenoma and cystadenocarcinoma because in many cases, intracystic haemorrhage, calcifications and septations are present in these lesions<sup>[2,62]</sup>.

Recent advances in technology have made diffusion-weighted magnetic resonance imaging (DWI) a promising MRI technique for liver lesion detection and characterisation<sup>[71]</sup>. DWI depicts the rate of diffusion of water molecules between tissues, given as the apparent diffusion coefficient (ADC)<sup>[72]</sup>. Generally, high ADC values are measured in cystic and necrotic tissue, which allow a relatively free diffusion of water, whereas low ADC values are an indication of cell-rich tissue (*e.g.*, tumour tissue)<sup>[22,73,74]</sup>. However, because of an overlap of ADC values, differentiating cystic neoplasms, echinococcosis and complicated cysts is not possible with DWI<sup>[75]</sup>. Therefore, additional immunodiagnostic tests are needed to rule out echinococcosis. Fine needle aspiration (FNA) could be of additional help to exclude complicated cysts<sup>[76]</sup>; however, due to the risk of malignancy, FNA is generally

not performed. In contrast, CEUS can be helpful in differentiating cystadenoma and cystadenocarcinoma from complicated cysts when USG, CT or MRI is inconclusive. CEUS characterises the vascular flow within septa in cystadenoma and cystadenocarcinoma, which is absent in complicated cysts<sup>[29-31]</sup>. Nonetheless, surgical resection remains the golden standard for diagnosing cystadenoma and cystadenocarcinoma when CEUS is not available.

### Therapy

The primary treatment of cystadenoma and cystadenocarcinoma is hepatic resection. A study in which 66 cases of cystadenocarcinoma were subjected to hepatic resection described a 3-year survival rate of 74%<sup>[5]</sup>.

## PCLD AND ADPKD

### Polycystic liver disease

Polycystic liver disease (PLD) is arbitrarily defined as the presence of > 20 liver cysts<sup>[77]</sup>. Autosomal dominant polycystic liver disease (PCLD) and autosomal dominant polycystic kidney disease (ADPKD) are two distinct genetic disorders associated with the development of polycystic livers<sup>[78]</sup>. Liver function, as judged by parameters of liver synthesis, is not affected in PLD, as functional hepatic tissue remains unaffected<sup>[77,79]</sup>.

### Pathogenesis

During embryogenesis, the intrahepatic bile ducts are formed from a cylindrical layer of cells (*i.e.*, ductal plate) surrounding each portal vein. Incorrect involution of the ductal plate results in ductal plate malformation (DPM)<sup>[80,81]</sup>. DPM consists of excess embryonic bile duct structures in a ductal plate configuration that does not communicate with the normally developed intrahepatic bile ducts. The progressive dilatation of these excess intrahepatic structures during life results in multiple liver cysts<sup>[82]</sup>. Similar to simple cysts, these cysts contain a clear, bile-like fluid and an inner lining of cholangiocytes<sup>[83]</sup>.

### Genetics

PCLD was historically considered a phenotypic variant of ADPKD<sup>[84]</sup>. However, the presence of PLD in the absence of renal cysts led to the belief that PCLD should be regarded as a separate entity<sup>[85]</sup>. The discovery of a familial form of PLD<sup>[86]</sup>, genetically distinct from the heterozygous mutation in genes *PKD1* and *PKD2* identified in ADPKD<sup>[87]</sup>, ultimately led to the identification of heterozygous mutations in genes encoding *SEC63* and *PRKCSH*<sup>[88-90]</sup>. Mutation analysis identified a heterozygous mutation in *PRKCSH* (15%) and *SEC63* (5%) in approximately 20% of studied PCLD cases<sup>[91]</sup>. In contrast, a *PKD1* mutation was found in 85% of cases of ADPKD, and a *PKD2* mutation was found in the remaining cases<sup>[92]</sup>.

*PRKCSH* and *SEC63* encode hepatocystin and SEC63 proteins, respectively. Hepatocystin acts in the folding process of proteins, while SEC63 acts as part of

the endoplasmic reticulum translocon<sup>[93]</sup>. Unfortunately, the exact mechanism of cystogenesis in PCLD remains unclear. Polycystin 1 and 2, encoded by *PKD1* and *PKD2*, respectively, are important for adequate functioning of the primary cilium<sup>[94]</sup>. It is therefore suggested that primary cilia play a central pathogenic role in the mechanism of hepatic cystogenesis in ADPKD<sup>[78]</sup>.

### Clinical features

The extra polarisation of 137 identified PCLD cases in a specific adherence region (the Netherlands) led to an estimated PCLD prevalence of 1 per 158000<sup>[77]</sup>. This number is most likely an underestimation of the true prevalence because only symptomatic patients referred to tertiary centres were included in this study, and PCLD often remains asymptomatic<sup>[95]</sup>. ADPKD is the most common monogenetic disorder, with a world-wide estimated prevalence of 0.10%-0.25%<sup>[96]</sup>, and it is responsible for approximately 8%-10% of cases with end-stage renal disease<sup>[97]</sup>. Although ADPKD is primarily characterised by the presence of renal cysts<sup>[98]</sup>, liver cysts are considered the most prevalent extra-renal manifestation of ADPKD<sup>[99,100]</sup>. Indeed, one study involving 230 ADPKD cases found an overall prevalence of 83%<sup>[101]</sup>. However, the exact prevalence of PLD in ADPKD is still unknown. PCLD is predominantly confined to the liver, but a few renal cysts can also be present, which leads to difficulties in the accurate differentiation between PCLD and ADPKD<sup>[79,99]</sup>. Although renal cysts in ADPKD ultimately lead to renal failure, renal function remains unaffected in the presence of PCLD-associated renal cysts<sup>[102]</sup>.

PLD is predominantly discovered during the fourth or fifth decade of life and is more severe in females<sup>[77,96,103,104]</sup>. PCLD tends to lead to a higher number and greater volume of liver cysts<sup>[79]</sup>. The number of pregnancies, increased age and severity of renal disease are considered additional risk factors for liver cyst growth in ADPKD<sup>[105]</sup>. PLD is mainly asymptomatic, but mechanical complaints can arise in a subset of patients<sup>[79,106]</sup>. Complications such as intracystic haemorrhage and infection are rare and typically occur in large cysts<sup>[106]</sup>.

### Laboratory findings

PLD causes increased  $\gamma$ GT and AP levels in both PCLD and ADPKD patients<sup>[77]</sup>. Occasionally, increased serum aspartate aminotransferase (AST) is also found in ADPKD<sup>[79,107]</sup>. Renal function remains intact in PCLD, whereas ADPKD patients show a rise in serum creatinine due to impaired renal function<sup>[102]</sup>.

### Diagnostic features

PLD is detected with the use of USG, CT or MRI. USG, which is accurate, non-invasive and low cost, is the preferred imaging modality for both PCLD and ADPKD<sup>[108,109]</sup>. Currently, there are no generally accepted USG criteria for PCLD. One study suggested that the diagnosis can be made in case of a positive family history of PCLD and the presence of > 4 liver cysts<sup>[78]</sup>. However,

**Table 3** Ultrasonography criteria for the diagnosis of autosomal dominant polycystic kidney disease

| Family history positive <sup>1</sup>   |                            |
|--|----------------------------|
| Unknown genotype   |                            |
| Age (yr)   |                            |
| ≥ 15 and ≤ 39  | ≥ 3 unilateral renal cysts |
| ≥ 40 and ≤ 59  | ≥ 2 bilateral renal cysts  |
| ≥ 60   | ≥ 4 bilateral renal cysts  |
| Family history negative  |                            |
| > 10 bilateral renal cysts, with the exclusion of renal or extra-renal disease causing renal cysts |                            |

<sup>1</sup>Exclude autosomal dominant polycystic kidney disease when < 2 unilateral renal cysts and ≥ 40 years of age.

diagnosing ADPKD is usually relatively straightforward when enlarged bilateral cystic kidneys are present in combination with a positive family history for ADPKD<sup>[102]</sup>. In case of a negative family history, screening direct family members with USG can be helpful to reveal asymptomatic ADPKD. Because mutation analysis for ADPKD has no clinical implications, its use is limited to family members of ADPKD patients involved in kidney donation programs. In 2009, the Pei USG criteria were developed because the original Ravine USG criteria for diagnosing ADPKD appeared to be insufficient<sup>[110,111]</sup>. Table 3 gives an overview of the USG criteria for diagnosing ADPKD when the causative gene is unknown. For example, in case of a positive ADPKD family history, diagnosis can be made when ≥ 3 renal cysts are unilaterally present in individuals aged 15 to 39 years<sup>[110]</sup>. ADPKD should be considered when there are > 10 bilateral renal cysts present in the absence of other renal or extra-renal disease that can cause renal cysts<sup>[108]</sup>. When PCLD or ADPKD criteria are not met, multiple simple cysts are most likely responsible for the hepatic cystic lesions.

ADPKD is characterised by an increased risk of developing vascular manifestations. Hypertension occurs in approximately 50%-70% of patients, and almost half of these hypertensive patients are reported to have left ventricular hypertrophy (LVH)<sup>[112]</sup>. Mitral valve prolapse is observed in 25% of patients and intracranial aneurysms in 4%-12% of patients<sup>[112]</sup>. As a result, magnetic resonance angiography (MRA) must be performed when ADPKD patients have a positive family history of intracranial aneurysms because the rupture of aneurysms is reported to be responsible for 4%-7% of deaths in affected ADPKD families<sup>[113]</sup>. In contrast to ADPKD, several studies have shown that PCLD patients do not appear to have an increased risk of vascular malformations. One study involving 19 PCLD cases reported hypertension in 10.5% of cases, mitral valve prolapse in 0% and aneurysms in 5.3%<sup>[79]</sup>. Another study involving 38 PCLD cases found mitral valve prolapse in 1 case (2.6%)<sup>[114]</sup>. Subsequently, targeted screening is not advised for PCLD.

### Therapy

The main objective of therapy is to reduce liver cyst

volume to diminish mass effect-related symptoms<sup>[115]</sup>. Hence, the only indication for reducing cyst volume is when a PLD patient reports symptoms that can be linked to the polycystic liver<sup>[116]</sup>.

Surgical procedures, such as aspiration-sclerotherapy and fenestration, are indicated when PLD consists of large cysts confined to a limited part of the liver. In more extensive disease, segmental hepatic resection or even liver transplantation is imperative to relieve symptoms<sup>[117]</sup>. Future medical therapies include somatostatin analogues, as several clinical trials with lanreotide and octreotide achieved polycystic liver volume reduction in PCLD and ADPKD<sup>[118-123]</sup>.

## CONCLUSION

Cystic lesions of the liver encompass a wide spectrum of disorders. As a result of the frequent use of abdominal imaging techniques in recent years, the incidence of so-called coincidental cysts has increased. Simple cysts are the most prevalent and have a tendency to follow a benign course. However, complicated cysts, echinococcosis and cystic neoplasms (*e.g.*, cystadenoma and cystadenocarcinoma), which cause a diagnostic enigma, demand accurate diagnosis in the early stage because specific treatment could be required. Furthermore, the presence of multiple hepatic cystic lesions must raise the suspicion of PCLD or ADPKD and requires further screening.

USG remains the most accurate, non-invasive and cost-effective imaging modality for diagnosing simple cysts. Despite recent advances (*e.g.*, contrast-enhanced CT and DWI), distinguishing complicated cysts from echinococcosis and cystic neoplasms remains impossible with USG, CT or MRI alone. Because of an ever-increasing spread of *Echinococcus* to previously non-endemic regions and its initial quiescent phase after primary infection, it is necessary to exclude echinococcosis. Serodiagnostic tests have high sensitivity and specificity to reveal *Echinococcus* antibodies. Subsequently, CEUS can be used to accurately and reliably exclude cystic neoplasms by demonstrating the absence of any enhancement within the hepatic cystic lesion. Therefore, when CEUS is available, it reduces the need for surgical resection.

The detection of multiple liver cysts requires USG screening of both kidneys and extensive family history taking regarding the occurrence of ADPKD or PCLD. When PCLD or ADPKD criteria are not met, multiple simple cysts are most likely responsible for the hepatic cystic lesions. PCLD or ADPKD could eventually be diagnosed through USG follow-up.

To summarise, we developed a diagnostic algorithm by integrating recent advances with conventional diagnostic tools (Figure 4). Our diagnostic algorithm facilitates evidence-based clinical decision making when clinicians are confronted with coincidental hepatic cystic lesions on USG. Further development of USG- and MRI-based techniques, such as CEUS and DWI, will probably lead to further improvement of hepatic cystic lesion characterisation.

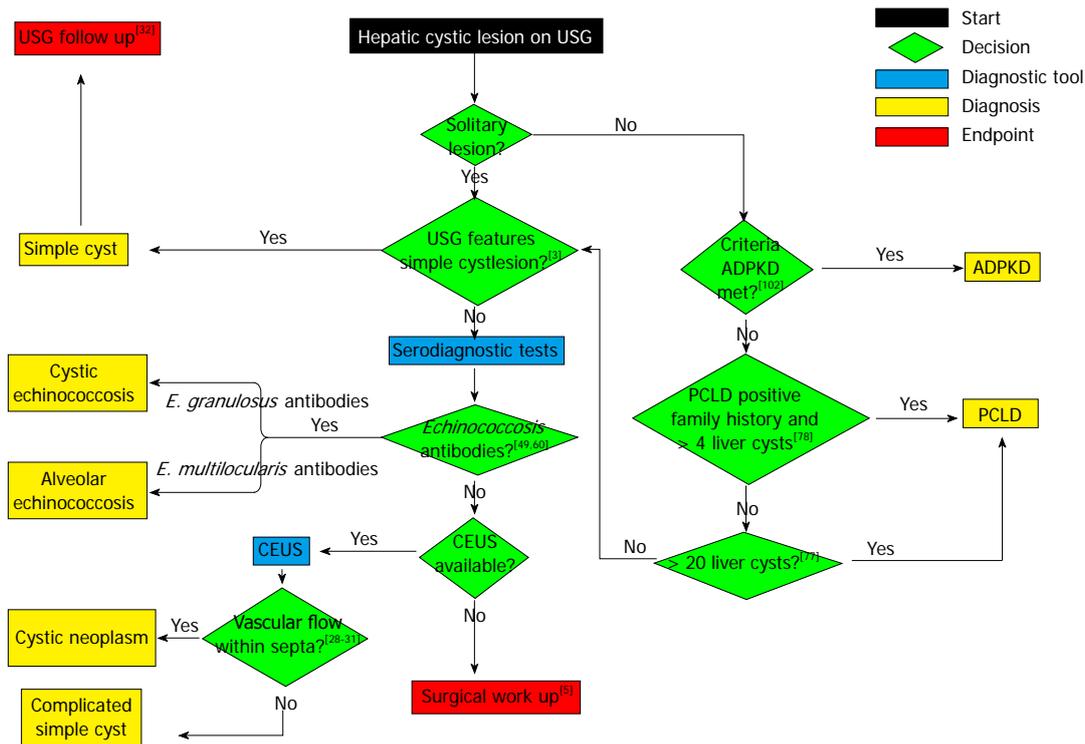


Figure 4 Diagnostic algorithm. Diagnosis of hepatic cystic lesions after detection on ultrasonography. *E. granulosus*: *Echinococcus granulosus*; *E. multilocularis*: *Echinococcus multilocularis*; CEUS: Contrast-enhanced ultrasound; PCLD: Polycystic liver disease; ADPKD: Autosomal dominant polycystic kidney disease.

## ACKNOWLEDGMENTS

The authors wish to thank Melissa Chrispijn from the Department of Gastroenterology and Hepatology Radboud University Nijmegen Medical Center, the Netherlands, for her expert advice.

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**P- Reviewers** de Oliveira C, Ramsay M, Silva ACS  
**S- Editor** Wen LL **L- Editor** A **E- Editor** Zhang DN



## Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review

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Supported by Natural Science Foundation of Ningbo City, No. 2012A610194; National Natural Science Foundation of China, No. 81071697; Natural Science Foundation of Guangdong Province, No. S2011040003694

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Received: January 2, 2013 Revised: March 15, 2013

Accepted: April 10, 2013

Published online: June 21, 2013

### Abstract

Ischemia/reperfusion (I/R) injury of the gut is a significant problem in a variety of clinical settings and is associated with a high morbidity and mortality. Although the mechanisms involved in the pathogenesis of gut I/R injury have not been fully elucidated, it is generally believed that oxidative stress with subsequent inflammatory injury plays an important role. Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme, followed by production of CO, biliverdin, and free iron. The HO system is believed to confer cytoprotection by inhibiting inflammation, oxidation, and apoptosis, and maintaining microcirculation. HO-1, an inducible form of HO, serves a vital metabolic function as the rate-limiting step in the heme degradation pathway, and affords protection in models of intestinal I/R injury. HO-1 system is an important player in intestinal

I/R injury condition, and may offer new targets for the management of this condition.

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**Key words:** Heme oxygenase; Ischemia/reperfusion injury; Oxidative stress; Cytoprotection; Gut

**Core tip:** In this review, we focused on the heme oxygenase (HO)-1 system and its possible roles and mechanisms in gut ischemia/reperfusion (I/R) injury studied to date. This review, for the first time, reviews in detail the relationship between HO-1 and gut I/R injury.

Liao YF, Zhu W, Li DP, Zhu X. Heme oxygenase-1 and gut ischemia/reperfusion injury: A short review. *World J Gastroenterol* 2013; 19(23): 3555-3561 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3555.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3555>

### INTRODUCTION

Ischemia/reperfusion (I/R) injury of the gut occurs frequently in a variety of clinical settings, including abdominal aortic aneurysm surgery, mesenteric artery occlusion, small bowel transplantation, cardiopulmonary bypass, strangulated hernias, trauma, and shock<sup>[1]</sup>; the exact mechanisms involved in the pathogenesis of which have not been fully elucidated. Gut I/R injury is associated with a substantial morbidity and mortality<sup>[2]</sup>.

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation, resulting in the formation of CO, biliverdin, and free iron<sup>[3]</sup>. There are three distinct HO isoforms (HO-1, HO-2, and HO-3) identified to date. HO-1 is the inducible form of the enzyme, and is expressed in relatively low amounts in most tissues<sup>[3]</sup>. HO-2 is the constitutive isoform, expressed mainly in brain and testis. HO-3 is identified only in rats, and its physiological

role remains unclear<sup>[4]</sup>.

HO-1, as an inducible form, also belongs to a member of the heat shock protein family and is highly inducible by a vast array of stimuli<sup>[3]</sup>. Many studies indicated that the induction of HO-1 plays a significant protective role against inflammatory processes and oxidative tissue injury<sup>[5-7]</sup>. In this review, we focus on the current understanding of the cytoprotective effects observed with the HO system during gut I/R injury. The implications for possible therapeutic manipulation of HO in gut I/R injury are elucidated.

## ISCHEMIA/REPERFUSION INJURY IN GUT

Interruption of blood supply results in ischemic injury which rapidly damages metabolically active tissues. Paradoxically, reintroduction of blood flow obtained following ischemia initiates a cascade of events that can potentially worsen the original injury. This effect is known as reperfusion injury<sup>[8]</sup>. The intestine is composed of labile cells that are very susceptible to I/R injury<sup>[9]</sup>. Multiple factors have been shown to be involved in the process of intestinal I/R injury. The primary pathophysiological events of this injury involve microcirculatory flow disturbances caused by the production of reactive oxygen species (ROS). Tissue ischemia and oxidative stress activate families of protein kinases that converge on specific transcriptional factors that regulate the expression of inflammatory genes. The resulting gene products include enzymes [*e.g.*, inducible nitric oxide synthase (iNOS); phospholipase A2, and cyclooxygenase-2 (COX-2)], cytokines [*e.g.*, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ); interleukin-1 (IL-1); interleukin-6 (IL-6)], prostaglandins (*e.g.*, PGE-2), and adhesion molecules [*e.g.*, intracellular adhesion molecule (ICAM-1); E-selectin]<sup>[10-14]</sup>. These initiate local inflammation, which is further amplified by the recruitment of circulating leukocytes<sup>[12]</sup>, which appear to be key effector cells in causing tissue injury. Furthermore, I/R injury induces widespread endothelial cell apoptosis and the loss of endothelial cells in the vessels serving the organ results in thrombosis<sup>[15,16]</sup> directly in the intestine<sup>[16]</sup>. This injury observed during I/R is believed to trigger a systemic inflammatory response leading to multiple organ failure<sup>[17,18]</sup>, which frequently involves the lungs<sup>[19,20]</sup> and liver<sup>[21]</sup>. Intestinal I/R injury is a complex, multifactorial pathophysiological process, dependent upon an understanding of which the optimal therapeutic approach is aimed at ameliorating I/R injury (Table 1). HO-1 system might be one of the most promising approaches among the potential therapeutic options.

## ROLE OF HO-1 IN ISCHEMIA/REPERFUSION INJURY IN GUT

HO-1 is expressed constitutively in normal gastric, intestinal, and colonic mucosa<sup>[22,23]</sup>, and is up-regulated in their inflamed tissue<sup>[23]</sup>. Many studies showed that HO-1 is involved in a variety of regulatory and protective cel-

lular mechanisms as a stress-responsive protein<sup>[5,6]</sup>. The normal expression and up-regulation of HO-1 suggest that activation of HO-1 could act as a natural defensive mechanism to alleviate inflammation and tissue injury in the gastrointestinal tract<sup>[24,25]</sup>. HO has been shown to have potent cytoprotective effects on intestinal I/R injury as well<sup>[26]</sup>. For example, induction of HO-1 by cobalt-protoporphyrin administration before intestinal I/R resulted in a significant reduction of intestinal tissue injury<sup>[27]</sup>. Another enhancer (pyrrolidine dithiocarbamate) of HO production improves intestinal microvascular perfusion and attenuates I/R injury of the intestine, possibly *via* HO production<sup>[28]</sup>. Similarly, administration with a HO inducer (hemin) results in lessened mucosal injury and improved intestinal transit following gut I/R<sup>[29]</sup>. Glutamine protects the intestine from warm ischemic injury, which was considered to be associated with inducible HO-1 expression through the interaction with cellular antioxidative activity and the inhibition of cytokines<sup>[30]</sup>. Several studies demonstrated that intraischemic hypothermia, hypertonic saline resuscitation, and whole-body hyperthermia decrease inflammation and protect against intestinal injury in a model of gut I/R<sup>[31-33]</sup>. Administration of IL-2, an immunoregulatory cytokine, resulted in clinical improvement of the study animals after intestinal I/R<sup>[34]</sup>. These protective interventions were associated with the induction of HO-1. Postischemic leukocyte-endothelial cell adhesive interactions are prevented by 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside preconditioning 24 h prior to I/R in the small intestine by HO-dependent mechanisms<sup>[35]</sup>. Furthermore, ischemic preconditioning of the intestine might prove to be an effective strategy for the amelioration of I/R injury, in which HO is involved<sup>[18,36,37]</sup>. Pretreatment with *Radix Paeoniae Rubra*<sup>[38]</sup>, or the anticancer drug doxorubicin<sup>[20]</sup>, can attenuate acute lung injury resulting from intestinal I/R. These results demonstrate that HO-1 is implicated in cytoprotection and may be an effective agent for the treatment of gut I/R.

## MECHANISMS OF ACTION

There is increasing evidence that HO-1 plays an important protective role in gut I/R injury. There are four factors that could be responsible for the protection of HO-1 in intestinal I/R, including: (1) removal of free heme; (2) CO; (3) biliverdin/bilirubin; and (4) Fe<sup>2+</sup>.

## REMOVAL OF FREE HEME

Heme, an essential iron chelate, is a potentially damaging species that not only provides a lipophilic form of iron, but also can directly attack and impair a multiplicity of intracellular targets<sup>[39]</sup>; production increases under conditions of oxidant stress, especially in I/R injury<sup>[39,40]</sup>. HO-1 is the key enzyme in heme degradation and plays a key role in regulating the intracellular heme level. HO-1 activity leads to rapid removal of free heme. Thus, in order

**Table 1** The role of heme oxygenase-1 for the protection of intestinal ischemia/reperfusion injury

|            | Treatment                   | I/R model                 | Ref.          |
|------------|-----------------------------|---------------------------|---------------|
| HO-1       | Glutamine                   | Warm ischemia             | [30]          |
|            | Ischemic preconditioning    | Resuscitation after shock | [37]          |
|            | Doxorubicin                 | Warm ischemia             | [20]          |
|            | Hypothermia                 | Warm ischemia             | [32]          |
|            | IL-2                        | Warm ischemia             | [34]          |
|            | Hemin                       | Warm ischemia             | [29]          |
|            | Hypertonic saline           | Warm ischemia             | [31]          |
|            | Pyrrolidine dithiocarbamate | Warm ischemia             | [28]          |
|            | Hyperthermia                | Warm ischemia             | [33]          |
|            | Ischemic preconditioning    | Warm ischemia             | [36]          |
|            | Cobalt-protoporphyrin       | Warm ischemia             | [27]          |
|            | Ischemic preconditioning    | Endotoxic shock           | [18]          |
|            | Radix Paeoniae Rubra        | Warm ischemia             | [38]          |
|            | AICAR preconditioning       | Warm ischemia             | [35]          |
| CO         | Gas inhalation              | Intestinal transplants    | [11]          |
|            | Gas inhalation              | Intestinal transplants    | [13]          |
|            | Gas inhalation              | Intestinal transplants    | [14]          |
|            | Gas inhalation              | Intestinal transplants    | [47]          |
|            | CO solution                 | Intestinal transplants    | [49]          |
|            | CORM preconditioning        | Warm ischemia             | [48]          |
|            | CORM preconditioning        | Warm ischemia             | [12]          |
|            | Biliverdin/bilirubin        | Bilirubin                 | Warm ischemia |
| Bilirubin  |                             | Warm ischemia             | [61]          |
| Biliverdin |                             | Intestinal transplants    | [47]          |

AICAR: 5-aminoimidazole-4-carboxamide 1-beta-D-ribofuranoside; CORM: CO-releasing molecules; IL-2: Interleukin-2; I/R: Ischemia/reperfusion; HO: Heme oxygenase.

to prevent heme from both extracellular and intracellular sources reacting and producing ROS, the heme degradation step is an important consideration in the cytoprotection afforded by the HO system<sup>[40]</sup>.

## CO

CO is one of the three products of heme degradation by HO-1 and has profound effects as a signaling molecule that culminates in anti-inflammatory, antiapoptotic, and vasodilating effects<sup>[41,42]</sup>. A number of studies have revealed that CO mediates potent cytoprotective and anti-inflammatory effects in models of I/R injury of the heart, lung, kidney, and liver<sup>[43-46]</sup>. Some studies demonstrate that the efficacy of CO gas inhalation for the prevention of cold intestinal I/R injury using a small intestinal transplantation model, in which CO is able to effectively inhibit an early up-regulation of proinflammatory mediators such as IL-6, IL-1, TNF- $\alpha$ , ICAM-1, iNOS, and COX-2<sup>[11,13,14,47]</sup>. It has been reported that pre-treatment with CO-releasing molecules also markedly reduced intestinal inflammation induced by surgical manipulation of the small intestine<sup>[48]</sup> or by occluding the superior mesenteric artery<sup>[12]</sup>. Similarly, one study showed that cold storage in a preservation solution that was bubbled with 5% CO significantly reduced I/R injury associated with intestinal transplantation, which reduced

inflammatory mediator up-regulation and improved graft blood flow<sup>[49]</sup>. Moreover, CO-treated animals showed early up-regulation of the anti-apoptotic molecule Bcl-2, and down-regulation of the proapoptotic signal Bax, and reduced *in vivo* apoptosis of both vascular endothelial cells and intestinal epithelial cells<sup>[11]</sup>. The protective effects of CO are arbitrated by activating one or both of the two key signaling pathways. One of the pathways is soluble guanylate cyclase/cyclic guanosine monophosphate and the other one is the p38 mitogen-activated protein kinase pathway which transduces oxidative stress and inflammatory signaling<sup>[11,48-53]</sup>, through which CO exerts significant cytoprotection due to its anti-inflammatory, vasodilating, and anti-apoptotic properties in gut I/R injury.

## BILIVERDIN/BILIRUBIN

HO degrades heme into equimolar quantity of biliverdin. Biliverdin is, in turn, very rapidly converted to bilirubin by the enzyme biliverdin reductase<sup>[3]</sup>. Biliverdin and its reduced product, bilirubin, scavenge various ROS and are hence considered potent antioxidants<sup>[54,55]</sup>, which have been shown to confer cytoprotection against oxidative stress conditions in various cell types<sup>[56]</sup>. Several studies have also demonstrated that the administration of biliverdin and/or bilirubin is potently cytoprotective in I/R injury of the liver and heart, and in organ transplantation<sup>[57-59]</sup>. Evidence from an experimental small intestinal I/R injury model in rats describes a protective effect for bilirubin<sup>[60]</sup>, in which the bilirubin is infused *via* the jugular vein. Similarly, another study showed that increased serum bilirubin ameliorates the extent of intestinal IR injury<sup>[61]</sup>. Recent studies have suggested that biliverdin, in addition to its antioxidant properties, may have anti-inflammatory action. For example, treatment with biliverdin can significantly decrease mRNA expression of iNOS, COX-2, and ICAM-1, as well as the inflammatory cytokines IL-6 and IL-1, and decreased neutrophil infiltration into the jejunal muscularis in rat syngeneic small intestinal transplants<sup>[47]</sup>. These results suggest that bilirubin possesses complex immune-modulatory and antioxidant effects.

## Fe<sup>2+</sup>

Though HO activity is generally associated with cellular protection, Fe<sup>2+</sup>, the third product of heme decomposition, participates in the Fenton reaction to promote the generation of ROS and is believed to have potential deleterious effects. Increased iron levels, on the one hand, can upregulate an iron-transporter pump that removes intracellular Fe<sup>2+</sup> from the cell<sup>[62]</sup>. On the other hand, iron release from HO activity induces the expression of ferritin (an iron storing protein)<sup>[63,64]</sup>. Expression of ferritin was originally reported to protect endothelial cells against oxidant damage *in vitro*<sup>[64]</sup>. In addition, over-expression of H-ferritin (heavy chain ferritin) has also been shown to protect cultured endothelial cells from undergoing apop-

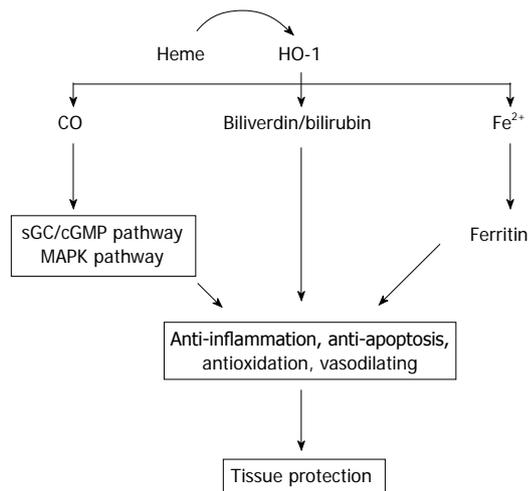


Figure 1 Schematic demonstration of the heme oxygenase-1 system and its biologic activities in gut ischemia/reperfusion injury. HO: Heme oxygenase.

osis and protects the liver from transplant-associated I/R injury<sup>[65]</sup>. Thus, ferritin seems to confer cytoprotection against oxidative challenge. There is no information about the roles of iron and ferritin in gut I/R injury, but in such a mechanism they could still be operative.

## CLINICAL EVIDENCE

As we mentioned above, the HO-1 system plays an important role in the cytoprotective process, up-regulation of which seems to be a potential therapeutic option for gut I/R injury. As far as we know, there have been no definitive trials designed to evaluate the efficacy of chemical HO-1 inducers in the clinical setting. Hemin, as an inducer of HO-1, has been used extensively in experimental studies, but has only been used by physicians experienced in the management of porphyrias clinically. However, there are increasing reports showing that hemin-induced HO-1 activity is a host defense mechanism in different animal models, such as the thrombosis vascular model<sup>[66]</sup>, in liver I/R injury<sup>[67]</sup>, acute pancreatitis with multi-organ failure<sup>[68,69]</sup>, human immunodeficiency virus-1 infection<sup>[70]</sup>, and spontaneously hypertension<sup>[71]</sup>. Such disease states share part or common physiopathological process with gut I/R injury, which suggests that hemin could offer a therapeutic benefit for gut I/R injury. A richer understanding of the cytoprotective mechanisms of hemin therapy will be necessary, which will also pave the way for clinical application in the treatment of gut I/R injury.

## CONCLUSION

Intestinal I/R injury is a complex, multifactorial pathophysiological process. Despite its complexity, the HO-1 system, owing to its antioxidative, anti-inflammatory, anti-apoptosis, and potent cytoprotective properties (Figure 1), may serve as promising potential therapeutic options for intestinal I/R injury.

The modulation of HO-1 expression using genetic or

pharmacological strategies may offer therapeutic strategies for intestinal I/R injury. Furthermore, HO-1-related molecules, including CO and biliverdin/bilirubin, might be employed as drugs in the management of intestinal I/R injury. More importantly, regulating the HO-1 system with different agents has already been demonstrated as important for attenuating I/R injury in other organs including the brain, liver, and kidney<sup>[40,72-74]</sup>. It is reasonable to assume that such a mechanism could also be operative in intestinal I/R injury. Research focused on the underlying mechanisms for the observed effects of HO-1 and its products will be necessary before their use can be evaluated in clinical applications for the prevention and/or treatment of human diseases such as intestinal I/R injury.

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**P-Reviewers** Camara CR, Kondo T, Wiley JW **S-Editor** Gou SX  
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## Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease

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**Supported by** The Polish Ministry of Science and Higher Education Grants No. 2 PO5A 094 29, 3 P05E 091 25, N N402 0861 and N N401 144638

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Received: December 18, 2012 Revised: March 5, 2013

Accepted: March 22, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To determine the features of *Enterococcus* that contribute to the development and maintenance of the inflammatory process in patients with inflammatory bowel disease (IBD).

**METHODS:** Multiplex polymerase chain reaction (PCR) was applied to assess the presence of genes that encode virulence factors [surface aggregating protein (*asa1*), gelatinase (*gelE*), cytolysin (*cy1A*), extracellular surface protein (*esp*) and hyaluronidase (*hyl*)] in the genomic DNA of 28 strains of *Enterococcus* isolated from the intestinal tissues of children with IBD ( $n =$

16) and of children without IBD (controls;  $n = 12$ ). Additionally, strains with confirmed presence of the *gelE* gene were tested by PCR for the presence of quorum sensing genes (*fsrA*, *fsrB*, *fsrC*) that control the gelatinase production. Gelatinase activity was tested on agar plates containing 1.6% gelatin. We also analysed the ability of *Enterococcus* strains to release and decompose hydrogen peroxide (using Analytical Merckoquant peroxide test strips) and tested their ability to adhere to Caco-2 human gut epithelium cells and form biofilms *in vitro*.

**RESULTS:** A comparison of the genomes of *Enterococcus* strains isolated from the inflamed mucosa of patients with IBD with those of the control group showed statistically significant differences in the frequency of the *asa1* gene and the *gelE* gene. Furthermore, the cumulative occurrence of different virulence genes in the genome of a single strain of *Enterococcus* isolated from the IBD patient group is greater than in a strain from the control group, although no significant difference was found. Statistically significant differences in the decomposition of hydrogen peroxide and adherence to the Caco-2 epithelial cell line between the strains from the patient group and control group were demonstrated. The results also showed that profuse biofilm production was more frequent among *Enterococcus* strains isolated from children with IBD than in control strains.

**CONCLUSION:** *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess antioxidant defence mechanisms seem to have the greatest influence on the inflammatory process.

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**Key words:** *Enterococcus*; Virulence factors; Inflammatory bowel disease; Hydrogen peroxide; Biofilm

**Core tip:** In this research we have attempted to show

which features make *Enterococcus* strains contributing to the development and maintenance of the inflammatory process in patients with inflammatory bowel disease. The outcome of this research may have an impact on better understanding of the pathomechanisms of this disease, as its etiology is not fully known. The study results suggest that *Enterococcus* strains which adhere strongly to the intestinal epithelium, form biofilm as well as possess the enzymatic mechanisms protecting them against the effects of reactive oxygen species, seem to have the highest chances to survive and influence the inflammatory process.

Golińska E, Tomusiak A, Gosiewski T, Więcek G, Machul A, Mikołajczyk D, Bulanda M, Heczko PB, Strus M. Virulence factors of *Enterococcus* strains isolated from patients with inflammatory bowel disease. *World J Gastroenterol* 2013; 19(23): 3562-3572 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3562.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3562>

## INTRODUCTION

Inflammatory bowel disease (IBD) refers to two disease entities: ulcerative colitis (UC) and Crohn's disease (CD). UC is a chronic inflammatory process of the mucous membranes of the rectum and colon. Inflammatory changes in the form of recurrent surface ulcerations are located along the whole length of the large intestine. The course of UC course ranges from mild disease activity, lasting for years, to severe disease that can result in death after only a few weeks. CD is a chronic inflammatory process that can affect any part of the alimentary system, from the oral cavity to the rectum.

The aetiology of IBD is not yet fully understood<sup>[1]</sup>. Based on numerous clinical observations and results of extensive *in vitro* and *in vivo* experiments, it is currently thought that this disease develops as a result of the concurrence of three factors: genetic predisposition, disorders of the immune system and the influence of the environmental factors. Among the environmental factors, changes in the composition of the bacterial flora that colonise the human alimentary tract are considered to be of great influence<sup>[2]</sup>. Consequently, a great deal of attention is currently devoted to the study of the bacterial species that constitute the normal alimentary tract flora compared to those found in the tract of the IBD patients.

From 2004 to 2006, research on the qualitative and quantitative changes in the microflora of the alimentary tracts of a group of children who had been diagnosed with CD or UC for the first time was performed in our department<sup>[3,4]</sup>. No single aetiological factor that could be responsible for the exacerbation of the progressing inflammatory process was confirmed based on the obtained results.

However, the analysis of the quantitative changes in the group of children with CD identified a significant

increase in the population of cocci (including *Streptococcus* and *Enterococcus*) and bacteria belonging to the genus *Lactobacillus* in the inflammatorily changed sites with a simultaneous decrease in the number of strictly anaerobic bacteria, particularly those belonging to the genus *Bifidobacterium*.

All of the above-mentioned bacteria are important members of the commensal flora of the human colon. Therefore, we must consider the mechanisms by which these bacteria may contribute to the development and/or maintenance of the inflammatory process in patients suffering from IBD. The virulence potential of the genus *Enterococcus* has been established in many publications, and it is this genus contributes to infections such as peritonitis, bloodstream and urinary tract infections and endocarditis<sup>[5,6]</sup>. Their potential role in the pathomechanisms leading to IBD has also been highlighted by research utilising *IL-10* gene knockout mice to show that *Enterococcus faecalis* (*E. faecalis*) can induce IBD<sup>[7]</sup>.

Recent studies on the pathogenicity of enterococci indicate that the genomes of strains that are able to cause tissue damage and inflammation contain a pathogenicity island that encodes aggregation substance (AS), gelatinase, extracellular surface proteins (Esp), cytolysin, hyaluronidase and other proteins<sup>[8,9]</sup>. Enterococci that express AS were found to resist phagocytosis significantly better than an isogenic AS-negative strain by inhibiting the respiratory burst of macrophages<sup>[10]</sup>. Gelatinase, a protease produced by enterococci, is capable of hydrolysing gelatin, collagen, casein, haemoglobin and other peptides<sup>[9]</sup>. The Esp enhance biofilm formation in *E. faecalis*<sup>[11]</sup>. Cytolysin produced by the enterococci is lethal for a broad range of prokaryotic and eukaryotic cells<sup>[12]</sup>. Hyaluronidase is mainly a degradative enzyme that is associated with tissue damage<sup>[13]</sup>.

Little attention has been devoted to the ability of enterococci to release hydrogen peroxide into the extracellular space<sup>[14]</sup>. Pursuant to the results obtained previously by our group, select members of several genera, including *Streptococcus*, *Enterococcus* and *Lactobacillus*, are, under aerobic conditions, able to produce amounts of hydrogen peroxide comparable to those released by cells of the immune system during the oxidative burst<sup>[15]</sup>. This additional source of hydrogen peroxide could help sustain, or even exacerbate, gut inflammation<sup>[16]</sup>. Notably, certain *Enterococcus* strains can defend themselves against the surplus of reactive oxygen species (ROS) by producing anti-oxidative enzymes to increase their chances of survival in unfavourable conditions<sup>[17]</sup>.

Thus, the main objective of this work was to compare the occurrence of genes encoding selected virulence factors [surface aggregating protein (*asa1*), gelatinase (*gelE*), cytolysin (*cytA*), extracellular surface protein (*esp*) and hyaluronidase (*hyl*)] in the genomes of *Enterococcus* strains isolated from patients suffering from IBD with the occurrence of these genes in the genomes of strains derived from the control group subjects. Additionally, strains confirmed positive for the *gelE* gene were tested for the presence of the quorum sensing genes *csrA-C*

that regulate gelatinase production, and gelatinase activity was tested on agar plates containing 1.6% gelatin. The adherence of the *Enterococcus* strains to Caco-2 epithelial cells and their ability to form biofilms were also tested. Furthermore, the ability of strains isolated from inflamed or non-inflamed gut mucosa to release and decompose extracellular hydrogen peroxide was assessed.

## MATERIALS AND METHODS

### Bacterial strains

*Enterococcus* strains were isolated from colon biopsies of 34 children who were diagnosed with IBD for the first time and 24 patients from the control group comprised of children with functional bowel disorders that were hospitalised in the same hospital during the same period of time. Neither the IBD patients nor the participants in the control group received antibiotics during the two weeks prior to the study. The diagnosis of CD or UC was based on endoscopic, histopathological, immunological and radiological criteria<sup>[3,4]</sup>. The biopsies were taken from both groups of patients during colonoscopy procedures carried out at the Clinic of Paediatrics, Gastroenterology and Nutrition of the Polish-American Institute of Paediatrics of the Jagiellonian University Medical College in Cracow pursuant to the approval of the Bioethical Committee (No. KBET/236/B/2002). The patients were prepared for the colonoscopy using routine washing procedures described by Gosiewski *et al.*<sup>[4]</sup>. During the colonoscopy, the location and intensity of the lesions was assessed, and tissue fragments were obtained for histopathological and microbiological examinations. The biopsy samples were transported to the microbiological laboratory in Schaedler broth (SAB, Difco, United States, + 10% glycerol) at -20 °C.

In the laboratory, the frozen samples were thawed, weighed and homogenised in 1 mL of SAB and quantitatively analysed for the main cultivable bacterial constituents of the colon microflora using differential media in aerobic and anaerobic conditions<sup>[3,4]</sup>. These steps were performed aseptically in an anaerobic chamber (MACS - MG 500 Work Station, DW Scientific, Shipley, United Kingdom) in N<sub>2</sub> (85%) + H<sub>2</sub> (10%) + CO<sub>2</sub> (5%) atmosphere. The homogenised samples were serially diluted with SAB and 100 µL aliquots plated on the following media: McConkey Agar (Oxoid, Basingstoke, United Kingdom) for *Enterobacteriaceae*, Columbia Blood Agar (Difco, Lawrence, Kansas, United States) with 5% sheep blood for streptococci, Enterococcosel agar (BBL, BD, Franklin Lakes, United States) for enterococci, MRS agar (Oxoid) for lactobacilli and other lactic acid bacteria, BL agar for bifidobacteria and Wilkins-Chalgren agar base (Difco) with supplements for *Bacteroides*.

The morphology of the grown colonies was analysed using a magnifying glass, and several colonies of each morphological type were subcultured on appropriate aerobic and anaerobic media and subsequently Gram-stained. After further incubation and culture purity checks, the phenotypic identification was conducted

with the use of commercial identification systems (API 20E, API20A, APIStaph, APIStrep, bioMerieux, Marcy l'Étoile, France; BBL Crystal ID System, BD, Franklin Lakes, United States). The identification was confirmed by species-specific multiplex polymerase chain reaction (PCR) as described by Jackson *et al.*<sup>[18]</sup>.

The selected strains were preserved at -80 °C on glass beads in BBL Nutrient Broth with the addition of 15% glycerol.

### Multiplex PCR

To detect presence of genes encoding selected virulence factors (*asa1*, *gelE*, *cylA*, *esp*, *hyl*) in the genomes of the tested strains, multiplex PCR was applied according to the methods of Vankerckhoven<sup>[19]</sup>.

### PCR

To investigate the presence of quorum sensing genes (*fsrA*, *fsrB*, *fsrC*) in the genomes of all the strains positive for the *gelE* gene, PCR was applied pursuant to Qin *et al.*<sup>[20]</sup>.

The reference strain *E. faecalis* ATTC29212 containing the gene *fsrC* was used as the positive control. The negative control was a reference strain of *E. faecium* (ATTC 35667) that did not possess any of the examined genes.

The product sizes for *fsrA*, *fsrB* and *fsrC* were 484 bp, 574 bp and 580 bp, respectively (BLAST).

### Detection of gelatinase activity

The production of gelatinase in *Enterococcus* strains was detected by the method described by Steck *et al.*<sup>[6]</sup>. Supernatants from overnight cultures were spotted onto tryptic soy agar supplemented with 0.5 g/L L-cysteine and 1.6% Difco gelatine. The zone of clearance was measured after 24 h of incubation.

### Adherence to human gut epithelium cells and evaluation of biofilm production

The ability of *Enterococcus* strains to adhere to Caco-2 human gut epithelium cells (ATCC HTB-37) was assessed using an *in vitro* assay according to Górska-Frączek<sup>[21]</sup>. Caco-2 cells were cultured for 24 h in a 12-well flat bottom tissue culture plate at a density of  $1 \times 10^6$  cells/mL (Iwaki, Japan) in Eagle's 1959 medium (MEM) with L-glutamine and NaHCO<sub>3</sub> (IITD, Wrocław, Poland) containing 5% foetal calf serum (Sigma-Aldrich Chemie, Germany) and antibiotics (penicillin 100 UI/mL, streptomycin 100 UI/mL, neomycin 200 µg/mL) (Sigma Aldrich Chemie, Germany) and were washed twice with PBS. Overnight cultures of bacteria were diluted with MRS+MEM to a concentration of about  $10^8$  CFU/mL. The Caco-2 cells were inoculated with the bacterial cultures. After incubating at 37 °C for 30 min, wells were washed twice with PBS to release unbound bacteria. Then, the cells were fixed with 3.7% formaldehyde for 1 h, washed twice with PBS and stained with crystal violet stain (Merck, Germany). The degree of adhesion was evaluated using a semiquantitative scoring system ranging from (-) to (+++). The adherent *Enterococcus* cells were

counted in 20 randomly selected microscopic fields, as we have previously described<sup>[22]</sup>.

Fifteen strains of enterococci collected from children with IBD and 10 strains from the control children were examined for biofilm formation in sterile plastic 96-well plates with an adherent surface (Greiner Bio-One, United States). Bacterial cells were grown in 10 mL of trypticase soy broth (TSB, Difco) at 37 °C for 24 h in aerobic conditions. The culture was then centrifuged (2000 r/min; 10 min) and washed with 10 mL of saline. A suspension of each strain ( $1 \times 10^5$  CFU/mL) was prepared by serial dilution of bacteria in saline using MacFarland's scale, and then 20 µL of the prepared suspension was added to a well followed by 180 µL of sterile TSB. The final concentration of bacteria was  $1 \times 10^4$  CFU/mL. The plates were centrifuged for 10 min at 2000 r/min to sediment the bacteria onto the bottom of each well and were then incubated for 48 h (37 °C, aerobic conditions). The biofilm quantity (total mass of bacterial polysaccharides) was measured using Congo red dye according to a modified procedure described by Reuter *et al.*<sup>[23]</sup>. Briefly, at two different time points (0 and 48 h), the plates were centrifuged, the culture medium was gently removed from the wells and 200 µL of 0.1% Congo red solution was immediately added. The plates were left at room temperature for 30 min and then washed twice with buffered saline to remove unbound dye. The absorbance was measured at a wavelength of 492 nm using a spectrophotometer (Awareness Technology, Inc.). All measurements were performed in triplicate, and the mean values  $\pm$  SD were calculated. The degree of biofilm production by the tested cocci was arbitrarily categorised as either highly positive ( $A_{492} \geq 0.81$ ) or weakly positive ( $A_{492} \leq 0.8$ ).

#### Measurement of hydrogen peroxide production

Analytical Merckoquant peroxide test strips (Merck, NJ, United States) were used to measure H<sub>2</sub>O<sub>2</sub> production by the tested strains on a detection scale between 0 and 100 mg/L. The tested bacteria were suspended in 2 mL of TSB broth (TSB) (Difco, Kansas, United States) and cultured at 37 °C in aerobic conditions. The measurements of H<sub>2</sub>O<sub>2</sub> were performed after 4 and 24 h according to the procedure provided by the manufacturer. The mean density of bacterial growth at 4 h was approximately  $3 \times 10^6$  CFU/mL and increased to  $1 \times 10^7$  CFU/mL after 24 h. Uninoculated TSB broth was used as a negative control. The amounts of H<sub>2</sub>O<sub>2</sub> are presented as mmol/L.

#### Kinetics of the decomposition of hydrogen peroxide

The bacterial strains were cultured in 10 mL of TSB for 24 h at 37 °C in aerobic conditions. Their final density was approximately 0.5 on the McFarland scale. After 24 h, chemically pure hydrogen peroxide (Sigma-Aldrich, United States) was added to each test culture at a final concentration of 2 mmol/L. The culture was incubated under the same conditions for 4 h, and the amount of hydrogen peroxide remaining in the test tube was then determined using Analytical Merckoquant peroxide test

strips (Merck, NJ, United States). The resulting amount of H<sub>2</sub>O<sub>2</sub> was converted from mg/L into mmol/L. Then, the number of bacterial cells in each culture was determined using the viable count method. These levels were comparable for all strains and equalled approximately  $8 \times 10^6$  CFU/mL. The negative control was sterile TSB containing 2 mmol/L H<sub>2</sub>O<sub>2</sub>.

#### Statistical analysis

The statistical analysis was focused on comparison of probabilities of the analyzed genes presence between the group of the bacteria isolated from IBD children and this from control group. Such comparison was done using frequency  $\chi^2$  test. If the given frequencies didn't fulfill the assumption of the test it's less strong equivalents: likelihood ratio of Fisher's exact test were used. The value  $P < 0.05$  was regarded as the threshold for statistical significance. All calculations were performed using JMP 7.0.2 (SAS, United States) software package.

## RESULTS

In total, 48 strains belonging to the genus *Enterococcus* were isolated. Of these, 34 strains originated from children diagnosed with IBD, including 21 from children diagnosed with CD and 13 from children with UC. In this group, 16 (47%) strains belonged to *E. faecalis*, 10 (29%) to *E. avium* and 8 (24%) to *E. faecium*. In the control group, 5 (36%) out of 14 strains were identified as *E. faecalis*, 4 (29%) as *E. avium*, 3 (21%) as *E. faecium* and 2 (14%) as *E. durans* (Table 1).

For further studies, 28 *Enterococcus* strains were randomly selected, including 16 strains from patients with IBD ( $n = 9$  CD,  $n = 7$  UC) and 12 strains from the control group (Tables 2, 3 and 4). Molecular typing of all *Enterococcus* isolates using PFGE procedure (not described here) was performed before selection to eliminate redundant pulsotypes. No clustering within individual species or across the entire set of strains was noted.

While identifying genes coding for selected virulence factors (*asa1*, *gelE*, *cylA*, *esp*, *hyl*) in the genomes of the *Enterococcus* strains isolated from the inflamed mucosa of IBD patients and from control group patients, statistically significant differences in the frequencies of the *asa1* gene, which encodes the surface aggregating proteins, and the *gelE* gene ( $P = 0.0091$ ), which encodes the gelatinase that is responsible for decomposing collagen and elastin, were confirmed (Tables 2 and 3; Figures 1 and 2).

The *cylA* gene, which encodes cytolysin, was detected in 2 strains of *E. faecalis* and 1 strain of *Enterococcus faecium* isolated from the inflamed sites. This gene was not detected in the control strains. The obtained results were at the limit of statistical significance ( $P = 0.0569$ ). The *esp* gene encoding the extracellular surface protein was present in 11 strains isolated from patients with IBD and in 5 strains isolated from the control group. None of the 28 strains examined, including strains from both research groups, contained the *hyl* gene that encodes hy-

**Table 1** List of *Enterococcus* strains used in the experiment

| Number of isolates | Strain number | Species (API 20 Strep/Multiplex PCR) | Disease entry/Patient number |
|--------------------|---------------|--------------------------------------|------------------------------|
| 1                  | 3A            | <i>Enterococcus faecium</i>          | CD/1                         |
| 2                  | 3B            | <i>Enterococcus faecium</i>          |                              |
| 3                  | 10A           | <i>Enterococcus avium</i>            | CD/2                         |
| 4                  | 12A/1         | <i>Enterococcus faecalis</i>         |                              |
| 5                  | 12A/2         | <i>Enterococcus avium</i>            | CD/3                         |
| 6                  | 12B           | <i>Enterococcus faecium</i>          |                              |
| 7                  | 25A           | <i>Enterococcus faecium</i>          | CD/4                         |
| 8                  | 25B           | <i>Enterococcus avium</i>            |                              |
| 9                  | 31B           | <i>Enterococcus faecalis</i>         | CD/5                         |
| 10                 | 42A/1         | <i>Enterococcus faecalis</i>         | CD/6                         |
| 11                 | 42A/2         | <i>Enterococcus faecium</i>          |                              |
| 12                 | 51A/1         | <i>Enterococcus faecalis</i>         | CD/7                         |
| 13                 | 51A/2         | <i>Enterococcus avium</i>            |                              |
| 14                 | 57A           | <i>Enterococcus faecium</i>          | CD/8                         |
| 15                 | 8A            | <i>Enterococcus faecalis</i>         | CD/9                         |
| 16                 | 8B/1          | <i>Enterococcus faecalis</i>         |                              |
| 17                 | 8B/2          | <i>Enterococcus avium</i>            |                              |
| 18                 | 19B           | <i>Enterococcus faecium</i>          | CD/10                        |
| 19                 | 22B           | <i>Enterococcus faecalis</i>         | CD/11                        |
| 20                 | 69A           | <i>Enterococcus faecalis</i>         | CD/12                        |
| 21                 | 79A           | <i>Enterococcus faecalis</i>         | CD/13                        |
| 22                 | 32B           | <i>Enterococcus faecalis</i>         | UC/1                         |
| 23                 | 35A           | <i>Enterococcus faecalis</i>         | UC/2                         |
| 24                 | 40A           | <i>Enterococcus avium</i>            | UC/3                         |
| 25                 | 40B           | <i>Enterococcus avium</i>            |                              |
| 26                 | 48A           | <i>Enterococcus faecalis</i>         | UC/4                         |
| 27                 | 58A           | <i>Enterococcus faecalis</i>         | UC/5                         |
| 28                 | 77A           | <i>Enterococcus faecalis</i>         | UC/6                         |
| 29                 | 33B           | <i>Enterococcus avium</i>            | UC/7                         |
| 30                 | 29B           | <i>Enterococcus avium</i>            | UC/8                         |
| 31                 | 34A/1         | <i>Enterococcus faecium</i>          | UC/9                         |
| 32                 | 34A/2         | <i>Enterococcus avium</i>            |                              |
| 33                 | 64B           | <i>Enterococcus faecalis</i>         | UC/10                        |
| 34                 | 65A           | <i>Enterococcus faecalis</i>         | UC/11                        |
| 35                 | 5B            | <i>Enterococcus faecalis</i>         | Control/1                    |
| 36                 | 7B            | <i>Enterococcus faecium</i>          | Control/2                    |
| 37                 | 15B/1         | <i>Enterococcus avium</i>            | Control/3                    |
| 38                 | 15B/2         | <i>Enterococcus durans</i>           | Control/4                    |
| 39                 | 16B/1         | <i>Enterococcus avium</i>            | Control/5                    |
| 40                 | 16B/2         | <i>Enterococcus durans</i>           |                              |
| 41                 | 27B/1         | <i>Enterococcus faecalis</i>         | Control/6                    |
| 42                 | 27B/2         | <i>Enterococcus faecium</i>          |                              |
| 43                 | 27B/3         | <i>Enterococcus avium</i>            |                              |
| 44                 | 30B/1         | <i>Enterococcus faecium</i>          | Control/7                    |
| 45                 | 30B/2         | <i>Enterococcus avium</i>            |                              |
| 46                 | 46B           | <i>Enterococcus faecalis</i>         | Control/8                    |
| 47                 | 55B           | <i>Enterococcus faecalis</i>         | Control/9                    |
| 48                 | 66B           | <i>Enterococcus faecalis</i>         | Control/10                   |

CD: Crohn's disease; UC: Ulcerative colitis; PCR: Polymerase chain reaction Species identification was conducted with the use of API Strep system (bioMérieux) and multiplex polymerase chain reaction assays.

aluronidase. No statistically significant differences were observed in the prevalence of *esp* or *hyl* between the two patient groups (Figures 1 and 2).

The cumulative occurrence of different virulence genes in the genome of a single strain of *Enterococcus* isolated from patients suffering from IBD was greater than that of a strain isolated from the control group (mean of 2.6 vs 2.0, respectively), but no significant difference was found. For instance, the strains *E. faecalis* 35A and *E. faecium* 57A were isolated from patients with IBD and pos-

sessed as many as 4 virulence genes (*cylA*, *esp*, *asa1* and *gelE*), but strains containing all four of these genes were not observed in *Enterococcus* isolates from the control group.

Further analysis was performed on strains in which the presence of the *gelE* gene was confirmed ( $n = 19$ ). Based on the results of a PCR assay for the presence of the regulator genes *fsrA-C*, statistically significant differences between the two groups were noted for the occurrence of *fsrC*. This gene was confirmed in 8 *Enterococcus* strains isolated from the study group, but it was detected in only 2 strains from the control group ( $P = 0.0195$ ). Furthermore, the presence of *fsrA* was confirmed in 3 strains from the study group and in 1 *Enterococcus* strain from the control group. The *fsrB* gene was detected in the genome of 3 strains isolated from the IBD children and in 2 strains from the control group children. These results are shown in Table 2.

Enterococci containing the gene encoding gelatinase also underwent an *in vitro* test in which the activity of gelatinase on agar plates containing 1.6% gelatin was examined. Zones of clearance were demonstrated only on plates inoculated with cultures of strains that had all three regulator genes (*fsrA*, *B* and *C*): 2 *Enterococcus* strains from the studied group and 1 strain from the control group. No statistically significant differences were shown between these two groups. The results are shown in Table 2.

While testing the ability of the enterococci to adhere to Caco-2 cells, statistically significant differences were demonstrated between the bacteria isolated from IBD children compared to controls. Ten of the 24 enterococcal strains from the IBD group strongly adhered to the Caco-2 cells ( $P = 0.0238$ ). Only 1 strain from the control group adhered to the epithelial cell line used in the study. It is important to note that adherence was demonstrated by all strains having the *asa1* gene, which encodes the surface aggregating protein and allows for increased adherence of the bacteria to the host's tissues. The results of this study are shown in Table 3. However, 6 strains from the control group and 1 strain from the study group with no confirmed presence of *asa1* gene also adhered to the cells. Therefore, there are other virulence factors that increase bacterial adherence to host tissues.

All of the tested *Enterococcus* strains were able to form biofilms structure within 48 h. However, the results demonstrated that profuse biofilm production was more frequent among strains isolated from children with IBD than in control strains. Among the enterococcal strains isolated from the studied group, 11 (73.3%) were abundant biofilm producers (high positive;  $A_{492} \geq 0.81$ ), and 4 (26.7%) were weak biofilm producers (low positive;  $A_{492} \leq 0.8$ ). Of the 10 strains isolated from the control group, 2 (20%) were classified as abundant biofilm producers and 8 (80%) were weak producers. The difference between the IBD and control groups was not statistically significant. The results of the biofilm formation analysis are shown in Table 3 and Figure 3.

While analysing the ability of the strains to produce

**Table 2** Presence of the *gelE* gene, quorum sensing genes (*fsrA*, *fsrB*, *fsrC*) and gelatinase activity in tested *Enterococcus* strains

| Source             | Strains                            | Detection of genes                 |             |             |             | Gelatinase activity on 16% gelatin plates |   |
|--------------------|------------------------------------|------------------------------------|-------------|-------------|-------------|---|---|
|                    |                                    | <i>gelE</i>                        | <i>fsrA</i> | <i>fsrB</i> | <i>fsrC</i> |   |   |
| Crohn's disease    | <i>Enterococcus faecalis</i> 12A/1 | +                                  | -           | -           | +           | -   |   |
|                    | <i>Enterococcus faecalis</i> 31B   | +                                  | -           | +           | +           | -   |   |
|                    | <i>Enterococcus faecalis</i> 51A/1 | +                                  | -           | -           | +           | -   |   |
|                    | <i>Enterococcus faecium</i> 3A     | +                                  | +           | +           | +           | +   |   |
|                    | <i>Enterococcus faecium</i> 57A    | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecium</i> 19B    | +                                  | +           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 51A/2    | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 25B      | +                                  | -           | -           | +           | -   |   |
|                    | <i>Enterococcus avium</i> 10A      | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecalis</i> 35A   | +                                  | -           | -           | -           | -   |   |
| Ulcerative colitis | <i>Enterococcus faecalis</i> 48A   | +                                  | -           | -           | +           | -   |   |
|                    | <i>Enterococcus faecalis</i> 77A   | +                                  | -           | -           | +           | -   |   |
|                    | <i>Enterococcus faecalis</i> 32B   | +                                  | +           | +           | +           | +   |   |
|                    | <i>Enterococcus avium</i> 40A      | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 34A/2    | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 40B      | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecalis</i> 5B    | +                                  | -           | -           | +           | -   |   |
| Control            | <i>Enterococcus faecalis</i> 27B/1 | +                                  | +           | +           | +           | +   |   |
|                    | <i>Enterococcus faecalis</i> 46B   | +                                  | -           | +           | -           | -   |   |
|                    | <i>Enterococcus faecalis</i> 55B   | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecalis</i> 66B   | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecium</i> 27B/2  | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus faecium</i> 30B    | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 15B/1    | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 16B/1    | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus avium</i> 27B/3    | +                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus durans</i> 15B/2   | -                                  | -           | -           | -           | -   |   |
|                    | <i>Enterococcus durans</i> 16B/2   | -                                  | -           | -           | -           | -   |   |
|                    | ATCC control                       | <i>Enterococcus faecalis</i> 29212 | +           | -           | -           | +   | - |
|                    |                                    | <i>Enterococcus faecium</i> 35667  | -           | -           | -           | -   | - |

+: Presence of gene and/or gelatinase activity; -: Lack of gene and/or gelatinase activity Denotes significant ( $P = 0.0195$  vs control group) differences in the occurrence of the *fsrC* gene between the study group and the control group.

extracellular hydrogen peroxide, we observed that only *E. faecium* and *E. avium* species released hydrogen peroxide in amounts equal to or higher than 0.3 mmol/L. In total, 5 of the 16 *Enterococcus* strains isolated from patients with IBD and 3 of the 12 strains isolated from the control group were able to produce extracellular hydrogen peroxide, but this difference was not statistically significant (Table 4).

On the other hand, significant differences in the ability of the strains to decompose H<sub>2</sub>O<sub>2</sub> were observed upon examination of the 28 strains of *Enterococcus* isolated from both groups. Two subgroups could be distinguished based on the amount of decomposed hydrogen peroxide. The first group contained enterococci that decomposed 2 mmol/L of hydrogen peroxide to a level of 0-1 mmol/L in 4 h. The second group comprised strains that decomposed hydrogen peroxide to a level higher than 1 mmol/L. The difference in hydrogen peroxide decomposition ability was statistically significant between the *Enterococcus* strains isolated from patients with IBD and the strains isolated from control group patients ( $P = 0.04$ ; Figure 4).

## DISCUSSION

Recently, an important role has been ascribed to the

quantitative changes in the composition of the intestinal flora during inflammation<sup>[15,24]</sup>. These changes are likely related to the alterations in structure and function of the gut epithelium under inflammatory conditions that affect oxygenation, acidity and the functions of many enzymes and may lead to the preferential selection of microbial strains that are able to adapt to and/or interact with the inflamed epithelium.

In this work, *Enterococcus* strains were chosen for further studies because of their potential to be highly virulent. We sought to detect the differences between the virulence potential of the *Enterococcus* strains that colonised the intestines of children with IBD and control strains isolated from children without signs of gut inflammation.

The virulence of *Enterococci* can be linked to the presence of specific virulence factors encoded by specific genes, including cytolytic toxin, extracellular surface protein and AS, serine protease, gelatinase, cell wall adhesins, collagen-binding proteins and capsular polysaccharides<sup>[19,25]</sup>.

In the presented study, statistically significant differences were demonstrated for the occurrence of the *asa1* gene in the population of *Enterococcus* strains isolated from gut biopsies taken from patients with IBD in comparison to those cultured from those of the control

**Table 3** Presence of *asa1* gene, degree of adhesion to human gut epithelium cells and biofilm production among *Enterococcus* strains

| Source             | Strains                            | <i>asa1</i> | Adherence | Biofilm production (after 48 h) $A_{492\text{ nm}} \pm \text{SD}$ |
|--------------------|------------------------------------|-------------|-----------|---|
| Crohn's disease    | <i>Enterococcus faecalis</i> 12A/1 | -           | +++       | 0.995 ± 0.351   |
|                    | <i>Enterococcus faecalis</i> 31B   | -           | +++       | 1.302 ± 0.585   |
|                    | <i>Enterococcus faecalis</i> 51A/1 | -           | ++        | 1.083 ± 0.413   |
|                    | <i>Enterococcus faecium</i> 3A     | -           | -         | 1.286 ± 0.088   |
|                    | <i>Enterococcus faecium</i> 57A    | +           | +++       | 0.698 ± 0.066   |
|                    | <i>Enterococcus faecium</i> 19B    | +           | +++       | 1.087 ± 0.368   |
|                    | <i>Enterococcus avium</i> 51A/2    | -           | -         | 2.101 ± 0.424   |
|                    | <i>Enterococcus avium</i> 25B      | -           | -         | 2.474 ± 0.683   |
|                    | <i>Enterococcus avium</i> 10A      | -           | -         | 1.423 ± 0.284   |
| Ulcerative colitis | <i>Enterococcus faecalis</i> 35A   | +           | +++       | 1.528 ± 0.326   |
|                    | <i>Enterococcus faecalis</i> 48A   | -           | +++       | 0.767 ± 0.582   |
|                    | <i>Enterococcus faecalis</i> 77A   | -           | ++        | 0.822 ± 0.196   |
|                    | <i>Enterococcus faecalis</i> 32B   | +           | ++        | 0.557 ± 0.344   |
|                    | <i>Enterococcus avium</i> 40A      | -           | +++       | 0.859 ± 0.152   |
|                    | <i>Enterococcus avium</i> 34A/2    | -           | -         | 0.282 ± 0.078   |
|                    | <i>Enterococcus avium</i> 40B      | -           | -         | 0.512 ± 0.065   |
| Control            | <i>Enterococcus faecalis</i> 5B    | -           | -         | 0.512 ± 0.065   |
|                    | <i>Enterococcus faecalis</i> 27B/1 | -           | -         | 0.934 ± 0.103   |
|                    | <i>Enterococcus faecalis</i> 46B   | -           | ++        | 0.507 ± 0.095   |
|                    | <i>Enterococcus faecalis</i> 55B   | -           | -         | 0.408 ± 0.242   |
|                    | <i>Enterococcus faecalis</i> 66B   | -           | -         | 0.172 ± 0.031   |
|                    | <i>Enterococcus faecium</i> 27B/2  | -           | -         | 0.917 ± 0.269   |
|                    | <i>Enterococcus faecium</i> 30B    | -           | -         | 0.359 ± 0.087   |
|                    | <i>Enterococcus avium</i> 15B/1    | -           | -         | 0.305 ± 0.104   |
|                    | <i>Enterococcus avium</i> 16B/1    | -           | -         | 0.657 ± 0.182   |
|                    | <i>Enterococcus durans</i> 15B/2   | -           | -         | 0.315 ± 0.246   |
|                    | <i>Enterococcus durans</i> 16B/2   | -           | -         | 0.877 ± 0.221   |
| ATCC control       | <i>Enterococcus faecalis</i> 29212 | +           | +++       | 0.877 ± 0.221   |
|                    | <i>Enterococcus faecium</i> 35667  | -           | ++        | 0.722 ± 0.371   |

Adhesion degree was assessed as +++ (strong adhesive abilities); ++ (average adhesive abilities) and - (lack of adhesive abilities). Biofilm formation was measured in triplicate at two time points (0 and 48 h). The differences between the time points were averaged, and the mean ± SD are shown.

group without gut inflammation. The *asa1* gene is responsible for bacterial aggregation on the surface of the host tissues (particularly enterocytes, neutrophil granulocytes and epithelial cells of the urinary tract), and it also enables conjugation between bacteria due to increased hydrophobicity of the cell wall surface<sup>[26]</sup>. Consequently, strains that possess the *asa1* gene can develop large aggregations of bacterial cells during an infection and may simultaneously increase the bacterial population<sup>[27]</sup>. This property is likely to be important in the pathomechanism underlying IBD because during episodes of diarrhoea, one of the main symptoms of IBD, the majority of the alimentary tract flora is removed with the intestinal contents. Enterococci that express the *asa1* gene are able to strongly adhere to intestinal surfaces lacking a mucous layer and are able to form biofilms for protection against unfavourable environmental factors.

Furthermore, experiments using a rat model of endocarditis have shown that enterococci equipped with the *asa1* gene are better able to survive inside stimulated immune cells, indicating that they may possess enzymatic mechanisms that protect them against the influence of ROS secreted by macrophages during the respiratory burst<sup>[10]</sup>.

The *gelE* gene was more frequently found in the genomes of enterococci isolated from the group of chil-

dren with IBD. However, the presence of the *gelE* gene alone is not sufficient for the production of gelatinase protein by bacteria. Therefore, we also detected the quorum sensing genes that regulate gelatinase production. Gelatinase is a zinc-dependent metalloproteinase that is capable of hydrolysing gelatin, collagen, casein, haemoglobin and human endothelin<sup>[28]</sup>. Its activity is one of the main causes of the pathological changes in the host's body upon infection with *E. faecalis*<sup>[25]</sup>. It is therefore possible that colonisation of the alimentary tract of patients with IBD with *Enterococcus* strains possessing the *gelE* gene can weaken the tight junction protein connections between the epithelial cells lining the intestinal walls of the host, thus leading to the disruption of the mucous barrier<sup>[6]</sup>.

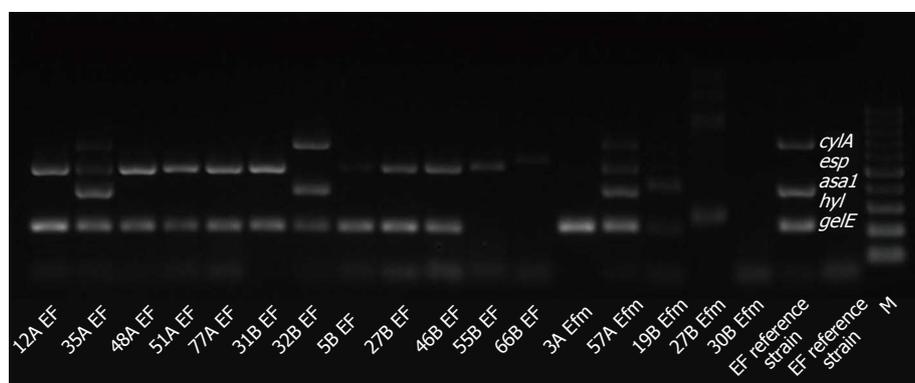
Cytolysin, encoded by the *cylA* gene, contributes to another pathomechanism of IBD that causes the excessive lysis of erythrocytes, leading to the uncontrolled release of large amounts of haemoglobin and subsequently heme and iron ions that influence the populations of *Enterobacteriaceae*, which are able to acquire iron<sup>[29]</sup>. Therefore, the increased number of enterococci that are able to decompose haemoglobin may be related to increases in the *E. coli* population observed in patients with IBD<sup>[30]</sup>.

Another *Enterococcus* virulence factor examined in this study was the *esp* gene, which encodes the extracellular

**Table 4** Mean amounts of hydrogen peroxide (in mmol/L) produced *in vitro* after 4 and 24 h by the tested strains

| Source                           | Strains                            | H <sub>2</sub> O <sub>2</sub> production (mmol/L) after 4 h | H <sub>2</sub> O <sub>2</sub> production (mmol/L) after 24 h |
|----------------------------------|------------------------------------|---|--|
| Crohn's disease                  | <i>Enterococcus faecalis</i> 12A/1 | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 31B   | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 51A/1 | 0   | 0  |
|                                  | <i>Enterococcus faecium</i> 3A     | 0   | 0  |
|                                  | <i>Enterococcus faecium</i> 57A    | 0   | 0  |
|                                  | <i>Enterococcus faecium</i> 19B    | 0   | 0.3  |
|                                  | <i>Enterococcus avium</i> 51A/2    | 0   | 0.3  |
|                                  | <i>Enterococcus avium</i> 25B      | 0   | 0.3  |
|                                  | <i>Enterococcus avium</i> 10A      | 0.3   | 1  |
|                                  | Ulcerative colitis                 | <i>Enterococcus faecalis</i> 35A                            | 0  |
| <i>Enterococcus faecalis</i> 48A |                                    | 0   | 0  |
| <i>Enterococcus faecalis</i> 77A |                                    | 0   | 0  |
| <i>Enterococcus faecalis</i> 32B |                                    | 0   | 0  |
| <i>Enterococcus avium</i> 40A    |                                    | 0   | 0  |
| <i>Enterococcus avium</i> 34A/2  |                                    | 0   | 0.3  |
| <i>Enterococcus avium</i> 40B    |                                    | 0   | 0  |
| Control                          | <i>Enterococcus faecalis</i> 5B    | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 27B/1 | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 46B   | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 55B   | 0   | 0  |
|                                  | <i>Enterococcus faecalis</i> 66B   | 0   | 0  |
|                                  | <i>Enterococcus faecium</i> 27B/2  | 0   | 0.3  |
|                                  | <i>Enterococcus faecium</i> 30B    | 0   | 0  |
|                                  | <i>Enterococcus avium</i> 15B/1    | 0.3   | 1  |
|                                  | <i>Enterococcus avium</i> 16B/1    | 0   | 0  |
|                                  | <i>Enterococcus avium</i> 27B/3    | 0   | 0.3  |
|                                  | <i>Enterococcus durans</i> 15B/2   | 0   | 0  |
|                                  | <i>Enterococcus durans</i> 16B/2   | 0   | 0  |

The measurement of H<sub>2</sub>O<sub>2</sub> was performed with the use of colorimetric method: Merckoquant peroxide test strips.



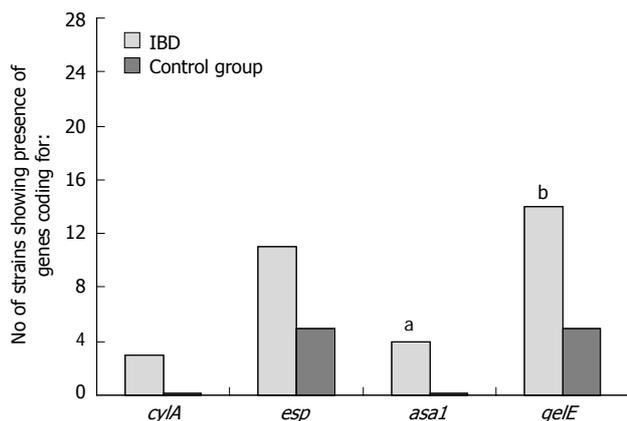
**Figure 1** Agarose gel electrophoresis of multiplex polymerase chain reaction amplification products from *Enterococcus* species isolates. The reference strain *Enterococcus faecalis* (*E. faecalis*) ATTC29212 (EF reference strain) possessing the genes *cylA*, *asa1* and *gelE* constituted the positive control. The negative control was a reference strain of *Enterococcus faecium* (*E. faecium*) ATTC 35667 Efm reference strain that did not possess any of the examined genes. Product sizes were as follows, *asa1*: 375 bp; *gelE*: 213 bp; *cylA*: 688 bp; *esp*: 510 bp; *hyl*: 276 bp. EF: Tested strains of *E. faecalis*; Efm: Tested strains of *E. faecium*; M: Mass marker 100-1000 bp.

surface protein (Esp). The *esp* gene was present in 11 strains of enterococci from the group of children with the IBD and in 5 strains from the control group. This finding may indicate that enterococci found in biopsy samples from ulcerations were able to adhere to and colonise the intestinal tissues more easily because Esp promotes primary attachment and biofilm formation in *E. faecalis*, as proved demonstrated by Toledo-Arana *et al*<sup>[31]</sup>.

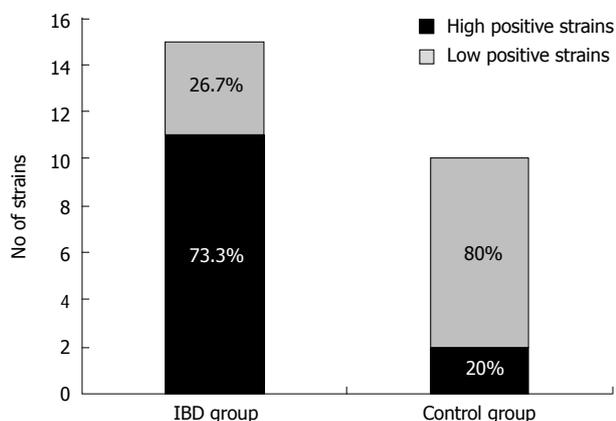
It should be noted here that our studies on the selected virulence factors were based only on the detection of

the specified genes that encode these factors, and more information could be obtained from the direct detection of these factors in bacteria using enterocytes in *in vitro* systems or in animal experiments because the expression of the studied genes may be specifically altered in inflamed tissues<sup>[32]</sup>.

In the alimentary tract of patients suffering from IBD, there can be a local accumulation of large amounts of ROS released by stimulated phagocytic cells, such as macrophages and neutrophils, due to the progressing

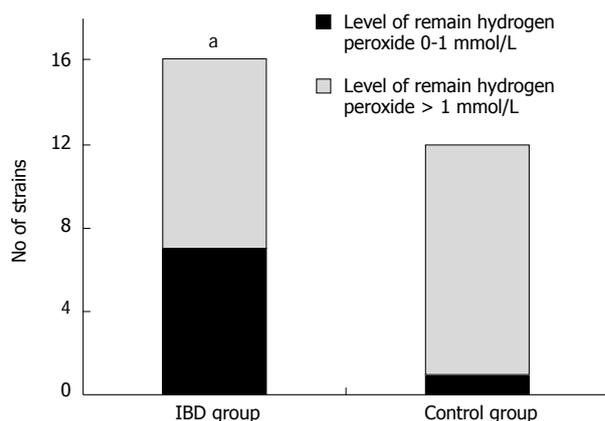


**Figure 2** Presence of virulence factors in *Enterococcus* strains. Significant differences in the occurrence of the *asa1* gene (<sup>a</sup>*P* = 0.0258 vs control group) and the *gelE* gene (<sup>b</sup>*P* = 0.0091 vs control group) were detected between strains isolated from children with inflammatory bowel disease (IBD) and strains from the participants in the control group.



**Figure 3** Comparison of the ability of the tested *Enterococcus* strains to form biofilms. The biofilm-forming abilities were assessed after 48 h, and all tested strains were classified as either abundant biofilm producers (high positive strains) or weakly adherent isolates (low positive strains). IBD: Inflammatory bowel disease.

acute and subsequently chronic inflammatory process. Additionally, some species of *Lactobacillus*, *Streptococcus* and *Enterococcus* that colonise the human intestinal tract are able to produce substantial amounts of superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) that are comparable to those released by immune cells<sup>[33,34]</sup>. However, as has been shown by Al-Mushrif and Jones, the oxygen concentration present in the specific ecological niche regulates the bacterial production of hydrogen peroxide<sup>[35]</sup>. As we have previously demonstrated and discussed elsewhere (Strus *et al*<sup>[15]</sup>), the oxygen tension in inflamed tissues during the course of IBD enables the propagation of facultative aerobes such as *Enterococcus* spp. and the production of considerable amounts of hydrogen peroxide and other metabolites. Selected *Enterococcus* strains belonging to the species *E. avium* and *E. faecium* isolated from patients with IBD and children from the control group were able to produce extracellular hydrogen peroxide in our studies. This result may indicate that the production of hydrogen



**Figure 4** Decomposition of hydrogen peroxide by the tested strains. Statistically significant differences (<sup>a</sup>*P* = 0.04 vs control group) in the decomposition of H<sub>2</sub>O<sub>2</sub> (to a level higher than 1 mmol/L) were detected between strains isolated from patients with inflammatory bowel disease and from participants in the control group.

peroxide is due to certain *Enterococcus* species rather than the inflammatory conditions at the site of their isolation.

Bacteria belonging to the genera *Streptococcus*, *Lactobacillus* and *Enterococcus* occur in increased numbers in the alimentary tracts patients with IBD and can produce significant amounts of H<sub>2</sub>O<sub>2</sub> that can consequently limit the population of anaerobic flora, stimulate the cells of the immune system to release pro-inflammatory cytokines and stimulate the apoptosis of intestinal epithelial cells deprived of their protective mucous layer<sup>[36]</sup>. Denning *et al*<sup>[16]</sup> showed that hydrogen peroxide concentrations ranging from 0.5 up to 2 mmol/L were able to induce apoptosis in the cells of the abdominal lining and intestinal epithelium; similar amounts of H<sub>2</sub>O<sub>2</sub> were produced *in vitro* by the *Enterococcus* strains examined in this study. These observations support the studies by Kruidenier *et al*<sup>[37]</sup> and underline the importance of oxidative stress in the pathogenesis of IBD.

During the inflammatory process, the intestinal epithelial cells are exposed to the effects of ROS. If this is a short-term process, no significant damage or disruption of host cell function are observed due to the production of enzymes that inactivate ROS, such as catalase and peroxidase, and DNA repair mechanisms. A significant risk for the intestinal epithelium occurs when ROS are generated at variable concentrations for a longer period of time. Then, not only can the host cells be damaged, but the qualitative and quantitative composition of the specific bacterial species that comprise the commensal flora changes as well<sup>[35]</sup>.

In this work, we showed that enterococci isolated from patients with IBD were able to decompose hydrogen peroxide considerably faster than those isolated from the control group. This may indicate that these strains survived the selective pressures of the inflammatory process. It is likely that only bacterial species possessing a system capable of enzymatic deactivation of ROS were able to survive and subsequently increase their populations on the surface of the damaged intestinal epithelial

cells in patients with IBD. Until recently, bacteria belonging to the genera *Enterococcus*, *Lactobacillus* and *Pediococcus* were regarded as catalase negative. However, it appears that these bacteria have a very efficient system of hydrogen peroxide deactivation based on manganese-containing catalase or haeme-dependent catalase<sup>[38]</sup>.

In this study, we present data that suggest that processes in the alimentary tract of patients with IBD (most probably based on inflammatory background of this disease) may lead to the selection of certain bacterial species or strains from the constituents of the commensal flora. Our observations have been supported by recent studies on microbiota from biopsies taken from patients with UC and controls performed by Fite *et al.*<sup>[39]</sup>, who detected statistically significant differences in the bacterial populations of the UC mucosa and in the control group that varied over the study period. High clinical activity indices and sigmoidoscopy scores were associated with enterobacteria, desulfovibrios, Type E *Clostridium perfringens* and *E. faecalis*. We believe that in case of *Enterococcus*, the strains that adhere strongly to the intestinal epithelium, build three-dimensional biofilm structures and possess enzymatic mechanisms to protect against the effects of ROS produced by the immune cells and other bacterial species have the highest chances of survival.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) is a chronic inflammation of all or part of the digestive tract. The aetiology of IBD is not fully known, but much attention is currently focused on the role of bacterial flora in the development and/or maintenance of the inflammatory process in patients suffering from this disease. Recent studies indicate that bacteria belonging to the genus *Enterococcus* play an important role in the pathomechanisms underlying IBD based on their virulence potential.

### Research frontiers

Changes in the composition of the bacterial flora colonising the human alimentary tract are considered influential in the maintenance of the inflammatory process in patients with IBD. The virulence potential the enterococci may be particularly important. It will be interesting to identify other factors that allow enterococci to develop and maintain the inflammatory process while increasing their chances of survival in unfavourable conditions.

### Innovations and breakthroughs

Recent studies have shown that no single aetiological factor could be responsible for the exacerbation of the inflammatory process in IBD. However, an analysis of the quantitative changes in the intestinal microflora of patients with Crohn's disease (CD) demonstrated a significant increase in the population of cocci (including *Enterococcus* and *Streptococcus*) at the sites of inflammation. In the present study, authors observed that virulence genes, such as *asa1* and *gelE*, occurred more frequently in the genomes of *Enterococcus* strains isolated from patients with IBD than in strains isolated from healthy patients. Moreover, authors demonstrated statistically significant differences in the ability of these strains to decompose hydrogen peroxide. Enterococci isolated from patients with IBD decomposed hydrogen peroxide considerably faster than those isolated from the control group. This result indicates that only select bacterial species with an efficient system of enzymatic deactivation of reactive oxygen species (ROS) are able to survive and subsequently increase their populations on the surface of the damaged intestinal epithelial cells.

### Applications

The results of this study suggest that *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess enzymatic mechanisms that protect against the effects of ROS have the highest chances surviving and influencing the inflammatory process. Their research improves our understand-

ing of the pathomechanisms underlying IBD.

### Peer review

An interesting publication in which authors determine the features of *Enterococcus* that contribute to the development and maintenance of the inflammatory process in patients with IBD. The results are interesting and suggest that *Enterococcus* strains that adhere strongly to the intestinal epithelium, form biofilms and possess antioxidant defence mechanisms seem to have the greatest influence on the inflammatory process.

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P- Reviewer Hays J S- Editor Wen LL L- Editor A  
E- Editor Zhang DN



## Aberrant glycosylation of the anti-Thomsen-Friedenreich glycotope immunoglobulin G in gastric cancer patients

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Supported by The Estonian Science foundation, No. 7317 and 8399

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Received: May 18, 2012 Revised: November 19, 2012

Accepted: December 5, 2012

Published online: June 21, 2013

### Abstract

**AIM:** To study whether alterations in the glycosylation of immunoglobulin G (IgG) specific to the Thomsen-Friedenreich glycotope (TF) have diagnostic and prognostic potential in gastric cancer.

**METHODS:** Serum samples were obtained from patients with histologically verified gastric carcinoma ( $n = 89$ ), healthy blood donors ( $n = 40$ ), and patients with benign stomach diseases ( $n = 22$ ). The lectin-enzyme-linked immunosorbent assay-based glycoprofiling of TF-specific IgG (anti-TF IgG) was performed using synthetic TF-polyacrylamide conjugate as antigen, total IgG purified by affinity chromatography on protein G sepharose, and lectins of various sugar specificities: mannose-specific concanavalin A (ConA), fucose-specific *Aleuria aurantia* lectin (AAL) and sialic acid-specific

*Sambucus nigra* agglutinin (SNA). The sensitivity and specificity of the differences between cancer patients and controls were evaluated by receiver operator characteristic (ROC) curve analysis. Overall survival was analyzed by the Kaplan-Meier method. Time-dependent ROC curve statistics were applied to determine cut-off values for survival analysis. All calculations and comparisons were performed using the GraphPad Prism 5 and SPSS 15.0 software.

**RESULTS:** The level of TF-specific IgG was significantly increased in cancer patients compared with non-cancer controls ( $P < 0.001$ ). This increase was pronounced mostly in stage 1 of the disease. Cancer patients showed a higher level of ConA binding to anti-TF-IgG ( $P < 0.05$ ) and a very low level of SNA lectin binding ( $P = 0.0001$ ). No appreciable stage-dependency of the binding of any lectin to anti-TF IgG was found. A strong positive correlation between the binding of AAL and SNA was found in all groups studied ( $r = 0.71-0.72$ ;  $P < 0.0001$ ). The changes in ConA reactivity were not related to those of the fucose- or sialic acid-specific lectin. Changes in the SNA binding index and the ConA/SNA binding ratio demonstrated good sensitivity and specificity for stomach cancer: sensitivity 78.79% (95%CI: 61.09-91.02) and 72.73% (95%CI: 57.21-85.04); specificity 79.17 (95%CI: 65.01-89.53) and 88.64% (95%CI: 71.8-96.6), for the SNA binding index and the ConA/SNA binding ratio, respectively. The other combinations of lectins did not improve the accuracy of the assay. The low level of ConA-positive anti-TF IgG was associated with a survival benefit in cancer patients (HR = 1.56; 95%CI: 0.78-3.09;  $P = 0.19$ ), especially in stages 3-4 of the disease (HR = 2.17; 95%CI: 0.98-4.79;  $P = 0.048$ ). A significantly better survival rate was found in all cancer patients with a low reactivity of anti-TF IgG to the fucose-specific AAL lectin (HR = 2.39; 95%CI: 1.0-5.7;  $P = 0.038$ ).

**CONCLUSION:** The changes in the TF-specific IgG glycosylation pattern can be used as a biomarker for

stomach cancer detection, and to predict patient survival.

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**Key words:** Thomsen-Friedenreich antigen; Anticarbhydrate antibodies; Stomach cancer; IgG glycosylation; Survival; Lectins

Kodar K, Izotova J, Klaamas K, Sergeyev B, Järvekülg L, Kurtenkov O. Aberrant glycosylation of the anti-Thomsen-Friedenreich glycotope immunoglobulin G in gastric cancer patients. *World J Gastroenterol* 2013; 19(23): 3573-3582 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3573.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3573>

## INTRODUCTION

The aberrant glycosylation often observed in cancer cells leads to the expression of tumor-associated carbohydrate antigens (TACA) which may be autoimmunogenic and recognized by autoantibodies<sup>[1-3]</sup>. This makes TACA a promising target for cancer immunotherapy. In cancer patients, an abnormal glycosylation pattern has also been observed for many circulating glycoconjugates, such as transferrin, MUC1 mucin, alpha1-acid glycoprotein, and immunoglobulins<sup>[6-10]</sup>. This suggests a systemic impact of malignancy on glycosylation machinery or possibly represents a specific feature of the host metabolism. In both cases, such changes might be considered as a biomarker of cancer, a premalignant state, or the disposition of the host to cancer (risk factors).

The Thomsen-Friedenreich antigen (TF, CD176, core-1) (Galβ1, 3GalNAcα/β-O-Ser/Thr) is expressed in many carcinomas and results from incomplete synthesis of O-linked glycans on glycoproteins and glycolipids<sup>[1,2]</sup>. The TF glycotope is known as a pancarcinoma antigen which is expressed in approximately 90% of all human cancers and in premalignant conditions<sup>[2,11]</sup>. TF expression is associated with more aggressive tumors and is related to the induction of invasion, metastasis and cancer surveillance mechanisms<sup>[12-16]</sup>. The TF antigen seems to play a crucial role in the adhesion of cancer cells to the endothelium through interaction with galectin-3, thereby promoting metastasis<sup>[17,18]</sup>.

Naturally-occurring TF antigen-specific immunoglobulin G (anti-TF IgG) autoantibodies are present in human serum in health and disease<sup>[3,19,20]</sup>. In cancer patients, their level is related to tumor progression and prognosis, being higher in patients with the early stages of the disease, in those with more differentiated tumors (G1-2), and in those with better survival<sup>[12,21,22]</sup>. This suggests an immediate impact of the humoral immune response on malignancy *via* direct or antibody-dependent cell-mediated effector pathways. However, the mechanisms behind these associations remain to be further elucidated.

Human serum IgG contains N-linked glycans attached to Asn297 on the fragment crystallizable (Fc) region. The Fc glycan structures are highly variable and differ in the level of terminal sialic acid, galactose (G0, G1, G2), core fucose and bisecting GlcNAc<sup>[23]</sup>. Changes in IgG Fc glycosylation strongly influence the Fc-receptor-mediated activities of antibody<sup>[23-25]</sup> and are associated with various pathologies, including cancer. However, little attention has been paid yet to the glycosylation of antibodies specific to tumor-associated antigens<sup>[26]</sup>. During the last decade, the diversity of IgG glycans has been thoroughly studied by the interaction of IgG with lectins<sup>[26-29]</sup>, as well as by mass spectrometry-based methodology<sup>[9,10,30]</sup>. Our recent studies demonstrated an increase in the level of the ConA lectin-positive glycoform of both total serum IgG and TF antigen-specific IgG in patients with cancer<sup>[8,31]</sup>. Moreover, the low level of this IgG glycoform was associated with an overall survival benefit in patients with gastric cancer<sup>[8]</sup>, indicating its functional relevance, as well as its potential clinical value. Similar changes in ConA reactivity have been reported for tumor-reactive IgG in patients with ovarian cancer<sup>[26]</sup>. However, the antigenic specificity of these antibodies remains unknown.

In an attempt to discover and evaluate potential biomarkers for stomach cancer diagnosis and patient prognosis, the TF antigen-specific IgG glycosylation profile was investigated using lectins of various sugar specificities. In this study, we demonstrate the aberrant glycosylation of anti-TF IgG in patients with stomach cancer, and the association of these changes with overall survival, indicating their potential clinical applicability.

## MATERIALS AND METHODS

### Subjects

Serum samples were obtained from healthy blood donors, patients with benign stomach diseases and patients with histologically verified gastric carcinoma (Table 1). The investigation was carried out in accordance with the ICH GCP Standards and approved by the Tallinn Medical Research Ethics Committee, Estonia. Written informed consent was obtained from each subject.

Tumor staging was based on the histopathological (pTNM) classification of malignant tumors. Serum samples were stored in aliquots at -20 °C until use.

### Serum IgG purification on protein G sepharose

To remove the anti-TF IgM and IgA isotype antibodies, preliminary purification of serum total IgG was performed on a Protein G HP Spin Trap column as described by the manufacturer (GE Healthcare, United States). The samples were immediately neutralized, dialyzed against phosphate buffered solution (PBS) - 0.1% NaN<sub>3</sub> and stored at + 4 °C until tested. About 8.5 mg of IgG was obtained from 1 mL of serum applied onto the Protein G Sepharose column.

**Table 1** Characteristics of the subjects tested<sup>1</sup>

| Groups                    | n  | Male | Female | M/F  | Median age (range), yr |
|---------------------------|----|------|--------|------|------------------------|
| Donors                    | 40 | 12   | 28     | 0.43 | 52.2 (23–70)           |
| Benign group <sup>2</sup> | 22 | 19   | 3      | 6.3  | 62.0 (44–76)           |
| Non-cancer <sup>3</sup>   | 62 | 31   | 31     | 1    | 59.5 (23–76)           |
| Cancer patients           | 89 | 53   | 36     | 1.47 | 66.0 (22–84)           |
| Stages 1–4                |    |      |        |      |                        |
| Stage 1                   | 18 | 8    | 10     | 0.86 | 65.0 (28–84)           |
| Stage 2                   | 19 | 14   | 5      | 1.67 | 66.5 (46–83)           |
| Stage 3                   | 42 | 22   | 20     | 0.83 | 65.0 (38–81)           |
| Stage 4                   | 10 | 9    | 1      | 9    | 65.0 (44–76)           |

<sup>1</sup>All subjects were tested for anti-Thomsen-Friedenreich glycotope IgG levels and the concanavalin A binding. *Aleuria aurantia* lectin and *Sambucus nigra* agglutinin binding test was performed in all donors and patients with benign gastric disease, and in 33 patients with stomach cancer (stage 1, *n* = 4; stage 2, *n* = 5; stage 3, *n* = 19; stage 4, *n* = 5); <sup>2</sup>Peptic ulcer disease, *n* = 6; chronic gastritis, *n* = 7; atrophic gastritis, *n* = 9; <sup>3</sup>Combined group of donors and patients with benign stomach disease. M/F: Male/female.

### Anti-TF IgG antibody assay

The anti-TF IgG level was determined by enzyme-linked immunosorbent assay (ELISA) as described elsewhere<sup>[21]</sup>, with minor modifications. Briefly, the plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with a synthetic TF-polyacrylamide conjugate (Lectinity, Russia, 10 mol% of carbohydrate) in carbonate buffer, pH 9.6, 5 µg per well. After overnight incubation, triple washing and blocking with a Superblock solution (Pierce, United States) for 15 min at 25 °C, the purified IgG samples (50 µg/well) in PBS-0.05% Tween (Tw) were applied for 1.5 h at 25 °C. After subsequent washing with PBS-Tw, the bound anti-TF IgG was detected with alkaline phosphatase conjugated goat anti-human IgG (Dako, United States) and p-nitrophenylphosphate disodium hexahydrate (Sigma, United States). The absorbance values were read at 405 nm (Tecan Reader, Austria). The relatively high doses of total IgG were applied because of the low concentration of anti-TF IgG in the serum. These IgG doses correspond approximately to the 1:25–1:50 serum dilution used in our previous studies<sup>[21]</sup>.

### Lectin reactivity of the TF-specific IgG

The lectin reactivity of the TF glycotope-specific IgG was measured in a similar way, except that the binding of mannose-specific concanavalin A (ConA), fucose-specific *Aleuria aurantia* lectin (AAL) and neuraminic acid (sialic acid)-specific *Sambucus nigra* agglutinin (SNA) to the absorbed anti-TF IgG was measured as described by Kodar *et al.*<sup>[8]</sup>. Biotinylated ConA (Sigma, United States) in the ConA binding buffer (0.05 mol/L Tris-HCl buffer, pH 7.2, containing 0.2 mol/L NaCl and 3 mmol/L CaCl<sub>2</sub>, MgCl<sub>2</sub> and MnCl<sub>2</sub> each), AAL (Vector Laboratories Inc., United States) in 10 mmol/L HEPES, 0.15 mol/L NaCl buffer, pH 7.5, and SNA (Vector Laboratories Inc., United States) in 10 mmol/L HEPES, 0.15 mol/L NaCl, 0.1 mmol/L CaCl<sub>2</sub>, pH 7.5, were each applied at a concentration of 5 µg/mL, for 1.5 h at 25 °C.

The bound lectins were detected with a streptavidin-alkaline phosphatase conjugate (Dako, United States) and p-nitrophenylphosphate (Sigma, United States). The optical density value (*A*) of control wells (blank: the Superblock solution instead of TF-PAA for anti-TF or no sample for lectin binding testing) was subtracted from that of the IgG-coated wells to determine the binding of both IgG and lectin. Each sample was analyzed in duplicate.

To standardize the assay, standard IgG was included in each plate for IgG determination and lectin binding measurement. The interassay variations were minimized by using the correction factor (CF): CF = 1/(standard serum *A* values - blank) × 100. The results were expressed in relative units (RU): RU = sample *A* value × CF. The lectin reactivity of anti-TF IgG was calculated as the lectin index: (sample lectin binding RU)/(sample anti-TF IgG binding RU).

### Statistical analysis

Comparisons between the groups were performed using the nonparametric Mann-Whitney *U* test for unpaired data or regression analysis with the Spearman test. Survival analysis was carried out using the Kaplan-Meier method. The receiver operator characteristic (ROC) curve analysis as generated in SPSS 15.0 was used to evaluate the sensitivity and specificity of the changes found for stomach cancer. The area under the ROC curve and the *P* value of the ROC curve were calculated. The time-dependent ROC curve statistics were applied to determine cut-off values for survival analysis. The difference between the groups was considered to be significant when *P* ≤ 0.05. All calculations were performed using the GraphPad Prism 5 and SPSS 15.0 software.

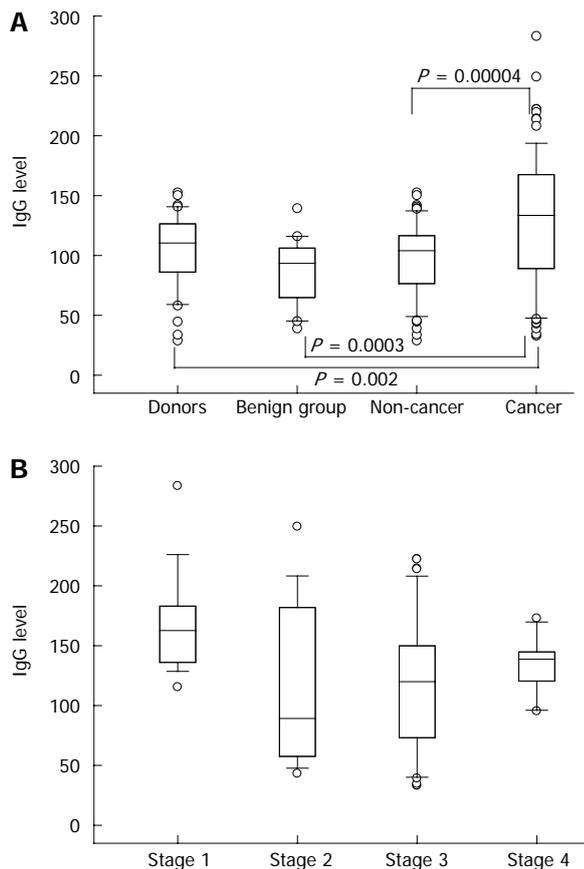
## RESULTS

### TF antigen-specific IgG antibody level in total IgG preparations

A significantly higher level of TF-specific IgG was found in purified total IgG preparations from the serum of patients with stomach cancer compared with that in controls: *P* = 0.002, 0.0003 and 0.00004 for donors, benign and combined non-cancer groups, respectively (Figure 1). This increase was mostly pronounced in stage 1 of the disease (*P* = 0.02, *P* = 0.0006, *P* = 0.01 compared with stages 2, 3 and 4, respectively). Up to 10-fold interindividual variations in anti-TF IgG antibody levels were observed in all the groups and especially in cancer patients.

### Lectin binding profile of TF-specific IgG

The anti-TF IgG of patients with cancer showed a significantly higher level of ConA-positive IgG glycoform than that of both controls: *P* = 0.013, 0.05 and 0.005 for donors, benign and non-cancer groups, respectively (Figures 2 and 3). In contrast, the binding of SNA was

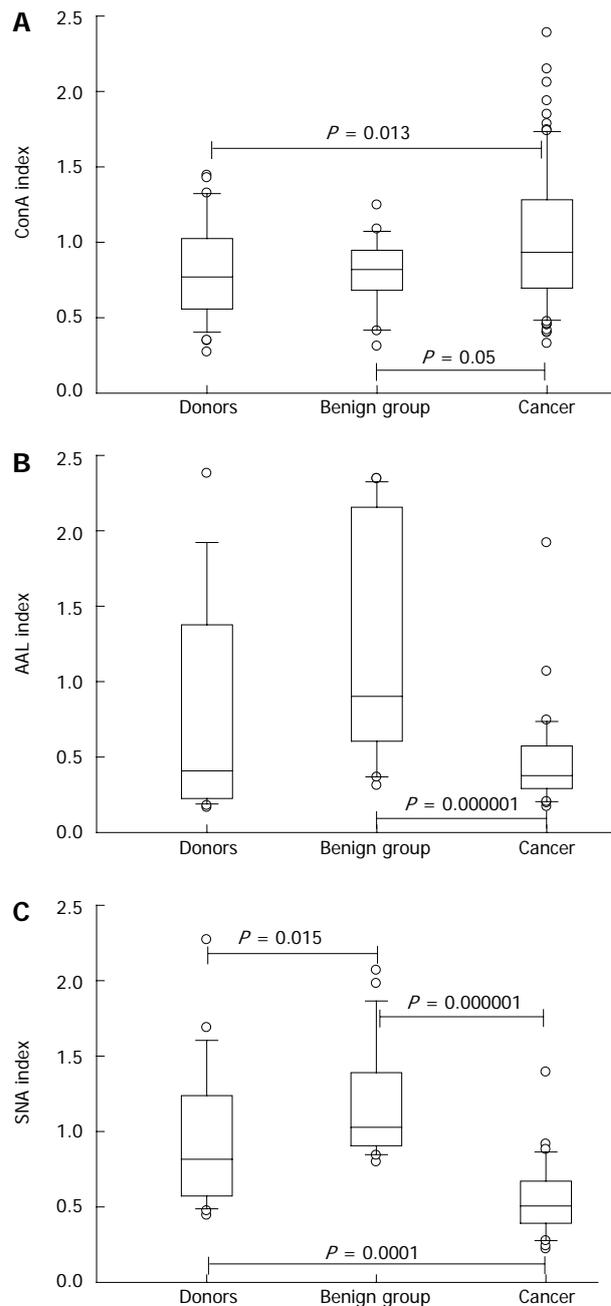


**Figure 1** Thomsen-Friedenreich glycotope-IgG level in patients with stomach cancer and controls. A: Box plots of anti-TF IgG levels (medians, ranges and quartiles) in controls and cancer patients; B: Anti-TF IgG levels in cancer patients by stage. Significantly higher in stage 1 compared with stages 2, 3 and 4 ( $P = 0.02$ ,  $0.001$  and  $0.01$ , respectively).  $P$  values were calculated by the Mann-Whitney  $U$  test.

significantly lower in cancer patients compared with that of blood donors and patients with benign gastric diseases ( $P < 0.0001$ ). In cancer patients, the binding of AAL did not differ from that of the donors ( $P = 0.64$ ), but was significantly lower than that of the benign group ( $P = 0.000008$ ) or non-cancer group ( $P = 0.005$ ). A group of patients with chronic gastritis ( $n = 7$ , one with atrophic gastritis), who showed very high AAL index values, accounted for this difference. This was the only exception in this study when the benign group differed significantly from the donors ( $P = 0.01$ ). Two of these patients also showed a high level of SNA binding. All patients with peptic ulcer disease ( $n = 6$ ) demonstrated a similar level of binding for all three lectins compared with that of blood donors.

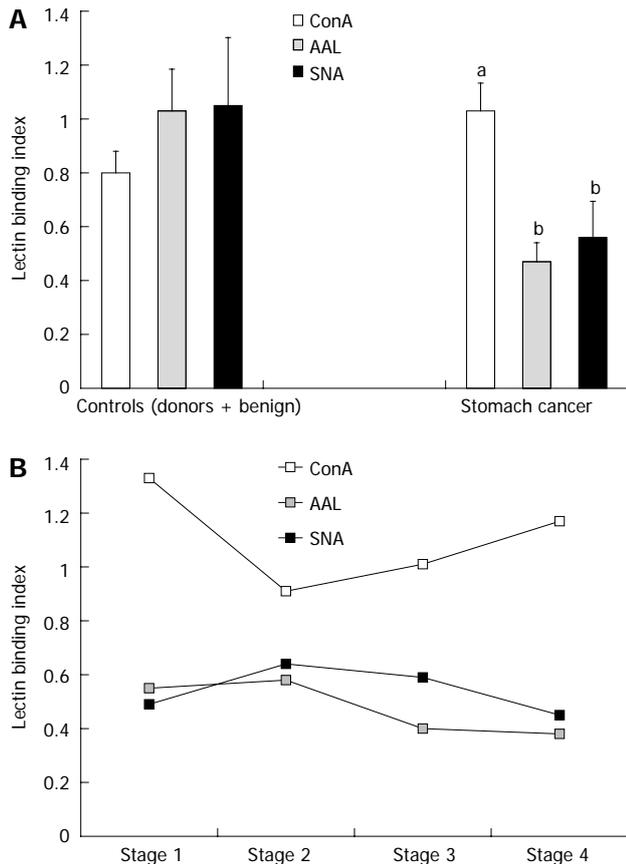
No appreciable stage-dependency of the binding of any lectin to the anti-TF IgG was found (Figure 3), though a slight trend towards higher ConA index values in stage 1 cancer patients was observed ( $P = 0.19$  compared with stage 3 patients).

A strong positive correlation between the reactivities of AAL and SNA was demonstrated in all groups: cancer



**Figure 2** Binding of lectins to the Thomsen-Friedenreich glycotope-specific IgG in gastric cancer patients and controls. A: Concanavalin A (ConA); B: *Aleuria aurantia* lectin (AAL); C: *Sambucus nigra* agglutinin (SNA). Box plots of lectin index values (medians, ranges and quartiles) in patients with stomach cancer, healthy blood donors and patients with benign stomach disease.  $P$  values were calculated by the Mann-Whitney  $U$  test and are shown for significant differences.

patients ( $r = 0.72$ ;  $P < 0.0001$ ); non-cancer group ( $r = 0.71$ ;  $P < 0.0001$ ) as well as the combined group of all tested subjects ( $r = 0.72$ ;  $P < 0.0001$ ). No significant correlation between the reactivities of ConA and the two other lectins was observed. Thus, the changes in ConA reactivity were not related to the modification of anti-TF IgG binding sites for the fucose- or sialic acid-specific lectins (AAL and SNA).

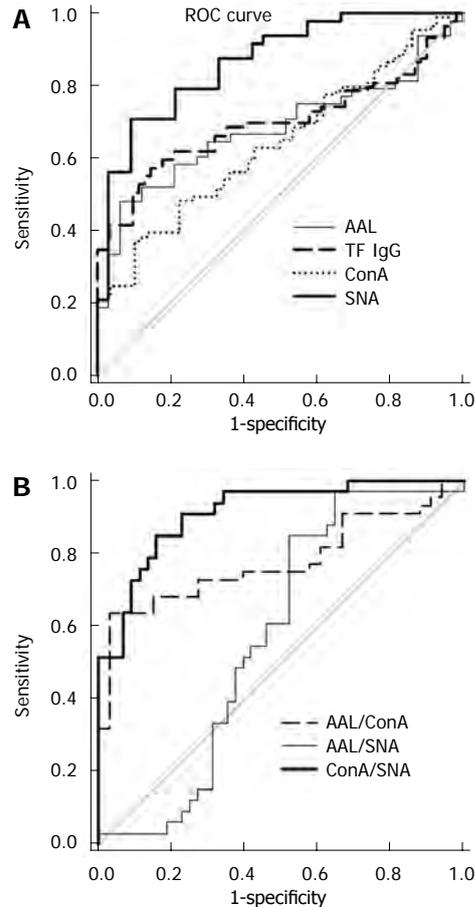


**Figure 3 Anti-Thomsen-Friedenreich glycotope-specific IgG glycosylation profile as defined by concanavalin A, *Aleuria aurantia* lectin and *Sambucus nigra* agglutinin binding.** A: Non-cancer group and cancer patients; B: Relation to the stage of cancer. The results are depicted as mean with error bars representing SEM. *P* values were calculated by the Mann-Whitney *U* test: <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs controls for all lectins. ConA: Concanavalin A; AAL: *Aleuria aurantia* lectin SNA: *Sambucus nigra* agglutinin.

### Anti-TF IgG level and lectin binding profile: Sensitivity and specificity in stomach cancer

ROC curve analysis was used to evaluate changes in the level and glycosylation profile of anti-TF-IgG as possible biomarkers. The diagnostic accuracy and ROC curve statistics are presented in Figure 4 and Table 2. In the absence of a correlation between the binding of ConA and the two other lectins, we investigated a possible interactive effect of lectin combinations using the ratios of ConA/SNA, ConA/AAL and AAL/SNA in the ROC analysis.

Despite the significant difference in anti-TF IgG levels between cancer patients and controls these changes showed a low sensitivity and specificity for cancer, possibly due to great variations within each group. The same was true for the ConA and AAL lectin index values. In contrast, changes in the SNA binding index and, especially, ConA/SNA ratio values demonstrated rather good sensitivity and specificity reaching 78.8%-88.6% (Table 2). Since no notable cancer stage dependency of lectin binding was observed, the sensitivity and specificity values are presented for the combined cancer group and non-cancer



**Figure 4 Sensitivity and specificity of anti-Thomsen-Friedenreich glycotope-specific IgG glycosylation changes in stomach cancer.** A: Receiver operator characteristic (ROC) curve analysis by lectin binding index values; B: ROC curve analysis by lectin combinations using the ratios of concanavalin A (ConA)/*Sambucus nigra* agglutinin (SNA), ConA/*Aleuria aurantia* lectin (AAL) and AAL/SNA.

controls (Figure 4 and Table 2). Using the combination of ConA/AAL and AAL/SNA lectin ratios did not improve the accuracy of the assay and showed lower sensitivity and specificity values, usually below 70% (Table 2).

### Lectin binding of TF-specific IgG and survival of cancer patients

The subgroups of cancer patients with high and low levels of lectin binding to anti-TF IgG were compared. The cut-off levels were calculated using time-dependent ROC curve analysis for each lectin. Despite the opposite changes in the binding of ConA and AAL or SNA lectin in cancer patients (increase vs decrease) a similar association with survival was demonstrated for all three lectins tested, with a common trend in the early and advanced stages of the disease (Figure 5).

The low level of ConA-positive anti-TF IgG was associated with a survival benefit in cancer patients (HR = 1.56; 95%CI: 0.78-3.09; *P* = 0.19), especially in those with stages 3-4 of the disease (HR = 2.17; 95%CI: 0.98-4.79; *P* = 0.048). A significantly better survival rate was found in all cancer patients with low reactivity

**Table 2** Anti-TF IgG level and lectin binding profile: Sensitivity and specificity for gastric cancer

| Parameter   | Sensitivity % (95%CI) | Specificity % (95%CI) | ROC statistics   |          | Sensitivity at specificity 90% |
|-------------|-----------------------|-----------------------|------------------|----------|--------------------------------|
|             |                       |                       | Area under curve | P value  |                                |
| Anti-TF IgG | 65.17 (51.34-76.26)   | 67.74 (56.66-76.98)   | 0.70             | < 0.0001 | 50.56                          |
| ConA        | 62.92 (48.37-74.49)   | 56.90 (46.37-67.74)   | 0.64             | 0.004    | 24.72                          |
| SNA         | 78.79 (61.09-91.02)   | 79.17 (65.01-89.53)   | 0.87             | < 0.0001 | 57.58                          |
| AAL         | 69.70 (51.29-84.41)   | 64.58 (49.46-77.84)   | 0.68             | 0.006    | 12.12                          |
| ConA/SNA    | 72.73 (57.21-85.04)   | 88.64 (71.80-96.60)   | 0.91             | < 0.0001 | 63.64                          |
| AAL/ConA    | 84.85 (68.10-94.89)   | 68.18 (52.42-81.39)   | 0.78             | < 0.0001 | 33.33                          |
| AAL/SNA     | 84.85 (72.24-93.93)   | 47.92 (30.80-66.46)   | 0.68             | 0.006    | 3.03                           |

ConA: Concanavalin A; AAL: *Aleuria aurantia* lectin; SNA: *Sambucus nigra* agglutinin; Anti-TF IgG: Anti-Thomsen-Friedenreich glycotope immunoglobulin G; ROC: Receiver operator characteristic.

of anti-TF IgG to the fucose-specific AAL lectin (HR = 2.39; 95%CI: 1.0-5.7;  $P = 0.038$ ). The association of SNA lectin reactivity with survival showed a similar trend. Considering that no correlation between the binding of ConA and the two other lectins was found, a possible interactive effect of the combination of the two lectins was investigated using the ratios of ConA/SNA, ConA/AAL and AAL/SNA. However, no additional information regarding association with survival was obtained (data not shown).

## DISCUSSION

The role of autoantibodies against tumor-associated glycans in cancer surveillance has been mostly considered for IgM<sup>[2,4]</sup>. These antibodies are not affinity-matured which argues in favor of their inherent natural origin. In contrast, the presence of IgG anti-glycan antibodies suggests an adaptive immune response. The origin of anti-glycan autoantibodies of both isotypes is still unclear though the available evidence suggests that at least anti-TF and anti-alpha Gal antigen-specific antibodies may be induced by bacterial glycans or, possibly, by cross-reactivity with these antigens<sup>[3,32]</sup>. In any case, large and unexplained interindividual variations in their level in health and disease exist<sup>[21,22]</sup>, possibly reflecting the distinct immunological histories of each individual. Moreover, the anti-TF IgG level is rather stable over time at an individual level in both patients and controls<sup>[22,33]</sup>.

In this study, a significantly higher level of TF-specific IgG in purified total IgG preparations from the serum of patients with stomach cancer than in both control groups was observed. This increase was mostly pronounced in stage 1 of the disease, suggesting that an adaptive immune response cannot be excluded in the early stages of tumor with a subsequent decrease in the anti-TF IgG level in advanced cancer as a result of tumor-induced immunodepression. If this is the case, the population of anti-TF IgG should be expected to be heterogeneous and to include both naturally-occurring TF glycotope-specific antibodies, whose presence precedes tumor development, and those triggered by disease, *i.e.*, induced by the tumor-derived TF glycotope. Because of

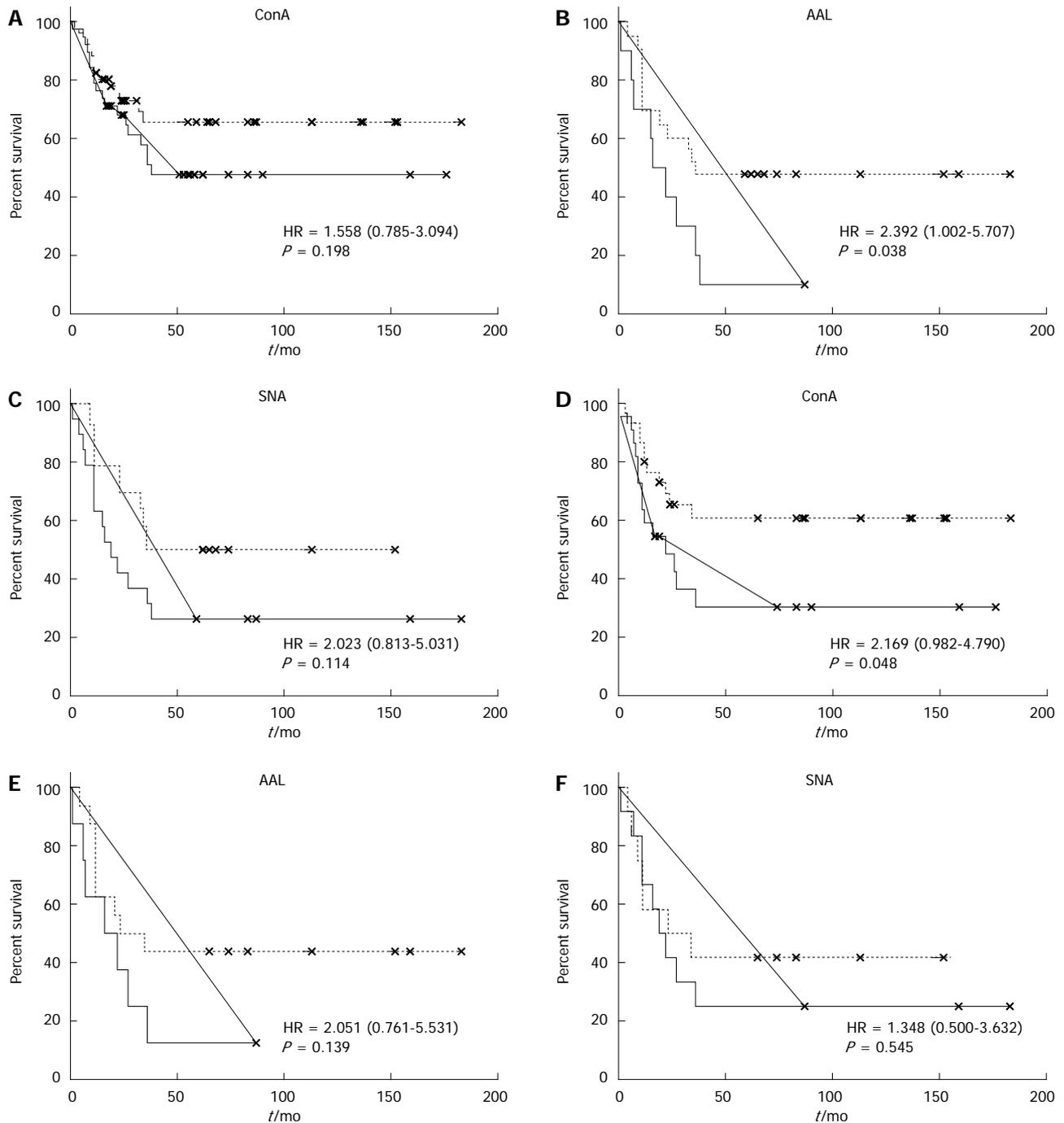
large interindividual variations in anti-TF IgG level, the human population may be divided into low and strong responders to the TF glycotope. Notably, donors and the benign group showed a more compact distribution. Further characterization of anti-TF IgG subpopulations is needed to determine their structural and functional diversities, and clinical significance.

A significantly higher binding of ConA ( $P = 0.005$ ) and a highly significant ( $P = 0.00000003$ ) decrease in SNA lectin binding was characteristic of the anti-TF IgG from the samples of cancer patients compared with those of the non-cancer group (Figure 3). The ConA binding index was higher in stage 1 patients, whereas the SNA and AAL lectin binding index values were low irrespective of the disease stage. We previously found similar changes in the binding of ConA to the total IgG from the serum of patients with gastric cancer.

The changes in anti-TF IgG glycosylation showed rather a high level of sensitivity and specificity in cancer and non-cancer group discrimination (Table 2). It appears that the SNA binding index and, in particular, the ConA/SNA ratio are promising as diagnostic markers to differentiate stomach cancer from controls, including benign gastric diseases. As there are no reliable markers for gastric cancer yet, these findings may be of clinical value.

We reported recently that the level of agalactosylated IgG (G0 glycoform) in the total IgG of gastric cancer patients was significantly higher than that of controls<sup>[9]</sup>. Interestingly, this shift positively correlated with the binding of ConA to the total serum IgG, and was also observed in purified TF-specific IgG samples (unpublished data). This indicates that IgG asialylation/agalactosylation is associated with an increased ConA binding possibly due to a better accessibility of the d-mannose residue to the ConA because of conformational changes in the Fc G0 glycoform. The absence of a correlation between the binding of ConA and SNA may be a reason for a positive interactive effect of using the ConA/SNA binding ratios for cancer *vs* non-cancer group discrimination.

Despite the opposite changes in the binding of ConA and the two other lectins to anti-TF IgG in pa-



**Figure 5 Binding of lectins to Thomsen-Friedenreich glycotopes-specific IgG and the survival of patients with stomach cancer.** The probability of survival (Kaplan-Meier method) of stomach cancer patients in relation to lectin binding to Thomsen-Friedenreich glycotopes (TF)-specific IgG. A: Concanavalin A (ConA) lectin binding of all cancer patients; B: *Aleuria aurantia* lectin (AAL) binding in all cancer patients; C: *Sambucus nigra* agglutinin (SNA) binding in all cancer patients; D: ConA binding in patients with stage 3-4 cancer; E: AAL binding in patients with stage 3-4 cancer; F: SNA binding of patients with stage 3-4 cancer. Patient lectin index values, which were either lower, equal to (dashed line) or higher (solid line) than the cut-off are compared. The cut-off levels were calculated using time-dependent receiver operator characteristic curve analysis.

tients with cancer (Figure 2), a better survival rate was associated with lower reactivity of anti-TF IgG to AAL and ConA. The SNA reactivity revealed no significant association with survival, though a similar slight trend was observed. It seems that IgG desialylation alone is not sufficient for the impact on survival, and further degalactosylation is needed to attain this effect. In this study, the patients were subjected to follow-up for more

than 10 years. The association of the binding of ConA and AAL with survival became evident after 2.5-3 years of observation, reaching a maximum after 5 years.

Several studies have demonstrated that agalactosylated IgGs show an increased inflammatory activity<sup>[34-37]</sup> that may promote tumor growth<sup>[38,39]</sup>. In addition, the IgG<sub>1</sub> lacking the branching fucose or with an additional bisecting GlcNAc shows an enhanced ADCC activity

through an increased interaction with Fc gamma receptors<sup>[36,40-42]</sup>. The association of the high level of the G0 IgG glycoform with a lower survival rate we reported recently<sup>[9]</sup>, and the decreased anti-TF IgG fucosylation associated with better survival in the present findings may be related to these mechanisms.

It has been shown that sialylated glycans predominate in glycosylated antibody binding fragments (Fab) whereas the Fc fragment N-glycans are mostly monosialylated<sup>[30,43]</sup>. Our findings cannot answer the question of whether the Fc or Fab fragment sialylation is responsible for the changes observed in anti-TF IgG glycosylation.

In addition, it remains unclear whether changes in the sialylation and core fucosylation of anti-TF IgG glycans in both IgG fragments may be independent or concordant events, despite the positive correlation observed between the binding of SNA and AAL lectin to the whole molecule of the TF-specific IgG. Given that an active immunization reduces the sialylation of IgG, especially the antigen-driven IgG<sup>[36]</sup>, we hypothesize that the decreased sialylation of anti-TF IgG observed in cancer patients may be an indicator of an adaptive immune response to tumor-derived TF antigen, in addition to naturally-occurring anti-TF IgG antibodies, which are present in every individual in different amounts.

In conclusion, the results show the glycosylation of TF-specific IgG antibodies in patients with gastric cancer to undergo significant changes when compared with that of controls. The appearance of these alterations already in the early stages of cancer and their association with survival suggest that they play a significant role in cancer development and progression. The lectin-based glycoprofiling ELISA assay is an informative and clinically applicable tool for the analysis of IgG glycans. The results imply that changes in the TF-specific IgG glycosylation have diagnostic and prognostic potential for stomach cancer. However, a further study is needed to support these findings on a larger scale using different control groups. Mass spectrometry-based methodology might help to further specify different subsets of anti-TF IgG of clinical importance.

## COMMENTS

### Background

Gastric cancer is the second leading cause of cancer deaths worldwide. Yet there are still no reliable serum biomarkers for gastric cancer diagnostics and prognostics. Previous studies have demonstrated that naturally-occurring antibodies (Ab) to tumor-associated glycans are involved in natural tumor immunity, being associated with tumor progression and cancer patients survival.

### Research frontiers

Recent findings about the impact of Ab glycosylation on their effect or functions suggest that the evaluation of not just the level of antibodies but rather their structural diversity might improve the clinical potential of the antibody-based approach in the disease diagnostics and prognostics. With this purpose in mind, a research team from Estonia led by Kurtenkov O aimed to evaluate whether the glycosylation profile of immunoglobulin G (IgG) antibody to the so-called tumor-associated Thomsen-Friedenreich antigen (TF) could serve as a marker of gastric cancer and the disease outcome. The study is based on the analysis of a long-term (for more than 10 years) survival of cancer patients.

## Innovations and breakthroughs

This study demonstrates for the first time that the glycosylation of anti-TF antibodies reveals gastric cancer-related changes with a diagnostic sensitivity and specificity of about 80%. It is striking that the stage of cancer had little effect on these parameters, thus allowing one to diagnose the disease in its early stages. Besides, the authors show that some Ab glycoforms may predict patient survival. Based on these results, the authors conclude that this serology-based approach may be of clinical importance for gastric cancer diagnostics and prognostics.

## Applications

The results indicate that changes in the TF-specific IgG glycosylation have diagnostic and prognostic potential for stomach cancer. The lectin-based glycoprofiling of anti-TF IgG antibodies is an informative and clinically applicable tool to specify sets of tumor-associated antibody glycoforms which can be used as a biomarker for stomach cancer detection and follow-up. It is plausible that the elevated level of a specific antibody glycoform might serve as a marker for a stronger immune response, providing protection against cancer. The findings may eventually lead to the development of novel forms of cancer immunotherapy based on vaccination with TF as target and the manipulation of Ab glycosylation machinery. This will need further work, in particular performance of functional tests using tumor cells and different Ab glycoforms, to understand the biological relevance of the results.

## Terminology

Glycans: The chains of sugars that coat the outer surface of cells or are attached to other molecules (proteins, lipids). Glycosylation is the enzymatic process in which sugars are attached to other molecules.

## Peer review

This important study extends findings from studies of the same authors published previously and make a great contribution to the understanding of the molecular mechanisms that underline aberrant glycosylation in gastric carcinogenesis, and to the evaluation of potential biomarkers for gastric cancer diagnosis and patient prognosis.

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**P- Reviewer** Vorobjova T **S- Editor** Gou SX  
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## Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption *via* a TNF- $\alpha$ -regulated mechanism

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Author contributions: Song HL and Shen ZY designed the research, analyzed and interpreted the data, and wrote the manuscript; Shen ZY, Zheng WP and Zhang J performed the research; Song HL and Zheng WP analyzed the data; all authors have read and approved the final manuscript.

Supported by Natural Science Foundation of China, No. 81270528; the Natural Science Foundation of Tianjin, No. 08JCY-BJC08400, No. 11JCZDJC27800 and No. 12JCZDJC25200; the Technology Foundation of Health Bureau in Tianjin, No. 2011KY11

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Received: December 30, 2012 Revised: March 21, 2013

Accepted: April 3, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To investigate the effect of bone-marrow mesenchymal stem cells (BM MSCs) on the intestinal mucosa barrier in ischemia/reperfusion (I/R) injury.

**METHODS:** BM MSCs were isolated from male Sprague-Dawley rats by density gradient centrifugation, cultured, and analyzed by flow cytometry. I/R injury was induced by occlusion of the superior mesenteric artery for 30 min. Rats were treated with saline, BM MSCs (*via* intramucosal injection) or tumor necrosis factor (TNF)- $\alpha$  blocking antibodies (*via* the tail vein). I/R injury was assessed using transmission electron microscopy, hematoxylin and eosin (HE) staining, immunohistochemistry, western blotting and enzyme linked immunosorbent assay.

**RESULTS:** Intestinal permeability increased, tight junctions (TJs) were disrupted, and zona occludens 1 (ZO-1) was downregulated after I/R injury. BM MSCs reduced intestinal mucosal barrier destruction, ZO-1 downregulation, and TJ disruption. The morphological abnormalities after intestinal I/R injury positively correlated with serum TNF- $\alpha$  levels. Administration of anti-TNF- $\alpha$  IgG or anti-TNF- $\alpha$  receptor 1 antibodies attenuated the intestinal ultrastructural changes, ZO-1 downregulation, and TJ disruption.

**CONCLUSION:** Altered serum TNF- $\alpha$  levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

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**Key words:** Bone marrow mesenchymal stem cells; Zona occludens 1; Ischemia-reperfusion injury; Intestinal mucosa; Tumor necrosis factor- $\alpha$

**Core tip:** Intestinal ischemia/reperfusion (I/R) injury is clinically important. Bone-marrow mesenchymal stem cells (BM MSCs) can protect against I/R injury; however, the mechanism is unclear. This study demonstrates that submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, *via* a mechanism linked to reduced serum tumor necrosis factor (TNF)- $\alpha$  levels and increased expression of the intestinal tight junction protein zona occludens 1. Altered serum TNF- $\alpha$  levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

Shen ZY, Zhang J, Song HL, Zheng WP. Bone-marrow mesenchymal stem cells reduce rat intestinal ischemia-reperfusion injury, ZO-1 downregulation and tight junction disruption *via* a TNF- $\alpha$ -regulated mechanism. *World J Gastroenterol* 2013;

19(23): 3583-3595 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3583.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3583>

## INTRODUCTION

Digestive organ transplantation and other abdominal surgical procedures can result in different degrees of intestinal ischemia/reperfusion (I/R) injury, which can delay patient recovery and lead to systemic organ failure. Therefore, intestinal I/R injury is an important clinical issue. The small intestine is composed of labile cells that are easily injured by I/R; however, the mechanisms responsible for intestinal I/R injury are unclear. Previous studies have reported that the serum level of tumor necrosis factor (TNF)- $\alpha$  is elevated in patients with severe intestinal I/R injury<sup>[1]</sup>. TNF- $\alpha$  is a cytokine with broad-spectrum physiological and pathological responsiveness, which is primarily secreted by monokaryons and macrophages. In addition to participating in the humoral and cellular immune responses, TNF- $\alpha$  also plays an important role in diseases such as severe hepatitis, septic shock and inflammatory bowel disease<sup>[2-6]</sup>; however, it is not known whether TNF- $\alpha$  affects the intestinal barrier function during I/R injury.

Bone-marrow mesenchymal stem cells (BM MSCs) are fibroblast-like, pluripotent adult stem cells. BM MSCs can adhere to plastic and grow readily in the laboratory. BM MSCs give rise to mesoderm cells<sup>[7,8]</sup>, and have been reported to differentiate into all three germ cell lines<sup>[9]</sup>, liver and neural cells<sup>[10,11]</sup>, which have potential to be used for the treatment of various diseases. Allogeneic MSCs were transplanted into primates *via* an intravenous route and distributed to the gastrointestinal tract where they proliferated<sup>[12]</sup>. MSCs have also been shown to have immunomodulatory capabilities due to the secretion of several growth factors<sup>[13,14]</sup>. BM MSCs reduce intestinal I/R injury in rats<sup>[15]</sup>. Studies in I/R rodent models have demonstrated that MSCs can beneficially produce paracrine growth factors and anti-inflammatory cytokines<sup>[16]</sup>. It should be noted that MSCs respond to TNF- $\alpha$ , but do not produce TNF- $\alpha$ <sup>[17]</sup>.

The intestinal mucosa is the physical and metabolic barrier against toxins and pathogens in the gut lumen. Tight junctions (TJs) are the main structures responsible for restricting the paracellular movement of compounds across the intestinal mucosa. Structurally, TJs are composed of cytoplasmic proteins, including the zona occludens proteins, ZO-1-3<sup>[18,19]</sup> and two distinct transmembrane proteins, occludin and claudin<sup>[20,21]</sup>, which are linked to the actin-based cytoskeleton<sup>[22]</sup>. TJs function as occlusion barriers by maintaining cellular polarity and homeostasis, and by regulating the permeability of paracellular spaces in the epithelium<sup>[23]</sup>. ZO-1, a member of the membrane-associated guanylate kinase family of proteins, acts as a scaffold for the organization

of transmembrane TJ proteins, and also recruits various signaling molecules and the actin cytoskeleton to TJs<sup>[24]</sup>. Although previous studies have provided an insight into the molecular structure of TJs, much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during I/R injury.

In this study, we used a rat model of intestinal I/R injury to investigate the effect of BM MSCs on intestinal mucosa ultrastructure, with an emphasis on the mechanisms of intestinal barrier dysfunction.

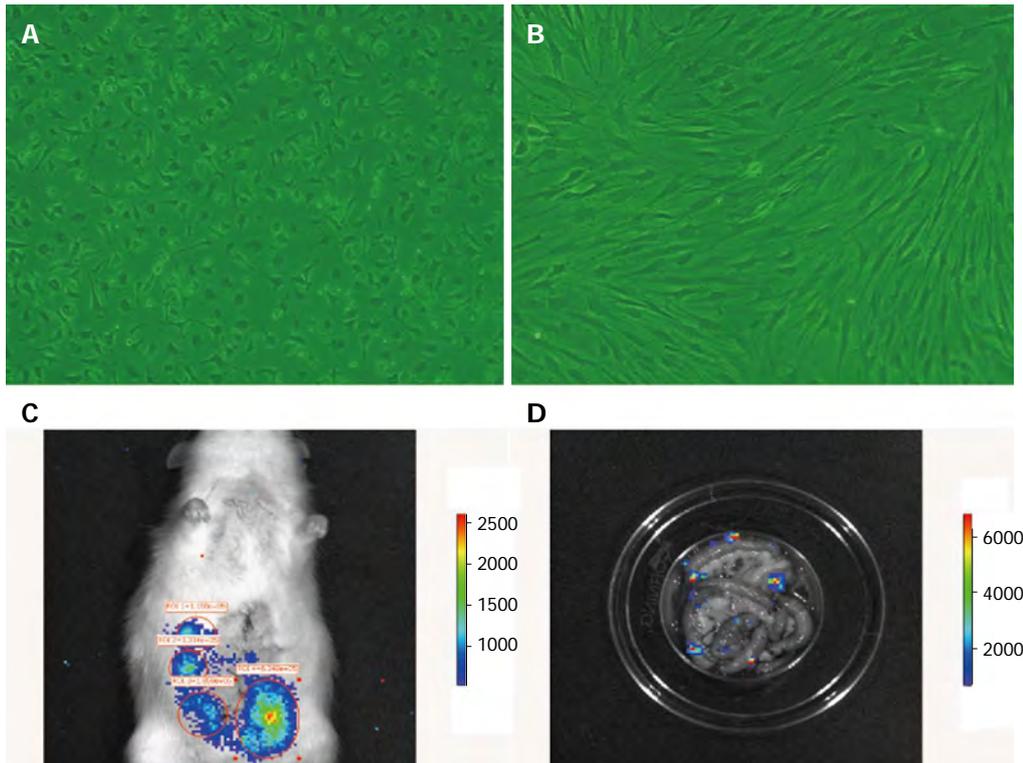
## MATERIALS AND METHODS

### Animals and I/R injury model

Male Sprague-Dawley rats (180-200 g) were obtained from the Military Medical Science Academy of China People's Liberation Army (PLA; Beijing, China), housed at a constant temperature and humidity, and provided with food and water *ad libitum*. All animal experimental procedures were approved by the Ethics Committee of the Military Medical Science Academy of the PLA before commencement of the study.

One-hundred and eight male rats were fasted for 12 h with free access to water before surgery and randomly assigned to five experimental groups. The operative procedures were performed using standard sterile technique under general anesthesia using 5% chloral hydrate (10 mL/kg). All rats were subjected to laparotomy using a midline incision that was approximately 3 cm, and the principal branches of the superior mesenteric artery (SMA) were identified. In the Sham group, the SMA was isolated using blunt dissection, without clamping the vessel. In the BM MSCs + I/R injury group, the SMA was occluded for 30 min using an atraumatic microvascular clamp. Immediately after the clamp was released,  $1 \times 10^7$  male rat BM MSCs suspended in 0.5 mL serum-free Dulbecco's Modified Eagle's Medium (DMEM) were injected into the intestinal submucosa at five different locations. Animals in the normal saline (NS) + I/R injury group underwent I/R followed by the injection of 0.5 mL normal saline into the intestinal submucosa at 10 different locations. The anti-TNF- $\alpha$  + I/R injury group and the anti-TNF- $\alpha$ R1-IgG + I/R injury group were administered with anti-TNF- $\alpha$  IgG (1000  $\mu$ g per rat; United States Biological, Swampscott, MA, United States) or anti-TNF- $\alpha$  R1 antibody (1000  $\mu$ g per rat; R and D Systems, Minneapolis, MN, United States), respectively. Injections were given *via* the tail vein after induction of I/R injury.

The abdomen was closed and the animals were allowed to recover with free access to tap water and standard pellet rat chow. Rats in the I/R injury, BMSCs + I/R injury and Sham groups were euthanized at 2, 6, 24, 72 and 144 h after I/R injury ( $n = 6$  at each time point). Rats in the anti-TNF- $\alpha$  IgG + I/R and anti-TNF- $\alpha$  R1



**Figure 1** Morphology of bone-marrow mesenchymal stem cells *in vitro*, and *in vivo* cell tracing of bone-marrow mesenchymal stem cells colonization in rat intestine. A: First-passage bone-marrow mesenchymal stem cells (BM MSCs); B: Third-passage BM MSCs ( $\times 200$ ); C: Homing of fluorescently labeled BM MSCs (B16-F10-Luc-G5) to the rat intestine 6 h after transplantation; D: After the intestine was removed and washed repeatedly, fluorescently labeled BM MSCs were still observed, confirming the cells homed to the intestine and survived.

antibody + I/R injury groups were euthanized at 6 h after I/R injury ( $n = 6$  each). Blood samples and approximately 5 cm of the ileum were collected from each rat. The plasma was separated by centrifugation and stored at  $-80^{\circ}\text{C}$  until analysis. The intestinal samples were fixed for histopathological analysis and transmission electron microscopy.

#### Isolation and characterization of BM MSCs

BM MSCs were isolated from the femur and tibia of male Sprague-Dawley rats (100-120 g). Red blood cells were lysed using 0.1 mol/L  $\text{NH}_4\text{Cl}$ , and the remaining cells were washed, resuspended, and cultured for 4 wk in DMEM/F12 (Gibco, Carlsbad, CA, United States) containing 100 U/mL penicillin, 100 mg/mL streptomycin, and 15% fetal bovine serum. BM MSCs were cultured in an incubator at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  with saturated humidity. The medium was changed every 72 h.

When the third-passage cells reached 80% confluence, the cells were trypsinized, washed, centrifuged, and resuspended at  $1 \times 10^7$  cells/mL in phosphate-buffered saline (PBS). BM MSCs were stained using antibodies against CD29, CD90, RT1A, CD45 and RT1B (Biolegend, San Diego, CA, United States) and CD34 (Santa Cruz Biotechnology, Santa Cruz, CA, United States). They were analyzed by flow cytometry (FACSCalibur; BD, Alaska, MN, United States). The proportion of CD29<sup>+</sup>, CD90<sup>+</sup> and RT1A-positive cells, and CD34<sup>-</sup>

CD45<sup>-</sup> and RT1B-negative cells was  $> 98\%$  (Figure 1A). BM MSCs were also confirmed as plastic-adherent cells with a spindle-shaped morphology under standard culture conditions by microscopy (Figure 1B). The purity of BM MSCs was  $> 95\%$ .

#### Detection of donor BM MSCs in recipient intestines

BM MSCs ( $1 \times 10^7$  cells) were incubated with  $3.5 \mu\text{g}/\text{mL}$  1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine iodide (DIR) in 10 mL PBS containing 0.5% ethanol for 30 min at  $37^{\circ}\text{C}$ , and washed twice with PBS. Sprague-Dawley rats weighing 180-200 g were anesthetized using 5% chloral hydrate, subjected to a midline laparotomy, and  $1 \times 10^7$  labeled BM MSCs suspended in 1 mL PBS were injected into the intestinal submucosa at five different points. At 2, 6, 24, 72 and 144 h later, luciferin was injected abdominally using a 25-gauge needle and, 7-8 min later, the animals were anesthetized and imaged using a high-sensitivity optical molecular imaging and high-resolution digital X-ray system (IVIS Lumina II, Alameda, CA, United States).

#### Histological measurement of intestinal mucosal injury

Serial 2-cm samples were taken from the terminal ileum and fixed with 10% neutral formalin. Tissues were processed, embedded, and stained with hematoxylin and eosin. Three paraffin sections were prepared from each tissue sample. Two pathologists who were blinded to the

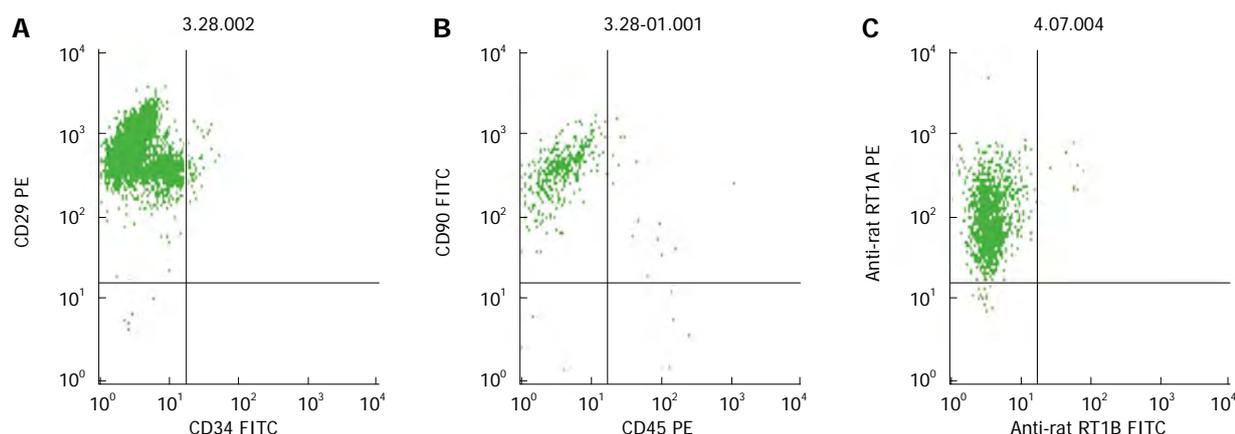


Figure 2 Flow cytometric analysis of third-passage bone-marrow mesenchymal stem cells. A: The proportion of CD29-positive and CD34-negative cells was approximately 96%; B: The proportion of CD90-positive and CD45-negative was approximately 98%; C: The proportion of RT1A-positive and RT1B-negative cells was > 98%.

source of the slides analyzed. The degree of histopathological changes was graded semiquantitatively using the histological injury scale previously described by Chiu *et al.*<sup>[25]</sup>, as follows: 0, normal mucosal villi; 1, development of a subepithelial space, usually at the apex of the villi with capillary congestion; 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3, massive epithelial lifting down the sides of the villi and ulceration at the villous tips; 4, denuded villi with dilated capillaries and increased cellularity of the lamina propria; and 5, degradation and disintegration of the lamina propria, hemorrhage, and ulceration. A minimum of six randomly chosen fields from each rat were evaluated and averaged to determine the degree of mucosal damage.

#### Serum D-lactate, diamine oxidase and TNF- $\alpha$ assay

The serum levels of TNF- $\alpha$ , D-lactate and diamine oxidase (DAO) were determined using enzyme linked immunosorbent assay kits (R and D Systems) according to the manufacturer's protocol.

#### Detection and observation of intestinal mucosal ultrastructure

Ultrathin (70-nm) intestinal sections were prepared using standard techniques and examined using a transmission electron microscope (Hitachi H-600, Tokyo, Japan).

#### Immunohistochemical detection of ZO-1 in frozen tissue sections

Frozen intestinal tissue sections (5  $\mu$ m) were fixed on glass slides by incubation in acetone for 10 min at 4  $^{\circ}$ C, and then incubated with 3% H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature, blocked in goat serum for 30 min at 37  $^{\circ}$ C, and then indirectly immunolabeled with a rabbit anti-mouse polyclonal ZO-1 antibody (1:50; Santa Cruz Biotechnology) using an ABC kit at 4  $^{\circ}$ C overnight (Takara, Dalian, China), according to the manufacturer's instructions. For the negative controls, the primary antibody was replaced with PBS. The sections were then incubated in biotinyl-

ated goat anti-rabbit IgG (1:300 in PBS; Histostain-Plus kit, Zymed Laboratories, South San Francisco, CA, United States) for 2 h at room temperature, rinsed in PBS, rinsed in distilled water, then the staining was developed using 3,3'-diaminobenzidine and the sections were counterstained using hematoxylin.

#### Western blotting of tissue ZO-1 content

Intestinal tissue samples were homogenized in lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 1% Triton  $\times$  100, 0.2 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mol/L DTT and 2 mol/L aprotinin]. The protein samples (50  $\mu$ g) were electrophoresed on 8% SDS-PAGE gels, transferred to nitrocellulose membranes, blocked with non-fat dried milk in TBS containing 0.05% Tween-20 (TTBS) for 1 h at room temperature, and incubated with a rabbit anti-mouse polyclonal ZO-1 antibody (1:400; Santa Cruz Biotechnology) at 4  $^{\circ}$ C overnight. After three washes in TTBS, the membranes were incubated with alkaline-phosphatase-labeled goat anti-rabbit IgG (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. The bands were visualized using  $\alpha$ -dianisidine and  $\beta$ -naphthyl acid phosphate (Sigma, St Louis, MO, United States).

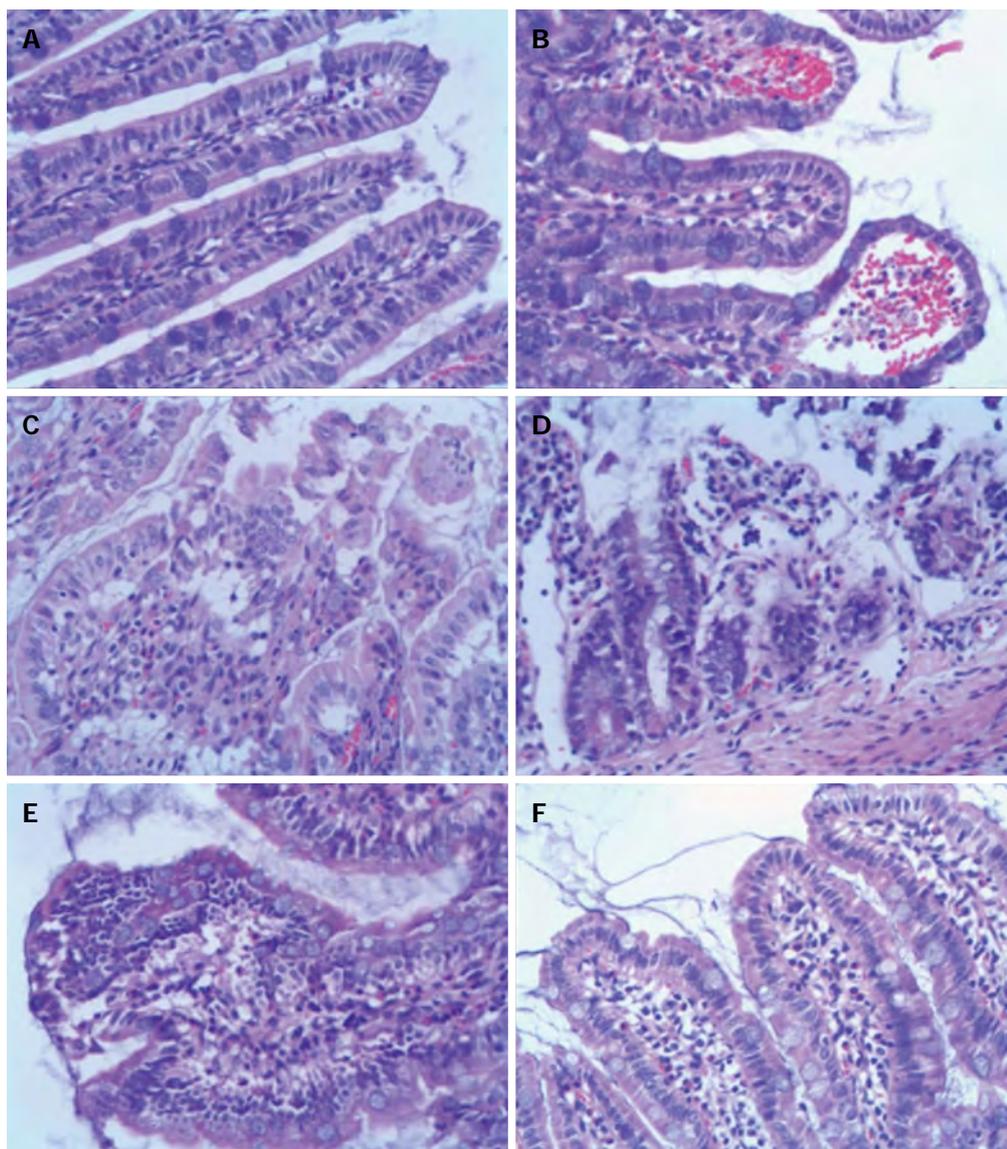
#### Statistical analysis

SPSS version 10.0 (SPSS, Chicago, IL, United States) was used for the statistical analysis. Normally distributed data were shown as the mean  $\pm$  SD. Different groups of data were compared by analysis of variance (ANOVA). The degree of relationship between TNF- $\alpha$  and the Chiu risk score was evaluated by a bivariate correlation. The results was statistically significant when  $P < 0.05$ , and was highly significant when  $P < 0.01$ .

## RESULTS

### Culture of BM MSCs

The cells were confirmed as BM MSCs based on their spindle-shaped morphology, adherence to plastic (Figure



**Figure 3** Histopathology of ileum sections at different time points after intestinal ischemia/reperfusion injury (hematoxylin and eosin,  $\times 200$ ). A: Sham group; the intestine showed normal villous architecture and glands, with no vascular congestion; B: In the ischemia/reperfusion (I/R) injury 2 h group, the degree of intestinal mucosa injury was marked with massive epithelial lifting down the sides of the villi and ulceration at the villous tips; C: At 6 h, there was intestinal mucosa degradation and disintegration of the lamina propria, hemorrhage, and ulceration; D: At 24 h, the damaged mucosa showed denuded villi with dilated capillaries and increased cellularity of the lamina propria; E: In the I/R injury group at 72 h, there was massive epithelial lifting down the sides of the villi and ulceration at the villous tips; F: However, at 144 h, the damaged mucosa had recovered.

1A and 1B), ability to differentiate hepatocytes *in vitro* (data not shown), and flow cytometry results (Figure 2). Most of the third-passage adherent cells were positive for CD90, CD29 and RT1A, and negative for the MSC markers, CD45, CD34 and RT1B. Furthermore, over the first three passages, the percentage of CD90<sup>+</sup> and CD45<sup>-</sup> cells rapidly increased from 80% to > 98% (Figure 2), which was in agreement with a previous study<sup>[26]</sup>.

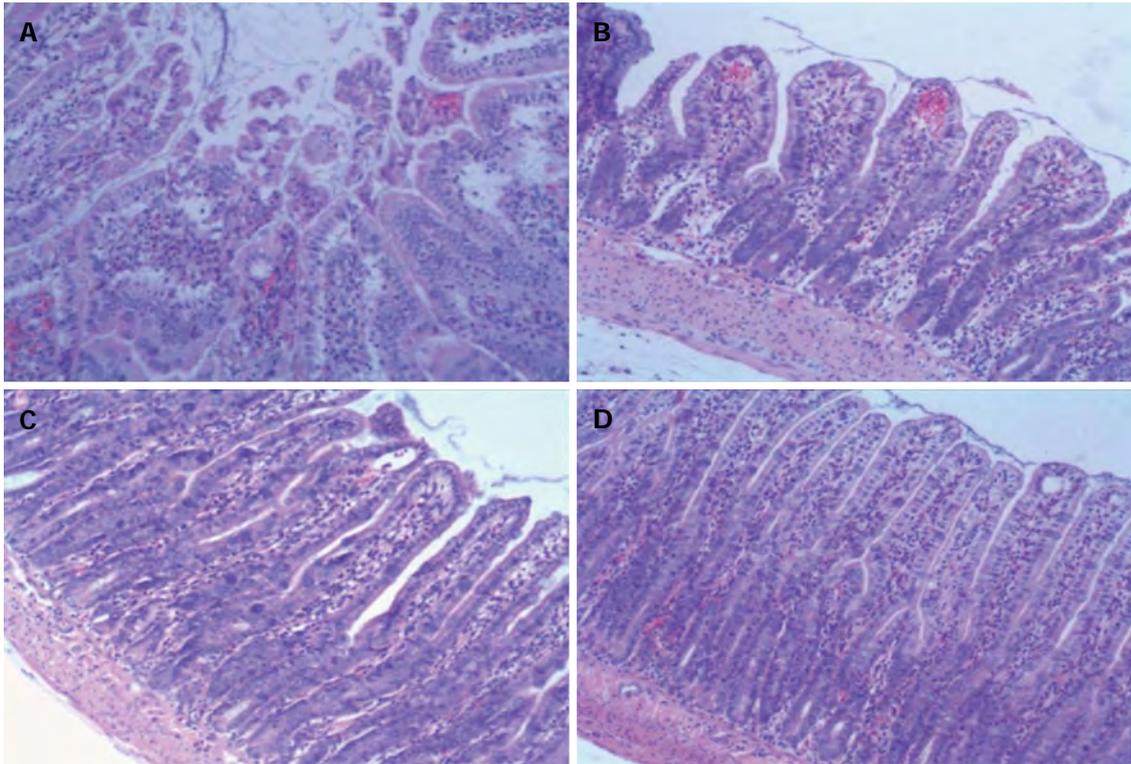
#### Confirmation of donor-derived BM MSCs

Labeled BM MSCs homing to the intestine were visible 2, 6, 24, 72 and 144 h after transplantation (Figure 1C). After the intestine was washed repeatedly with PBS, the labeled BM MSCs were still visible (Figure 1D), which indicated that the transplanted BM MSCs could home to

the intestine and survive long term.

#### Histopathological examination

The histopathological findings showed intact villi with no epithelial disruption in the Sham groups. In the NS + I/R injury group, massive destruction of the villi and inflammatory cell infiltration into the lamina propria were evident. In contrast, intestinal samples in the BM MSCs + I/R injury group (BM MSCs group) had significantly less damage in the small intestine. Major pathological changes observed were slight hyperemia, edema, and inflammatory cell infiltration in the mucosa and submucosa, with most of the intestinal villi intact (Figures 3 and 4). Chiu's grade scores of the three groups are shown in Table 1.



**Figure 4** Histopathology of ileum sections of different groups at 6 h after ischemia/reperfusion injury (hematoxylin and eosin, × 100). A: In the ischemia/reperfusion (I/R) injury group there was marked intestinal mucosa injury at 6 h, with intestinal mucosa degradation and disintegration of the lamina propria, hemorrhage, and ulceration. B: In the bone-marrow mesenchymal stem cells + I/R injury group at 6 h, the damaged mucosa had recovered and there was extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria, massive epithelial lifting down the sides of the villi, and ulceration at the villous tips. C and D: In the anti-tumor necrosis factor (TNF)-α + I/R injury group and the anti-TNF-αR1-IgG + I/R injury group at 6 h, the damaged mucosa had almost recovered to resemble that in the Sham control group.

**Table 1** Grade of intestinal mucosal injury after intestinal ischemia/reperfusion in the different groups

| Group                         | Chiu's score              |                           |                           |                          |                          |
|-------------------------------|---------------------------|---------------------------|---------------------------|--------------------------|--------------------------|
|                               | 2 h                       | 6 h                       | 24 h                      | 72 h                     | 144 h                    |
| Sham group                    | 0.5 ± 0.8                 | 0.5 ± 0.5                 | 0.7 ± 0.8                 | 0.3 ± 0.8                | 0.7 ± 0.8                |
| NS + I/R injury               | 18.0 ± 3.9 <sup>b</sup>   | 27.8 ± 2.3 <sup>b</sup>   | 23.7 ± 5.2 <sup>b</sup>   | 19.0 ± 3.8 <sup>b</sup>  | 11.0 ± 3.0 <sup>b</sup>  |
| BM MSCs + I/R injury          | 13.7 ± 3.3 <sup>b,d</sup> | 15.2 ± 2.9 <sup>b,d</sup> | 13.7 ± 1.5 <sup>b,d</sup> | 8.3 ± 2.3 <sup>b,d</sup> | 5.8 ± 2.6 <sup>b,d</sup> |
| Anti-TNF-α + I/R injury       |                           | 6.5 ± 1.2 <sup>d,f</sup>  |                           |                          |                          |
| Anti-TNF-αR1-IgG + I/R injury |                           | 7.7 ± 1.2 <sup>d,f</sup>  |                           |                          |                          |

All values are mean ± SD (*n* = 6, three paraffin sections were prepared from each tissue sample. Two pathologists who were blinded to the source of the slides analyzed each slide); <sup>b</sup>*P* < 0.01 *vs* the Sham group, <sup>d</sup>*P* < 0.01 *vs* the saline (NS) + ischemia/reperfusion (I/R) injury group, <sup>f</sup>*P* < 0.01 *vs* bone-marrow mesenchymal stem cells (BM MSCs) + I/R injury group. TNF-α: Tumor necrosis factor-α.

### Serum D-lactate and DAO

The levels of D-lactate and DAO significantly increased, reaching a peak at 6 h in the NS + I/R injury and BM MSCs + I/R injury groups, compared to the Sham group. This confirmed that I/R injury increased intestinal permeability.

The serum D-lactate and DAO levels in the NS + I/R injury group increased more than twofold compared to the Sham group at 2, 6 and 24 h after I/R (*P* < 0.01). However, the serum DAO levels in the BM MSCs + I/R injury group were significantly lower than in the NS + I/R group at 2, 6 and 24 h, and the serum D-lactate levels in the BM MSCs + I/R injury group were significantly

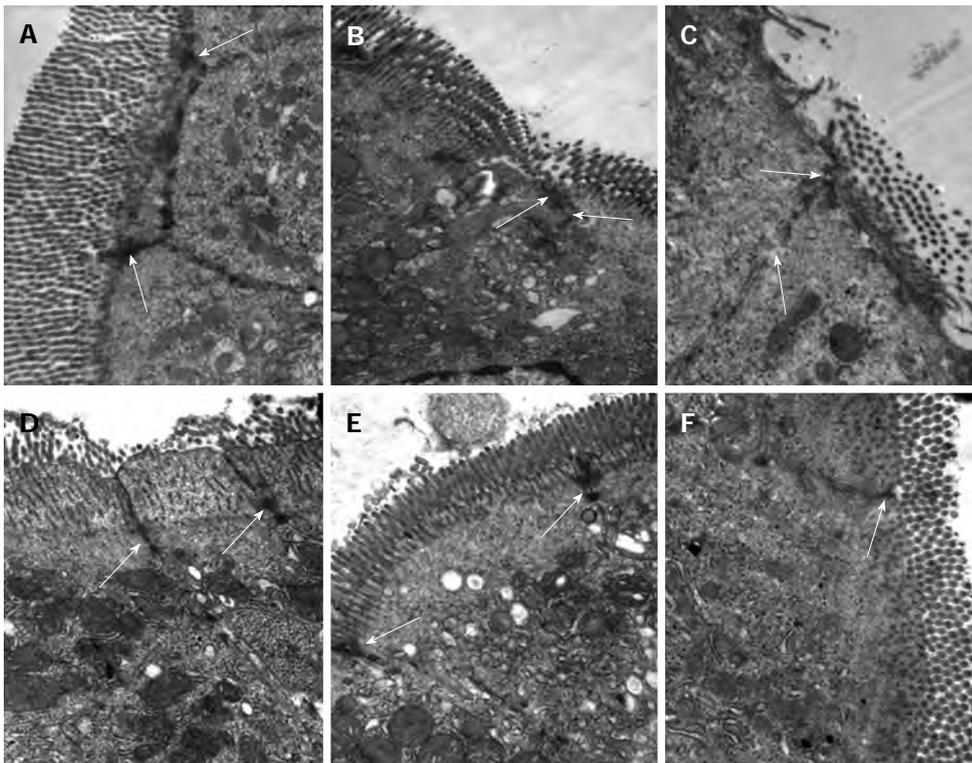
lower than in the NS + I/R group at 6 and 24 h. At 6 h, the serum D-lactate and DAO levels in the anti-TNF-α + I/R injury and anti-TNF-αR-IgG + I/R injury groups were lower than in the NS + I/R injury group (*P* < 0.01; Table 2). At 72 and 144 h, the serum DAO levels in the NS + I/R and BM MSCs + I/R injury groups had reduced, but remained higher than in the Sham group, whereas D-lactate levels were not significantly different in the NS + I/R, BM MSCs + I/R and Sham groups at 144 h.

These data indicate that serum DAO is a more sensitive marker of intestinal permeability than D-lactate, and also that the administration BM MSCs or TNF-α block-

**Table 2 Serum levels of diamine oxidase and D-lactate in a rat model of ischemia/reperfusion injury**

| Group                                  | DAO (IU/mL)                    |                                 |                                |                              |                              | D-lactate ( $\mu\text{g/mL}$ ) |                                 |                                |                              |                 |
|--|--------------------------------|---------------------------------|--------------------------------|------------------------------|------------------------------|--------------------------------|---------------------------------|--------------------------------|------------------------------|-----------------|
|  | 2 h                            | 6 h                             | 24 h                           | 72 h                         | 144 h                        | 2 h                            | 6 h                             | 24 h                           | 72 h                         | 144 h           |
| Sham group                             | 2.08 $\pm$ 0.16                | 2.08 $\pm$ 0.75                 | 2.03 $\pm$ 0.46                | 1.95 $\pm$ 0.36              | 2.24 $\pm$ 0.62              | 5.11 $\pm$ 0.24                | 5.30 $\pm$ 0.44                 | 5.38 $\pm$ 0.45                | 5.46 $\pm$ 0.42              | 5.54 $\pm$ 0.69 |
| NS + I/R injury                        | 11.04 $\pm$ 0.59 <sup>b</sup>  | 14.58 $\pm$ 2.01 <sup>b</sup>   | 7.36 $\pm$ 1.28 <sup>b</sup>   | 5.12 $\pm$ 0.66 <sup>b</sup> | 3.91 $\pm$ 0.59 <sup>b</sup> | 14.73 $\pm$ 1.37 <sup>b</sup>  | 17.85 $\pm$ 1.86 <sup>b</sup>   | 12.73 $\pm$ 0.56 <sup>b</sup>  | 8.22 $\pm$ 1.78              | 6.54 $\pm$ 1.04 |
| BM MSCs + I/R injury                   | 8.16 $\pm$ 0.71 <sup>b,d</sup> | 11.36 $\pm$ 1.89 <sup>b,d</sup> | 5.04 $\pm$ 1.04 <sup>b,d</sup> | 4.93 $\pm$ 0.69 <sup>b</sup> | 3.55 $\pm$ 0.59 <sup>a</sup> | 12.62 $\pm$ 2.24 <sup>b</sup>  | 13.40 $\pm$ 1.53 <sup>a,b</sup> | 9.80 $\pm$ 1.20 <sup>b,d</sup> | 6.82 $\pm$ 0.80 <sup>b</sup> | 6.44 $\pm$ 0.83 |
| Anti-TNF- $\alpha$ + I/R injury        | -                              | 7.99 $\pm$ 1.70 <sup>d</sup>    | -                              | -                            | -                            | -                              | 12.77 $\pm$ 1.44 <sup>d</sup>   | -                              | -                            | -               |
| Anti-TNF- $\alpha$ R1-IgG + I/R injury | -                              | 7.83 $\pm$ 1.28 <sup>d</sup>    | -                              | -                            | -                            | -                              | 12.16 $\pm$ 1.47 <sup>d</sup>   | -                              | -                            | -               |

All values are mean  $\pm$  SD ( $n = 6$ ). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs Sham group; <sup>d</sup> $P < 0.01$  vs the saline (NS) + ischemia/reperfusion (I/R) injury group.



**Figure 5** Bone-marrow mesenchymal stem cells and tumor necrosis factor- $\alpha$  blockade prevent ultrastructural pathological damage after intestinal ischemia/reperfusion injury. Transmission electron microscopy of the rat intestine after ischemia/reperfusion (I/R) injury. A: Epithelial cells and tight junctions (TJs) (arrows) were intact in the Sham group,  $\times 30000$ ; B: At 2 h after I/R injury, epithelial cells were swollen and shrunken, microvilli and organelles were normal, and TJs (arrows) were disrupted in the saline (NS) + I/R injury group,  $\times 25000$ ; C: At 6 h after I/R injury in the NS + I/R injury group, some microvilli were loose, TJs (arrows) were disrupted, and organelles were swollen with reduced electron density,  $\times 30000$ ; D: At 6 h after I/R injury and administration of bone-marrow mesenchymal stem cells, the microvilli and mitochondria of the endothelial cells were almost normal and TJs (arrows) were not disrupted,  $\times 30000$ ; E and F: TJs (arrows) between endothelial cells were intact 6 h after I/R injury in rats that received anti-tumor necrosis factor (TNF)- $\alpha$  IgG + I/R antibody (E,  $\times 25000$ ) or anti-TNF- $\alpha$  R1 antibody; (F,  $\times 30000$ ) before I/R injury.

ade reduced the permeability of the small intestine and accelerated the recovery of intestinal barrier function after I/R injury in rats.

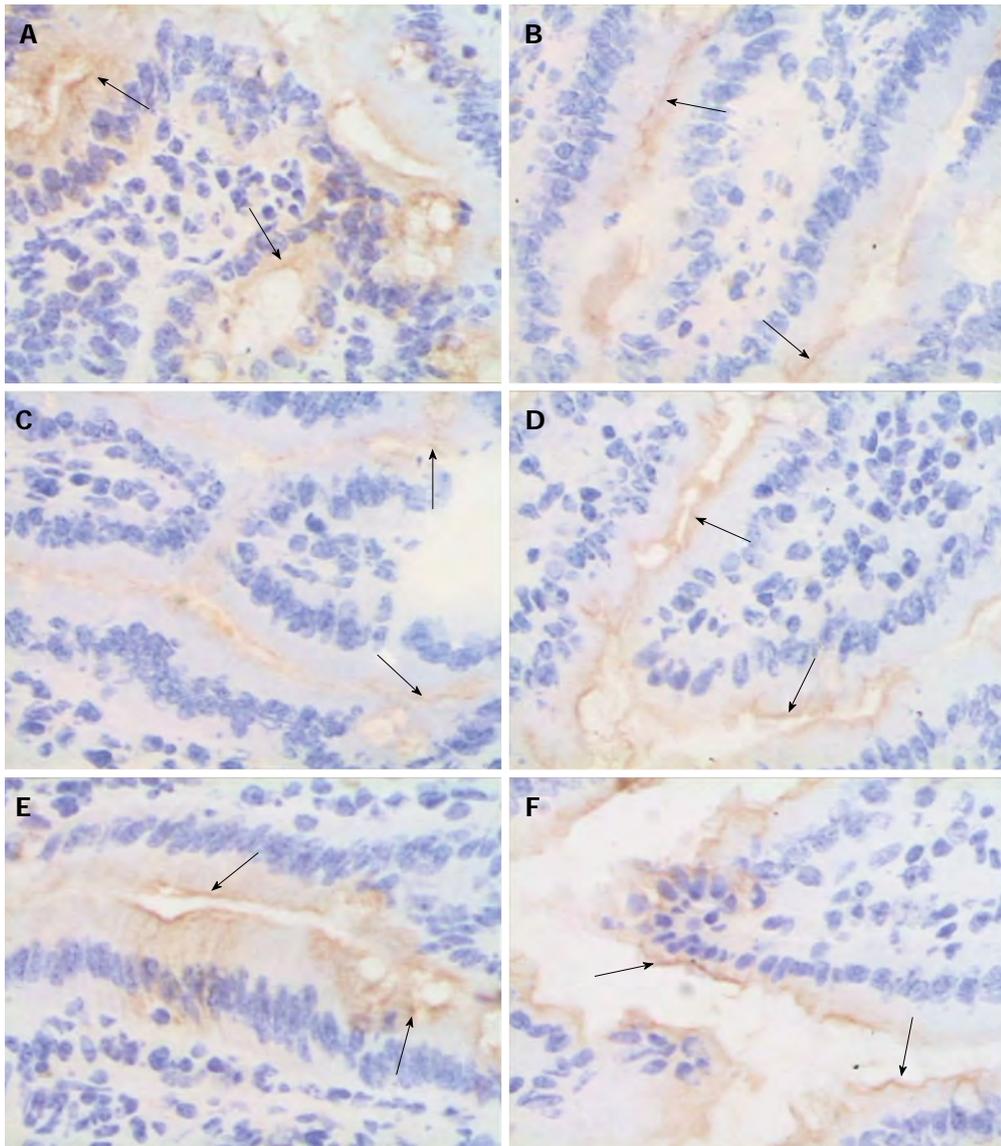
#### Ultrastructural characteristics of the intestinal mucosa

Compared to the Sham group, we observed obvious ultrastructural changes in the intestinal mucosa after I/R injury in the rats from the NS + I/R group. Epithelial cell microvilli were sparsely distributed, disarranged and distorted, and the epithelial cells were swollen or shrunken. The mitochondrial matrices were swollen, cristae were broken, and numerous TJs were disrupted.

There was no disruption of TJs in the BM MSCs + I/R injury group, and only swelling of the epithelial cells was observed. The ultrastructural pathological changes in the groups treated with anti-TNF- $\alpha$  and anti-TNF- $\alpha$ R-IgG were also less severe than in the NS + I/R injury group (Figure 5).

#### Expression of ZO-1 protein

Immunohistochemical analysis revealed strong ZO-1 expression in the intestinal tissue of the Sham group. In the intestinal tissue of the NS + I/R injury group, ZO-1 was expressed at low levels 2 h after injury, slightly in-



**Figure 6** Bone-marrow mesenchymal stem cells and tumor necrosis factor- $\alpha$  blockade attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. The mucosal tissue sections after ischemia/reperfusion (I/R) injury were immunohistochemically labeled for zona occludens 1 (ZO-1) (brown) and counterstained with hematoxylin (blue). A: Sham group; B and C: In the normal saline + I/R injury group, decreased ZO-1 staining (arrows) was observed in the epithelial cells 2 h (B) and 6 h (C) after I/R; D-F: ZO-1 was not obviously affected in the bone-marrow mesenchymal stem cells + I/R injury group (D), anti-tumor necrosis factor (TNF)- $\alpha$  + I/R injury group (E), or anti-TNF- $\alpha$  R1 antibody + I/R injury group (F) at 6 h; original magnification,  $\times 400$ .

creased at 6 and 24 h, and by 72 h, ZO-1-positive signals were detected throughout the entire intestine (Figure 6). Western blot analysis confirmed that ZO-1 expression decreased more significantly in the NS + I/R group than the BM MSCs + I/R injury group, particularly at 6 h (Figure 7). Consistent with the immunohistochemical results, western blotting indicated that ZO-1 expression was significantly higher in the BM MSCs + I/R injury group and the two antibody-treated groups at 6 h than in the NS + I/R injury group (Figure 8).

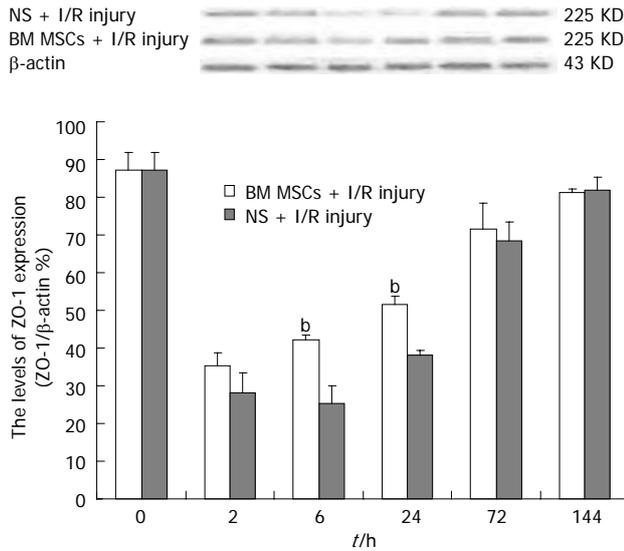
#### **Effect of I/R injury on serum TNF- $\alpha$ levels**

Compared to the Sham group, the serum TNF- $\alpha$  levels increased significantly, peaking at 6 h, in the NS + I/R injury group. The serum level of TNF- $\alpha$  was significant-

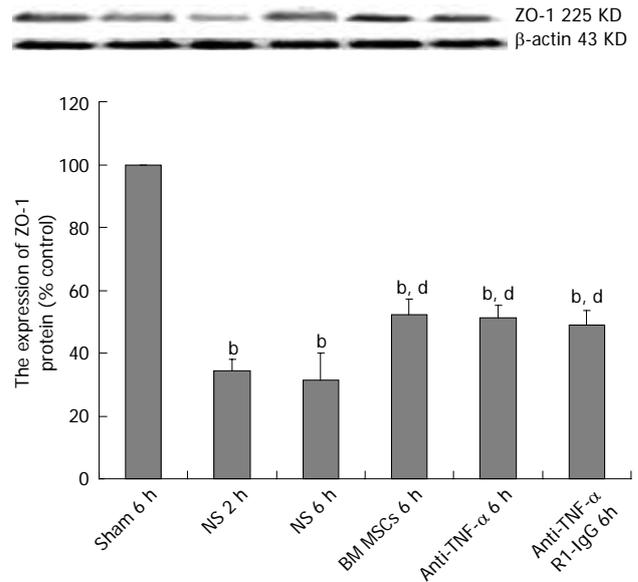
ly lower in the BM MSCs+ I/R injury group at 6 and 24 h than the NS + I/R injury group ( $P < 0.05$ , Table 3). The morphological abnormalities after intestinal I/R injury were positively correlated with serum TNF- $\alpha$  levels (Table 4).

## **DISCUSSION**

I/R injury to the gut is a common event in a variety of clinical conditions, such as trauma, burn injuries, septic shock, heart and aortic surgery, and liver and small bowel transplantation<sup>[27,28]</sup>. Intestinal I/R results in edema, apoptosis, necrosis of epithelial cells, disruption of mucosal integrity and small intestine function, which in turn increases mucosal and vascular permeability, bacte-



**Figure 7** Bone-marrow mesenchymal stem cells attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. Representative western blots and quantification of zona occludens 1 (ZO-1) protein expression in the intestinal mucosa. ZO-1 expression was significantly lower in the saline (NS) + ischemia/reperfusion (I/R) injury group than the bone-marrow mesenchymal stem cells (BM MSCs) + I/R injury group at 6 h ( $25.35 \pm 4.58\%$  vs  $42.32 \pm 1.26\%$ ;  $P < 0.01$ ). Actin was used as a loading control. Values are shown as the mean  $\pm$  SD ( $n = 3$  rats per group); <sup>b</sup> $P < 0.01$  BM MSCs +I/R injury group vs the NS + I/R injury group [one-way ANOVA followed by the least significant difference (LSD) test].



**Figure 8** Bone-marrow mesenchymal stem cells and tumor necrosis factor- $\alpha$  blockade attenuate zona occludens 1 downregulation after intestinal ischemia/reperfusion injury. Representative western blots and quantification of zona occludens 1 (ZO-1) protein expression in the intestinal mucosa. Actin was used as a loading control. Values are the mean  $\pm$  SD ( $n = 3$  rats per group); <sup>b</sup> $P < 0.01$  vs Sham group; <sup>d</sup> $P < 0.01$  vs the saline (NS) + ischemia/reperfusion (I/R) injury group (one-way analysis of variance followed by the LSD test). BM MSCs: Bone-marrow mesenchymal stem cells; Anti-TNF: Anti-tumor necrosis factor.

rial translocation, as well as the risk of systemic inflammation response syndrome, multiple organ dysfunction and death<sup>[29,30]</sup>. Until recently, no effective treatments existed for intestinal I/R injury. Research has suggested that BM MSCs could possibly play a role in the treatment of I/R injury in the heart, kidney and brain<sup>[31-33]</sup>; however, studies of the effects of BM MSCs in intestinal disorders are scarce. In the current study, the therapeutic potential of BM MSCs was evaluated in an experimental rat model of I/R injury, which led to disruption of intestinal mechanical barrier function. The results of this study suggest that BM MSCs can effectively reduce both the intestinal permeability and pathological damage associated with I/R injury. BM MSCs have the potential for multidirectional differentiation. They participate in colonic mucosal regeneration<sup>[34]</sup>. In this study, intestinal I/R injury lead to necrosis and the loss of a large number of intestinal epithelial cells. BM MSCs could reduce I/R injury and protect the intestine.

Stem cell homing processes are thought to play a crucial role in the success of cell therapy for organ function disorders. Intravenous or intra-arterial infusions of BM MSCs often result in the entrapment of the administered cells in organ capillary beds, especially in the lung and the liver<sup>[35]</sup>. The transplantation of BM MSCs by intravenous or intra-arterial routes usually results in a low engraftment rate; therefore, increasing the number of MSCs within the injured area would improve the efficacy of cell therapy. Zhang *et al*<sup>[36]</sup> used gene-modified MSCs to enhance the homing rate of BM MSCs to the

irradiated intestine by 20% using an intravenous delivery route. However, using viral vectors to transfect MSCs may decrease the viability of MSCs. In this study, we directly injected MSCs into the wall of the intestine after I/R injury, which significantly increased the homing of MSCs into the I/R damaged intestinal mucosa. This indicated that the direct injection of BM MSCs into the intestine may provide a better method to enhance the homing rate.

The intestinal mucosal barrier is composed of mucosal fluid, microvilli, epithelial mucosal cell TJs, and other special structures. TJs are the most important structures in the mucosal barrier. The mechanisms responsible for intestinal I/R injury include cytotoxic effects and alterations in the structure of the intestinal mucosa<sup>[15]</sup>. However, few studies have examined the intestinal mucosa and TJ ultrastructure during I/R injury, and the role and mechanism of action of BM MSCs in intestinal I/R injury are unclear. In the present study, we found that severe intestinal mucosal damage occurred 2, 6 and 24 h after I/R injury. The morphological alterations to the intestinal mucosa included the shedding of epithelial cells, fracturing of villi, fusion of adjacent villi, mucosal atrophy and edema. Disruption of TJs between enterocytes, and damage to the mitochondria and endoplasm were also observed. Although damage to the intestinal mucosa plays a significant role in the permeability of the intestine, the mechanisms which cause this damage are poorly characterized. Moreover, we observed that the intestinal permeability increased 2, 6 and 24 h after I/R

**Table 3 Serum levels of tumor necrosis factor- $\alpha$  in a rat model of ischemia/reperfusion injury**

| Group                | Tumor necrosis factor- $\alpha$ (pg/mL) |                                   |                                  |                                |                                |
|----------------------|---|-----------------------------------|----------------------------------|--------------------------------|--------------------------------|
|                      | 2 h                                     | 6 h                               | 24 h                             | 72 h                           | 144 h                          |
| Sham group           | 87.07 $\pm$ 6.47                        | 84.45 $\pm$ 4.18                  | 87.60 $\pm$ 6.25                 | 83.91 $\pm$ 6.67               | 86.54 $\pm$ 5.62               |
| NS + I/R injury      | 226.32 $\pm$ 11.94 <sup>b</sup>         | 332.95 $\pm$ 49.03 <sup>d</sup>   | 221.80 $\pm$ 16.06 <sup>d</sup>  | 180.87 $\pm$ 8.63 <sup>b</sup> | 134.84 $\pm$ 7.18 <sup>b</sup> |
| BM MSCs + I/R injury | 214.21 $\pm$ 17.77 <sup>b</sup>         | 236.76 $\pm$ 20.66 <sup>b,d</sup> | 190.39 $\pm$ 4.24 <sup>a,d</sup> | 177.00 $\pm$ 2.52 <sup>b</sup> | 91.67 $\pm$ 3.84 <sup>b</sup>  |

All values are mean  $\pm$  SD ( $n = 6$ ). <sup>b</sup> $P < 0.01$  vs Sham group; <sup>d</sup> $P < 0.01$  vs the saline (NS) + ischemia/reperfusion (I/R) injury group. BM MSCs: Bone-marrow mesenchymal stem cells.

**Table 4 Serum levels of tumor necrosis factor- $\alpha$  and intestinal mucosal injury grade after intestinal ischemia/reperfusion**

| Group                     | TNF- $\alpha$ (pg/mL) |        |        |        |        | Chiu's grade scores |       |       |       |       |
|---------------------------|-----------------------|--------|--------|--------|--------|---------------------|-------|-------|-------|-------|
|                           | 2 h                   | 6 h    | 24 h   | 72 h   | 144 h  | 2 h                 | 6 h   | 24 h  | 72 h  | 144 h |
| Saline + I/R injury group | 243.27                | 290.41 | 210.51 | 186.58 | 132.55 | 19                  | 28    | 18    | 14    | 10    |
|                           | 212.21                | 286.35 | 241.42 | 189.44 | 125.39 | 14                  | 27    | 21    | 14    | 8     |
|                           | 221.76                | 283.66 | 203.19 | 174.82 | 137.45 | 17                  | 30    | 17    | 13    | 10    |
|                           | 235.42                | 295.87 | 210.37 | 175.76 | 129.57 | 18                  | 28    | 19    | 13    | 9     |
|                           | 229.54                | 345.78 | 226.96 | 189.26 | 145.57 | 18                  | 29    | 20    | 14    | 12    |
|                           | 215.74                | 395.61 | 238.32 | 169.34 | 138.51 | 15                  | 30    | 21    | 12    | 10    |
| Spearman                  |                       |        |        |        |        | 0.947               | 0.931 | 0.961 | 0.971 | 0.956 |
| <i>P</i>                  |                       |        |        |        |        | 0.004               | 0.011 | 0.002 | 0.001 | 0.003 |

The degree of relationship between tumor necrosis factor (TNF)- $\alpha$  and the Chiu risk score was evaluated by bivariate correlation. The results were statistically significant when  $P < 0.05$ , and was highly significant when  $P < 0.01$ . I/R: Ischemia/reperfusion.

injury, with simultaneous disruption in TJ integrity. Additionally, the administration of BM MSCs significantly attenuated the histological damage due to I/R injury (Figure 4) and reduced intestinal permeability (Table 2), compared with the NS + I/R injury group. Therefore, we hypothesized that changes in intestinal permeability may occur due to the disruption of TJs between intestinal mucosa epithelial cells.

To understand the mechanism of TJ disruption, we investigated the expression of ZO-1. ZO-1 was the first TJ-related protein to be identified<sup>[37]</sup>, and it connects the actin cytoskeleton to the transmembrane occludin proteins<sup>[38]</sup>. ZO-1 plays a vital role in the maintenance of intestinal mucosal barrier integrity and TJs during pathological insults<sup>[39]</sup>. In this study, ZO-1 expression in the intestinal mucosa significantly decreased after I/R injury; thus, we concluded that decreased ZO-1 expression lead to TJ disruption and possibly increased gut permeability.

Next, we examined the mechanism of TJ disruption and reduced ZO-1 protein expression during I/R injury. We observed that TNF- $\alpha$  increased at 2, 6 and 24 h after I/R injury, and correlated with ZO-1 downregulation and TJ disruption. The pathophysiological processes of I/R injury *in vivo* are complex, and it is thought that TNF- $\alpha$  may play an important role. Inflammation involves the sequential activation of signaling pathways which result in the production of pro- and anti-inflammatory mediators during I/R injury. Amongst the proinflammatory mediators, the TNF- $\alpha$  and TNF- $\alpha$ R1 systems play central roles in the physiological regulation of intestinal barrier function<sup>[40,41]</sup>, and both TNF- $\alpha$  and interferon (IFN)- $\gamma$  can induce intestinal epithelial barrier

dysfunction<sup>[42]</sup>. Some cytokines can induce endocytosis<sup>[43]</sup> and internalization of epithelial TJ proteins<sup>[44]</sup>. In mice with fulminant hepatic failure, reduced expression of occludin in intestinal epithelial cells was linked to increased TNF- $\alpha$  production<sup>[4]</sup>. TNF- $\alpha$  can also induce an increase in Caco-2 cell TJ permeability *via* nuclear factor- $\kappa$ B activation, leading to downregulation of ZO-1 protein expression and altered junctional localization<sup>[38,45]</sup>. We hypothesize that TNF- $\alpha$  acts as an initiator, which can induce expression of other cytokines such as IL-6 and IFN- $\gamma$ , which then initiate and aggravate the development of I/R injury, and disrupt intestinal TJs.

After the transplantation of BM MSCs, the serum TNF- $\alpha$  level significantly decreased, the damaged mucosa recovered, ZO-1 expression increased and intestinal permeability significantly improved. TNF- $\alpha$  is known to inhibit the expression of ZO-1<sup>[44]</sup>, and if the TJs are damaged, intestinal barrier dysfunction will occur. Research has confirmed that BM MSCs can inhibit the generation of TNF- $\alpha$  in dendritic cells *in vitro*<sup>[46,47]</sup>, and therefore we hypothesized that BM MSCs could repair intestinal I/R injury by inhibiting the release of TNF- $\alpha$ . In order to further study the role of TNF- $\alpha$ , we used anti-TNF- $\alpha$  and anti-TNFR antibodies. The TNF- $\alpha$  antibody neutralizes TNF- $\alpha$ , whereas the anti-TNFR antibody blocks the binding of TNF- $\alpha$  to the TNF- $\alpha$  receptor. TNF- $\alpha$  blockade significantly decreased the severity of I/R injury, which indicates that TNF- $\alpha$  is an important mediator of intestinal mucosa damage during I/R injury. These findings suggest that I/R injury increases TNF- $\alpha$ , leading to downregulation of ZO-1 protein expression, whereas BM MSCs can inhibit pro-

duction of TNF- $\alpha$ , leading to increased expression of ZO-1 and reduced intestinal mucosa damage. These effects were observed over a relatively short observation period, and long-term studies are required to elucidate if TNF- $\alpha$  exerts long-lasting effects during I/R injury.

In summary, this study demonstrates that the submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, in a mechanism linked to reduced serum TNF- $\alpha$  levels and the increased expression of the intestinal TJ protein ZO-1. Future studies using exogenous or autologous BM MSCs to prevent or modulate intestinal I/R injuries are required to assess the clinical potential of BM MSCs. The mechanisms by which BM MSCs and TNF- $\alpha$  blockade protect against I/R-induced disruption of intestinal barrier function remain to be further investigated.

Disruption of the intestinal mucosa and the consequent increase in permeability after I/R injury may be due to reduced levels of the TJ-associated protein, ZO-1. BM MSCs restored the epithelial structure, promoted the recovery of intestinal permeability, increased ZO-1 protein expression and protected against intestinal I/R injury. TNF- $\alpha$  plays an important role in the ability of BM MSCs to protect against intestinal I/R injury, as the epithelial structure remained normal, and changes in intestinal permeability and ZO-1 protein expression were reduced when rats were treated with anti-TNF- $\alpha$  IgG antibody or anti-TNF- $\alpha$  R1 antibodies before I/R injury. This study confirms that high levels of TNF- $\alpha$  damage TJs and downregulate ZO-1 protein expression *in vivo*. The mechanism of TNF- $\alpha$ -induced change during I/R injury is complex and requires further study.

## COMMENTS

### Background

Digestive organ transplantation and other abdominal surgical procedures can result in different degrees of intestinal ischemia/reperfusion (I/R) injury, which can delay patient recovery and lead to systemic organ failure. Therefore, intestinal I/R injury is an important clinical issue. Bone-marrow mesenchymal stem cells (BM MSCs) can protect against I/R injury; however, the mechanism is unclear. Although previous studies have provided an insight into the molecular structure of tight junctions (TJs), much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during intestinal I/R injury. In this study, the authors used a rat model of intestinal I/R injury to investigate the effect of BM MSCs on the intestinal mucosa ultrastructure, with an emphasis on the mechanisms of intestinal barrier dysfunction.

### Research frontiers

In this study, the authors demonstrated that the submucosal infusion of BM MSCs decreased intestinal permeability and preserved intestinal mechanical barrier function after I/R injury in rats, in a mechanism linked to reduced serum tumor necrosis factor (TNF)- $\alpha$  levels and the increased expression of the intestinal TJ protein zona occludens (ZO)-1. Altered serum TNF- $\alpha$  levels play an important role in the ability of BM MSCs to protect against intestinal I/R injury.

### Innovations and breakthroughs

Recent reports have highlighted the importance of BM MSCs reducing intestinal I/R injury in rats. Although previous studies have provided an insight into the molecular structure of TJs, much less is known about TJ functionality under physiological or pathophysiological conditions. Few studies have described the intestinal mucosa ultrastructure or changes in TJs during intestinal I/R injury.

This is believed to be the first study to report that BM MSCs reduce rat intestinal I/R injury, ZO-1 downregulation, and TJ disruption *via* a TNF- $\alpha$ -regulated mechanism.

### Applications

By understanding how BM MSCs reduce rat intestinal I/R injury, this study may represent a future strategy for therapeutic intervention in the treatment of patients with digestive organ transplantation and other abdominal surgical procedures that result in different degrees of intestinal I/R injury, which can delay patient recovery and lead to systemic organ failure.

### Terminology

TJs are the main structures responsible for restricting the paracellular movement of compounds across the intestinal mucosa. Structurally, TJs are composed of cytoplasmic proteins, including ZO-1-3 and two distinct transmembrane proteins, occludin and claudin, which are linked to the actin-based cytoskeleton. ZO-1, as a scaffold for the organization of transmembrane TJ proteins, also recruits various signaling molecules and the actin cytoskeleton to TJs.

### Peer review

This paper shows the impact of BM MSCs on rat intestinal I/R injury. This study will be of interest and the paper is clearly written.

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**P- Reviewer** Fujino Y **S- Editor** Zhai HH **L- Editor** Kerr C  
**E- Editor** Zhang DN



## Risk factors for colonoscopic perforation: A population-based study of 80118 cases

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Author contributions: Hamdani U made the contribution to conception and designed research, acquisition of data and interpretation of data, wrote the paper; Naeem R, Haider F, and Bansal P made the contribution to data gathering and study design and drafting the manuscript; Komar M and Diehl DL made the contribution to revising it critically for important intellectual content; Kirchner HL made the contribution to statistical analysis, study design and methods.

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Received: November 19, 2012 Revised: January 12, 2013

Accepted: April 13, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To assess the incidence and risk factors associated with colonic perforation due to colonoscopy.

**METHODS:** This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System during the period from January 1, 2002 to August 25, 2010. Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables. Baseline comparisons across those with and without a perforation were made using the two-sample *t*-test and Pearson's  $\chi^2$  test, as appropriate.

**RESULTS:** A total of 50 perforations were diagnosed out of 80118 colonoscopies, which corresponded to an incidence of 0.06% (95%CI: 0.05-0.08) or a rate of 6.2 per 10000 colonoscopies. All possible risk factors associated with colonic perforation with a *P*-value < 0.1 were checked for inclusion in a multivariable log-binomial regression model predicting 7-d colonic perforation. The final model resulted in the following risk factors which were significantly associated with risk of colonic perforation: age, gender, body mass index, albumin level, intensive care unit (ICU) patients, inpatient setting, and abdominal pain and Crohn's disease as indications for colonoscopy.

**CONCLUSION:** The cumulative 7 d incidence of colonic perforation in this cohort was 0.06%. Advanced age and female gender were significantly more likely to have perforation. Increasing albumin and BMI resulted in decreased risk of colonic perforation. Having a colonoscopy indication of abdominal pain or Crohn's disease resulted in a higher risk of colonic perforation. Colonoscopies performed in inpatients and particularly the ICU setting had substantially greater odds of perforation. Biopsy and polypectomy did not increase the risk of perforation and only three perforations occurred with screening colonoscopy.

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**Key words:** Colonoscopic perforation; Colon cancer; Endoscopy

**Core tip:** This study is unique because we have used state of the art electronic medical records to collect information about risk factors which can predispose patient to a high risk of perforation. We have looked into multiple risk factors including but not limited to serum albumin, serum creatinine, body mass index (BMI), inpatient and outpatient colonoscopy and intensive care unit (ICU) patients. Limited literature is available about the above mentioned risk factors and there propensity

to cause perforation. The important findings deduced from this research can have important implication in day to day practice of colonoscopy. The findings of Albumin, BMI, and Inpatient and out patient colonoscopy particularly performing colonoscopy in ICU setting predisposing to higher risk of perforation are crucial piece of information that can help physician in considering available alternatives which in turn may help to reduce the number of colonoscopic perforations.

Hamdani U, Naem R, Haider F, Bansal P, Komar M, Diehl DL, Kirchner HL. Risk factors for colonoscopic perforation: A population-based study of 80118 cases. *World J Gastroenterol* 2013; 19(23): 3596-3601 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3596.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3596>

## INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer and the second leading cause of cancer-related death in the United States<sup>[1]</sup>. Early detection benefits patients and increases their quality of life, but also reduces health care expenditures. The ability of colonoscopy to detect polyps and colorectal cancer has been shown to reduce mortality and morbidity associated with this cancer<sup>[2,3]</sup>. In July 2001, Medicare began covering screening colonoscopy for individuals over the age of 50 at average risk for colorectal cancer and depending on the detection of polyps, at variable intervals thereafter. Since that time, the use of colonoscopy has been increasing<sup>[4-6]</sup>.

Colonoscopy is generally regarded as a safe procedure; potential complications include perforation, post-polypectomy bleeding and post-polypectomy syndrome<sup>[7]</sup>. The incidence of colonic perforation ranges from 0.005% and 0.63% with the majority of patients requiring laparotomy for repair<sup>[8-12]</sup>. Colonoscopic perforation occurs due to one of three mechanisms; mechanical forces from the endoscope, barotrauma from air insufflation, or as a direct result of a therapeutic procedure (*e.g.*, polypectomy). To better understand the risk factors associated with colonoscopic perforation, we conducted a large cross-sectional study to estimate the incidence of this serious complication, and to examine potential contributing effects of demographic and medical characteristics of patients.

## MATERIALS AND METHODS

### Study cohort, design, and setting

This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System (GHS) during the period from January 1, 2002 to August 25, 2010. GHS is a primary care and multispecialty medical practice located in central and northeast Pennsylvania and is the largest rural not-

for-profit health system in the nation. GHS uses health information technology infrastructure for managing and using patient data. Colonoscopy procedures were identified by the presence of current procedural terminology (CPT) 2005 codes 45378-45387, 45391, and 45392.

The study outcome was the diagnosis of colonic perforation using International Classification of Disease, 9<sup>th</sup> revision (ICD-9) codes 569.83 and 998.2, defined as perforation of intestine and accidental puncture or laceration during a procedure, 7 d after the day of colonoscopy. We specifically looked at the 7 d post colonoscopy for perforation since previous studies have shown that almost all post-colonoscopy perforations were detected within this time frame<sup>[13,14]</sup>. Both inpatient and outpatient procedures were included.

Variables obtained from the electronic health record included age at colonoscopy, gender, body mass index (BMI), albumin, serum creatinine, operator specialty (surgeon or gastroenterologist), and indications for the colonoscopy (identified by procedure codes in the colonoscopy report). Race was not assessed for analysis since the primary care population seen in Geisinger Health System is > 95% Caucasian.

Data on comorbid health conditions were also collected including history of coronary artery disease, congestive heart failure, peripheral arterial disease, cerebrovascular disease, dementia, chronic obstructive pulmonary disease, connective tissue disease, peptic ulcer disease, liver disease, diabetes mellitus, hemiplegia, chronic kidney disease, leukemia, lymphoma, metastatic cancer, and acquired immune deficiency syndrome.

### Statistical analysis

Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables. Baseline comparisons across those with and without a perforation were made using the two-sample *t*-test and Pearson's  $\chi^2$  test, as appropriate. The incidence of 7-d post-colonoscopy perforations was calculated as the number of colonic perforations divided by the total number of colonoscopies, and expressed as both percentage and as an incidence rate (*e.g.*, number of perforations per 10000 colonoscopies). The count of comorbid conditions was summarized as a general indicator of health.

The log-binomial model was used to estimate the incident rate ratios (IRR) for risk factors found to vary across the two groups. A fully adjusted model was then developed to identify those risk factors predictive of perforation. Variables were considered for inclusion in the model if they were found to vary between groups at a significance level of  $P < 0.10$ . Backward elimination was performed to obtain a final model that retained clinically meaningful predictors. Results are presented as RR and corresponding 95%CI. The analysis was performed using SAS v9.2 (SAS Institute, Inc., Cary, NC, United States) and R v2.13 (R Development Core Team, [www.r-project.org](http://www.r-project.org))<sup>[15]</sup>.

**Table 1** Potential risk factors stratified by colonic perforation *n* (%)

|   | No perforation<br>( <i>n</i> = 80068)    | Perforation<br>( <i>n</i> = 50)       | <i>P</i> -value |
|---|--|---------------------------------------|-----------------|
| Age category (yr)                                 |  |                                       | < 0.0001        |
| 18-50   | 13698 (17.11)                            | 5 (10.00)                             |                 |
| 50-65   | 38695 (48.33)                            | 10 (20.00)                            |                 |
| 65-80   | 22954 (28.67)                            | 20 (40.00)                            |                 |
| 80+   | 4271 (5.90)                              | 15 (30.00)                            |                 |
| Gender  | <i>n</i> = 80059                         | <i>n</i> = 50                         | 0.0183          |
| Male  | 38972 (46.68)                            | 16 (32.00)                            |                 |
| Female  | 41087 (51.32)                            | 34 (68.00)                            |                 |
| BMI (median, IQR)<br>(5.35% unknown) <sup>3</sup> | 28.66 (25.14, 32.92)<br><i>n</i> = 79615 | 26.27 (20.70, 28.55)<br><i>n</i> = 48 | 0.0002          |
| Operator specialty <sup>4</sup>                   | <i>n</i> = 78421                         | <i>n</i> = 46                         | 0.2290          |
| Surgery   | 13826 (17.63)                            | 5 (10.87)                             |                 |
| Gastroenterology                                  | 64595 (82.37)                            | 41 (89.13)                            |                 |
| Type of colonoscopy <sup>2</sup>                  |  |                                       | < 0.0001        |
| Therapeutic                                       | 37867 (47.29)                            | 13 (26.53)                            |                 |
| Polypectomy                                       | 16367 (20.44)                            | 6 (12.42)                             |                 |
| Dilation  | 97 (0.12)                                | 2 (4.08)                              |                 |
| Biopsy  | 18807 (23.49)                            | 2 (4.08)                              |                 |
| Other <sup>1</sup>                                | 2596 (3.2)                               | 3 (6.1)                               |                 |
| Screening   | 29898 (37.34)                            | 3 (6.12)                              |                 |
| Diagnostic  | 12303 (15.37)                            | 33 (67.35)                            |                 |
| Number of Interventions performed <sup>5</sup>    |  |                                       | 0.0342          |
| 1   | 73919 (92.32)                            | 45 (90.00)                            |                 |
| 2   | 5527 (6.90)                              | 3 (6.00)                              |                 |
| 3   | 622 (0.78)                               | 2 (4.00)                              |                 |
| Count of morbidities                              |  |                                       | 0.2004          |
| 0   | 55601 (69.4)                             | 31 (62.0)                             |                 |
| 1   | 17330 (21.6)                             | 11 (22.0)                             |                 |
| 2+  | 7137 (8.9)                               | 8 (16.0)                              |                 |
| Setting   |  |                                       | < 0.0001        |
| Inpatient   | 4132 (5.2)                               | 23 (46.0)                             |                 |
| Outpatient  | 75936 (94.8)                             | 27 (54.0)                             |                 |
| ICU   | 85 (0.1)                                 | 9 (18.0)                              | < 0.0001        |
| Indications for CP                                |  |                                       |                 |
| Abdominal pain                                    | 3623 (4.52)                              | 7 (14.00)                             | 0.0070          |
| Anemia  | 1875 (2.34)                              | 5 (10.00)                             | 0.0063          |
| Bleeding  | 3615 (4.51)                              | 7 (14.00)                             | 0.0070          |
| Crohn's disease                                   | 329 (0.41)                               | 2 (4.00)                              | 0.0183          |
| Diarrhea  | 2565 (3.20)                              | 0 (0)                                 | 0.4115          |
| Diverticulosis of colon                           | 15635 (19.53)                            | 10 (20.00)                            | 0.9328          |
| Obstruction                                       | 416 (0.52)                               | 0 (0)                                 | 0.9999          |
| Ulcerative colitis                                | 920 (1.15)                               | 1 (2.00)                              | 0.4391          |
| Weight loss                                       | 464 (0.58)                               | 1 (2.00)                              | 0.2526          |
| Creatinine (median,<br>IQR) (12% unknown)         | 0.9 (0.7, 1.0)                           | 0.9 (0.7, 1.1)                        | 0.9824          |

<sup>1</sup>Includes foreign body removal, hemostasis; <sup>2</sup>Info to ascertain type of colonoscopy in one perforation not available; <sup>3</sup>Two body mass index (BMI) in perforation group not available; <sup>4</sup>Operator specialty of 4 perforations are other than gastroenterology and general surgery; <sup>5</sup>Number of interventions includes biopsy, polypectomy, dilations and hemostasis. ICU: Intensive care unit; CP: Cerebral palsy; IQR: Interquartile range.

## RESULTS

A total of 50 perforations were diagnosed out of 80118 colonoscopies, which corresponded to an incidence of 0.06% (95%CI: 0.05-0.08) or a rate of 6.2 per 10000 colonoscopies. Thirty-nine patients underwent emergent exploratory laparotomy and 11 were managed conservatively.

Patients that had a perforation within 7 d of their procedures were more likely to be older, female gender,

lower BMI, have more comorbidities, and a lower albumin value (Table 1). Indications for colonoscopy including abdominal pain, anemia, and bleeding were reported more in those with a perforation as compared to the non-perforation group. Operator specialty and creatinine values were not found to vary between groups. The presence of end stage renal disease (ESRD) and prior abdominal surgeries also were not associated with colonic perforations in our cohort.

Based on the findings in Table 1, those risk factors meeting the criteria of a *P* < 0.1 were further described. Table 2 reports the number of perforations and the incidence rate per 10000 patients stratified by these important factors. For every year increase in age, the risk of a perforation increased by 7% (95%CI: 5-9) with the incidence of perforation increasing from 2.6 cases per 10000 in the 50-64 year old age group to 31.7 cases per 10000 in the 80+ year old age group. Females were twice as likely to have a perforation compared to males. Lower BMI resulted in a higher risk of perforation. Decreased albumin levels ( $\leq 4.0$ ) (closest prior to colonoscopy) were associated with an increased risk of colonic perforation (IRR = 7.8, 95%CI: 4.1-14.6). There was also a significant difference of perforation rate between colonoscopy performed in an inpatient and outpatient setting. Inpatients were much more likely to have perforation compared with outpatients (55.4 and 3.6 cases per 10000, respectively). Similarly, the risk of perforation increased in intensive care unit (ICU)-patients compared to non-ICU patients.

All possible risk factors associated with colonic perforation with a *P*-value < 0.1 were checked for inclusion in a multivariable log-binomial regression model predicting 7-d colonic perforation. The final model resulted in the following risk factors which were significantly associated with risk of colonic perforation: age, gender, BMI, albumin level, ICU patients, inpatient setting, and abdominal pain and Crohn's disease as indications for colonoscopy. Approximately 21% of the patients did not have an albumin laboratory result available for analysis. Therefore, a model was fit with and without including albumin. Also, based on the descriptive results albumin was categorized at 4.0. The estimates from the final models are displayed in Table 3.

## DISCUSSION

In reviewing literature from 1975 onward, we observed that the incidence rate of colonic perforation ranges between 0.005% to 0.63%. We noticed a gradual decline in the incidence of colonic perforation which has reached a plateau in the last 10 years. The differences in the incidence rates in this study compared to those in the literature can possibly be attributed to the way the studies were conducted. For example Sieg *et al*<sup>[16]</sup> and Rathgeber *et al*<sup>[17]</sup> both reported low incidence rate of perforation (0.005 and 0.01 respectively). Sieg *et al*<sup>[16]</sup> looked prospectively at 82, 16 colonoscopies but there was a selection

**Table 2** Incidence of 7-d colonic perforation risk by important risk factors

| Patient variable                     | Frequency | Perforations | Incidence per 10000 | Incident rate ratio (95%CI)       |
|--------------------------------------|-----------|--------------|---------------------|-----------------------------------|
| Total                                | 80118     | 50           | 6.2                 | -                                 |
| Age (yr)                             |           |              |                     | 1.07 <sup>1</sup><br>(1.05, 1.09) |
| 18-49                                | 13703     | 5            | 3.6                 |                                   |
| 50-64                                | 38705     | 10           | 2.6                 |                                   |
| 65-79                                | 22974     | 20           | 8.7                 |                                   |
| 80+                                  | 4736      | 15           | 31.7                |                                   |
| Gender                               |           |              |                     |                                   |
| Female                               | 41121     | 34           | 8.3                 | -                                 |
| Male                                 | 38988     | 16           | 4.1                 | 0.50<br>(0.27, 0.90)              |
| BMI (kg/m <sup>2</sup> )             |           |              |                     | 0.91 <sup>1</sup><br>(0.86, 0.96) |
| < 24 (normal weight)                 | 18019     | 21           | 11.7                |                                   |
| 25-29 (overweight)                   | 26873     | 17           | 6.3                 |                                   |
| 30+ (obese)                          | 30873     | 10           | 3.2                 |                                   |
| Type of Colonoscopy                  |           |              |                     |                                   |
| Therapeutic                          | 37880     | 13           | 3.4                 | -                                 |
| Screening                            | 29901     | 3            | 1.0                 | 0.29<br>(0.08, 1.03)              |
| Diagnostic                           | 12336     | 33           | 26.8                | 7.79<br>(4.10, 14.80)             |
| Albumin result (percentile cut-offs) |           |              |                     | 0.15 <sup>1</sup><br>(0.12, 0.20) |
| ≤ 4.0                                | 16537     | 36           | 21.8                | 7.76<br>(4.12, 14.64)             |
| > 4.1                                | 46366     | 13           | 2.8                 | -                                 |
| ICU patients                         | 94        | 9            | 957.4               | 186.9<br>(93.5, 373.5)            |
| Non-ICU patients                     | 80024     | 41           | 5.1                 | -                                 |
| Inpatients                           | 4155      | 23           | 55.4                | 15.6<br>(8.9, 27.1)               |
| Outpatients                          | 75963     | 27           | 3.6                 | -                                 |
| Indications for CP                   |           |              |                     |                                   |
| Abdominal pain                       |           |              |                     | 3.4                               |
| Yes                                  | 3630      | 7            | 19.3                | (1.5, 7.6)                        |
| No                                   | 76488     | 43           | 5.6                 | -                                 |
| Anemia                               |           |              |                     | 4.6                               |
| Yes                                  | 1880      | 5            | 26.6                | (1.8, 11.6)                       |
| No                                   | 78238     | 45           | 5.8                 | -                                 |
| Bleeding                             |           |              |                     | 3.4                               |
| Yes                                  | 3622      | 7            | 19.3                | (1.5, 7.6)                        |
| No                                   | 76496     | 43           | 5.6                 | -                                 |
| Crohn's disease                      |           |              |                     |                                   |
| Yes                                  | 331       | 2            | 60.4                | 10.0                              |
| No                                   | 79787     | 48           | 6                   | (2.5, 41.2)                       |

<sup>1</sup>Variable was treated as continuous in the estimation of the incident rate ratio. ICU: Intensive care unit; CP: Cerebral palsy; BMI: Body mass index.

bias since only perforations that required surgical intervention were included in the study. Similarly, Rathgaber and Wick's study of 12407 colonoscopies, complications were gathered by monthly retrospective review of all hospitalizations and patient phone calls. Anderson *et al*<sup>[18]</sup> reported an incidence 0.19% in 10486 colonoscopies and Gatto *et al*<sup>[13]</sup> found 0.196% in 39286 colonoscopies. Both studies primarily looked at an older patient population which may have contributed to the higher rate of colonoscopic perforations.

This study looked at patients 18 years or older. By

**Table 3** Multivariate log-binomial regression results predicting 7-d post colonoscopic perforation

| Risk factor         | Model without albumin | Model with albumin  |
|---------------------|-----------------------|---------------------|
| Age                 | 1.04 (1.01, 1.06)     | 1.03 (1.01, 1.05)   |
| BMI                 | 0.96 (0.91, 1.00)     | 0.94 (0.90, 0.99)   |
| ICU                 | 9.37 (4.42, 19.88)    | 5.83 (2.80, 12.14)  |
| Inpatient           | 18.08 (8.58, 38.17)   | 11.05 (5.14, 23.75) |
| Type of colonoscopy |                       |                     |
| Therapeutic         | -                     | -                   |
| Screening           | 0.25 (0.07, 0.87)     | 0.17 (0.04, 0.76)   |
| Diagnostic          | 12.93 (6.65, 25.13)   | 15.33 (7.79, 30.18) |
| Abdominal pain      | 5.32 (2.40, 11.82)    | 5.79 (2.64, 12.74)  |
| Crohn's disease     | 11.26 (3.88, 32.70)   | 5.16 (1.79, 14.88)  |
| Albumin (≤ 4.0)     | -                     | 3.58 (1.72, 7.47)   |

BMI: Body mass index; ICU: Intensive care unit.

including a wider range of patients, the current findings are likely to be more representative of the true incidence of perforation. We found that age greater than 65 years was a significant predictor for risk of perforation. This finding is in congruence with other studies<sup>[12-14,19]</sup> that found increased age as an independent risk factor for perforation.

We found that the female gender is predisposed to a higher risk of perforation as compared to the male gender. Anderson *et al*<sup>[18]</sup> and Korman *et al*<sup>[12]</sup> also found female gender to be an independent risk factor for perforation. In contrast, Arora *et al*<sup>[19]</sup> did not find female gender as a significant risk factor for perforation in 277434 colonoscopies.

We found lower BMI to be another statistically significant predictor of perforation. Literature on the relation between BMI and risk of colonic perforation is sparse. Takahashi *et al*<sup>[20]</sup> postulated lower BMI as a predictor of pain and difficult colonic intubation during colonoscopy. Patients with low BMI may have sharper angulation of the sigmoid colon which theoretically can predispose these patients to a higher chance of mechanical injury during colonoscopy.

Increasing number of comorbidities resulted in increased risk of colonic perforation. Our findings are in congruence with other studies Gatto *et al*<sup>[13]</sup> and Arora *et al*<sup>[19]</sup> that demonstrated an increased risk of perforation with two or more co-morbidities.

Imai *et al*<sup>[21]</sup> studied the risk of perforation in patients with ESRD on hemodialysis (HD) undergoing colonoscopy. The study looked at 1106 HD patients and 13992 controls, and the authors found a higher risk of perforation among HD patients. Our study looked at patients with ESRD on hemodialysis, and also at patients with chronic kidney disease who were not on HD. There were no perforations among the 321 patients with ESRD in our cohort. We did not find any statistically significant relationship with increasing creatinine level and risk of perforation.

Low albumin has been shown to be a predictor for failure to complete colonoscopy<sup>[22]</sup>. Hypoalbuminemia is

a well-documented marker of morbidity and is a strong predictor of mortality in elderly patients<sup>[22,23]</sup>. We found low albumin level to be associated with a higher risk for perforation. It is possible that a low albumin may decrease the tensile strength of the colonic wall and also generally indicates poor health status that can theoretically predispose to higher risk for perforation.

We did not find any significant difference in the rate of perforation between colonoscopies performed by gastroenterologists or surgeons. This is in congruence with a prospective study of 13580 colonoscopies done by surgeons Wexner *et al.*<sup>[24]</sup>, which found that colonoscopy performed by surgeons are safe with low morbidity and mortality.

We did not find performance of biopsy or polypectomy as significant risk factors for perforation. Similar findings were noted by Arora *et al.*<sup>[19]</sup>, but are in contrast to Levin *et al.*<sup>[4]</sup> and Misra *et al.*<sup>[25]</sup> who found increased risk of perforation after polypectomy. We found that the performance of invasive procedures such as foreign body removal, hemostasis increase the risk of perforation, similar findings were noted by Arora *et al.*<sup>[19]</sup>. We also found dilation as a significant risk factor for perforation in our cohort.

A potential limitation of this study is the validity of coding and capturing of all perforations. We used ICD-9 and CPT codes to capture perforations and co-morbidities. It is possible that we may have missed perforations due to incorrect coding. Also, if a patient went outside of our health care system, then some perforations would not have been reported and thus, not identified. Therefore, underestimation of the incidence of perforation is possible in this study.

In conclusion, the cumulative 7 d incidence of colonic perforation in this cohort was 0.06%. Advanced age and female gender were significantly more likely to have perforation. Increasing albumin and BMI resulted in decreased risk of colonic perforation. Having a colonoscopy indication of abdominal pain or Crohn's disease resulted in a higher risk of colonic perforation. Colonoscopies performed in inpatients and particularly the ICU setting had substantially greater odds of perforation. Biopsy and polypectomy did not increase the risk of perforation and only three perforations occurred with screening colonoscopy.

The increased risk of perforation during inpatient colonoscopy among the elderly and very elderly (> 80 years), and ICU patients is not inconsequential. On the basis of this data, we have restricted inexperienced operators (such as first year gastroenterology fellows) from performing these types of cases. Additionally those over 80 years referred for diagnostic colonoscopy should also be advised of their increased risk of perforation. By understanding which patient populations are at greatest risk for colonoscopic perforation, considering available alternatives, and adjusting patient selection criteria balancing for those at highest risk, may help to reduce the number of colonoscopic perforations.

## COMMENTS

### Background

This study is unique because we have used state of the art electronic medical records to collect information about risk factors which can predispose patient to a high risk of perforation. The authors have looked into multiple risk factors including but not limited to serum albumin, serum creatinine, body mass index (BMI), inpatient and outpatient colonoscopy and intensive care unit patients. Limited literature is available about the above mentioned risk factors and there propensity to cause perforation. The important findings deduced from this research can have important implication in day to day practice of colonoscopy.

### Research frontiers

Authors found lower BMI to be another statistically significant predictor of perforation. Literature on the relation between BMI and risk of colonic perforation is sparse. Yuuichi postulated lower BMI as a predictor of pain and difficult colonic intubation during colonoscopy. Patients with low BMI may have sharper angulation of the sigmoid colon which theoretically can predispose these patients to a higher chance of mechanical injury during colonoscopy.

### Innovations and breakthroughs

This was a retrospective cross-sectional study. Patients were retrospectively eligible for inclusion if they were 18 years and older and had an inpatient or outpatient colonoscopy procedure code in any facility within the Geisinger Health System during the period from January 1, 2002 to August 25, 2010. Data are presented as median and inter-quartile range, for continuous variables, and as frequency and percentage for categorical variables.

### Peer review

This is an interesting paper on a clinically important topic and with good numbers. By understanding which patient populations are at greatest risk for colonoscopic perforation, considering available alternatives, and adjusting patient selection criteria balancing for those at highest risk, may help to reduce the number of colonoscopic perforations.

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**P- Reviewer** Myreliid PE **S- Editor** Gou SX **L- Editor** A  
**E- Editor** Lu YJ



## Evaluation of enterochromaffin cells and melatonin secretion exponents in ulcerative colitis

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**Supported by** The Ministry of Science and High Education of Poland, No. NN402 4221/38

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Received: October 29, 2012 Revised: April 1, 2013

Accepted: April 18, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To study an assessment of the number of enterochromaffin cells and expression of hydroxyindole-O-methyltransferase in colonic mucosa and urine excretion of 6-sulfatoxymelatonin in patients with ulcerative colitis.

**METHODS:** The study included 30 healthy subjects (group I -C), 30 patients with ulcerative proctitis [group II -ulcerative proctitis (UP)] and 30 patients with ulcerative colitis [group III -ulcerative colitis (UC)] in acute phases of these diseases. The number of enterochromaffin cells (EC) was estimated in rectal and colonic mucosa. Biopsies were assembled from many different parts of the large intestine. Immunoreactive cells

collected from various parts of the colon were counted according to the Eurovision DAKO (Dako A/S, Copenhagen, Denmark) System in the range of 10 fields in each biopsate at  $\times 200$  magnification. The level of mRNA expression of hydroxyindole-O-methyltransferase (HIOMT) in colonic mucosa was estimated with RT-PCR. Urine 6-sulfatoxymelatonin (6-HMS) excretion was determined immunoenzymatically using an IBL (IBL International GmbH, Hamburg, Germany) kit (RE 54031).

**RESULTS:** The number of EC cells in healthy subjects (C) was  $132.40 \pm 31.26$ . In patients of group II (UP) and group III (UC) the number of these cells was higher -  $225.40 \pm 37.35$  ( $P < 0.001$ ) and -  $225.24 \pm 40.50$  ( $P < 0.001$ ) respectively. Similar differences were related to HIOMT expression, which was  $1.04 \pm 0.36$  in group C,  $1.56 \pm 0.56$  ( $P < 0.01$ ) in group UP and  $2.00 \pm 0.35$  ( $P < 0.001$ ) in group UC. Twenty-four hour 6-HMS urinary excretion was as follows: C -  $16.32 \pm 4.95$   $\mu\text{g}/24$  h, UP -  $26.30 \pm 7.29$   $\mu\text{g}/24$  h ( $P < 0.01$ ), UC -  $42.30 \pm 12.56$   $\mu\text{g}/24$  h ( $P < 0.001$ ). A correlation between number of EC cells and 6-HMS excretion was noted in all groups:  $r = 0.766$  in patients with UP,  $r = 0.703$  with UC and  $r = 0.8551$  in the control group; the correlation between the results is statistically significant.

**CONCLUSION:** In the acute phases of both UP and UC, proliferation of EC cells and high expression of HIOMT and urine excretion of 6-HMS is noted. These changes may represent a beneficial response in the anti-inflammatory and defense mechanism.

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**Key words:** Enterochromaffin cells; 5-hydroxyindole-O-methyltransferase; 6-sulfatoxymelatonin; Ulcerative proctitis; Ulcerative colitis

**Core tip:** In the gastrointestinal tract melatonin is secreted mainly by enterochromaffin cells (EC). It appears

that hydroxyindole-O-methyltransferase (HIOMT) is enzyme determining melatonin synthesis. This indoleamine exerts, antioxidant, and anti-inflammatory effects. Its synthesis can be disturbed by pro-inflammatory cytokines. The obtained results noted the proliferation of EC cells and the increase of HIOMT expression in ulcerative colitis (UC). Positive correlation between the amount of EC cells and urinary 6-sulfatoxymelatonin excretion points to the increase of melatonin secretion in the colon, but its beneficial anti-inflammatory effect was insufficient. The results indicate that the supplementation of melatonin may be useful in complex treatment of UC.

Chojnacki C, Wiśniewska-Jarosińska M, Kulig G, Majsterek I, Reiter RJ, Chojnacki J. Evaluation of enterochromaffin cells and melatonin secretion exponents in ulcerative colitis. *World J Gastroenterol* 2013; 19(23): 3602-3607 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3602.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3602>

## INTRODUCTION

Ulcerative colitis (UC) is a chronic disease with periods of exacerbation and remission. Its pathogenesis is complex and inflammatory and immune factors, as well as toxic forms of oxygen produced in this condition, play a major role in the destruction of colonic mucosa<sup>[1]</sup>.

The phase of exacerbation is characterized by, inter alia, the increased production of pro-inflammatory cytokines, cell adhesion molecules and acute-phase proteins. In addition, various defense mechanisms are activated, including increased production of specific antibodies and antioxidants. Melatonin is also a potent agent in antioxidative defense through its antioxidant and anti-inflammatory properties<sup>[2,3]</sup>.

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced by the cells of the neuroendocrine system located throughout the body amine precursor uptake and decarboxylation (APUD). It is produced from L-tryptophan, an exogenous amino acid, which is first converted in the body into serotonin with the involvement of tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase. The serotonin is then converted to melatonin by arylalkylamine N-acetyltransferase (AANAT) and 5-hydroxyindole-O-methyltransferase hydroxyindole-O-methyltransferase (HIOMT)<sup>[4]</sup>.

This indoleamine is secreted by pineal cells according to circadian rhythms, which are regulated mainly by light stimuli<sup>[5]</sup> and by enterochromaffin cells (EC) which are distributed throughout the whole gastrointestinal tract<sup>[6]</sup>. Melatonin from the gut is transported *via* the portal vein to the liver, where it is metabolized to 6-sulfatoxymelatonin (6-HMS)<sup>[7]</sup>. The amounts of urinary 6-HMS excretion are known to be indices of the synthesis and metabolism of this hormone<sup>[8]</sup>.

Melatonin is secreted from the gastrointestinal tract under the influence of a range of stimuli, including nutritional factors, but the precise mechanisms regulating its release have not been recognized thoroughly. This hormone, released from enterochromaffin cells, fulfills important enteroprotective functions *via* the paraendocrine mechanism<sup>[9]</sup>.

In inflammatory processes, melatonin inhibits nitric oxide and cyclooxygenase-2 synthesis and decreases the concentration of endogenous oxidants<sup>[10]</sup>. Furthermore, it induces other systems which in turn reduce oxygen free radicals: Superoxide dismutase and glutathione peroxidase. It also exerts a beneficial effect on microcirculation and tissue blood supply by inhibiting COX-2<sup>[11]</sup>.

In experimental colitis in animals, the administration of exogenous melatonin decreased lipid, protein and nucleic acid oxidation, and also ameliorated epithelial damage and inflammatory infiltration<sup>[12,13]</sup>. In our studies, carried out on patients with ulcerative colitis, melatonin was demonstrated to reduce DNA damage and to stimulate the repair of hydrogen-peroxide-induced oxidative DNA damage in enterocytes<sup>[14]</sup>. Melatonin also has an influence on beneficial immune processes. Melatonin receptors have been identified on immunologically active cells. It also stimulates Th lymphocytes to produce IL-2 and IFN- $\gamma$ , monocytes to produce IL-1, IL-6 and IL-12 and decreases TNF- $\alpha$  concentration and the expression of adhesion molecules (ICAM-1, P-selectin and MAdCAM-1)<sup>[15,16]</sup>.

All these properties of melatonin can have a beneficial effect on the course of ulcerative colitis, as the mentioned pro-inflammatory factors play a significant role in the pathogenesis of this disease. Melatonin deficiency has been suggested to be one of the causes of upper digestive tract mucosal defects<sup>[17]</sup>, whereas the results related to the colon are not consistent. The amount of melatonin secreted in an organism depends on the number of EC cells and their activity. Spiller *et al.*<sup>[18]</sup> found an increased number of EC cells in rectal mucosa in patients with diarrhea predominant IBS. Similar observations were made by Osadchuk *et al.*<sup>[19]</sup>. However, El-Salhy *et al.*<sup>[20]</sup> demonstrated that patients with constipation-predominant IBS have a decreased number of EC cells in the colonic mucosa.

Some authors have tried to relate the changes observed in IBS patients to inflammatory bowel diseases but no reliable results have been obtained, either. Most of the researchers demonstrated that the number of EC cells increases in colonic mucosa in patients with ulcerative colitis<sup>[21-23]</sup>. In turn, others have detected a decreased number of EC cells in this group of patients<sup>[24,25]</sup>. These differences may result from a heterogeneous clinical and morphological evaluation of colonic mucosa.

The aim of the present study was to evaluate the number of EC cells, HIOMT expression in colonic mucosa and urinary 6-HMS excretion in patients with acute phase of ulcerative proctitis (UP) and ulcerative colitis (UC).

## MATERIALS AND METHODS

### Patients

The study included 30 healthy subjects (control group I (C)-aged  $32.4 \pm 9.8$  years), 26 patients with an acute phase of ulcerative proctitis [group II (UP)-aged  $31.9 \pm 11.6$  years] and 30 patients with ulcerative colitis (group III (UC)-aged  $33.0 \pm 16.9$  years). The severity of the disease was classified according to modified Mayo Clinic Score<sup>[26]</sup>. Biopsates were collected from many different parts (10-16) of the rectum and colon close to erosions and ulcerations. Histopathological activity was expressed according to the criteria of truelove and richards<sup>[27]</sup>.

### Methods

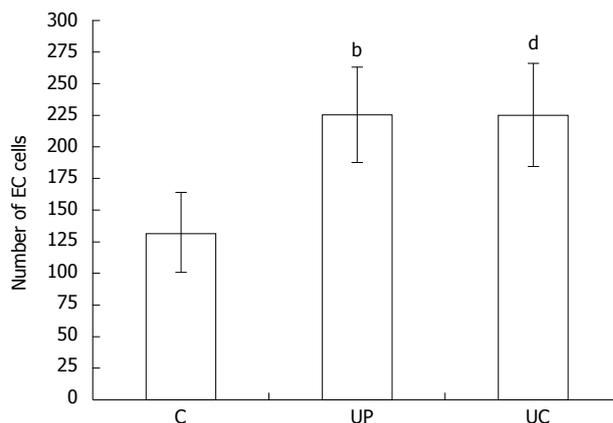
To determine the number of enterochromaffin cells in the biopsates, an immunohistochemical method was used with rabbit polyclonal antibodies (PSE) at a dilution of 1:200 (Eurodiagnostica). Immunoreactive cells were counted with a computer according to the Eurovision DAKO system, in the range of 10 fields in each biopsate at  $\times 200$  magnification.

The level of mRNA expression of HIOMT was estimated with RT-PCR, and for this purpose, 50 mg of colonic tissue was used. Total RNA was isolated with trizol (Gibco) reagents, and then purified with DNase using gigagen *RNeasy* mini kit. The quantity and quality of RNA was estimated by using spectrophotometry. The obtained extract was used as a matrix in analyses of gene expression. cDNA synthesis was performed with Oligo (dT) 12-18 in an MJ Research PTG-1000 thermocycler. cDNA was obtained in reverse transcription and applied as the matrix for a PCR reaction incorporating selected fragments of the analyzed gene. The hypoxanthine phosphoribosyltransferase gene was the quantitative marker for the evaluation of the activity of the selected genes.

The reaction products were first separated on 6%-10% polyacrylamide gel stained with ethidium bromide and then subjected to densitometry to determine the reaction efficacy and the level of mRNA of the investigated genes. The expression of the investigated genes was compared to *HPRT* gene product to normalize the expression.

The urine concentration of 6-HMS was determined immunoenzymatically using an IBL kit (RE54031).

On the day of testing excretion of 6-HMS through the urine, the patients remained in a room in which white light was off at night, and they received condensed liquid meals (Nutridrink-400 mL three times a day) with a total energy value of 1800 kcal and drank 1500 mL non-carbonated isotonic mineral water. After completion of 24-hour urine collection, the urine was centrifuged and the samples were stored at  $-70^\circ\text{C}$ . The 6-HMS concentration of the urine was determined immunoenzymatically using an Immuno-Biological Laboratories kit (No. RE54031). The measurements were performed by photometry at a wavelength of 450 nm using an Expert 96 reader (Biogent).



**Figure 1** Number of enterochromaffin cells in colonic mucosa in healthy subjects (C), patients with ulcerative proctitis and ulcerative colitis. The number of enterochromaffin cells was counted in 10 fields in each biopsate at  $\times 200$  magnification; Healthy subjects [control group (C);  $n = 30$ ]; Patients with acute phase of ulcerative proctitis (UP;  $n = 30$ ); Patients with acute phase of ulcerative colitis (UC;  $n = 30$ ); Differences between group C and UP <sup>b</sup> $P < 0.01$ ; Differences between group C and UC <sup>d</sup> $P < 0.01$ . EC: Enterochromaffin cells.

### Ethics

The study was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. Written consent was obtained from each patient enrolled in the study and the study protocol was approved by Bioethics Committee of the Medical University of Lodz (RNN/242/06/KB).

### Statistical analysis

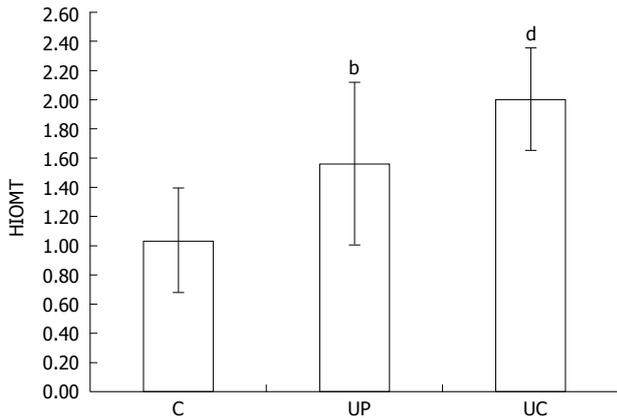
The non-parametric Krushal-Wallis test was used to evaluate the number of enterochromaffin cells, as well as the expression of HIOMT and urinary 6-HMS excretion in the three groups: C, UP and UC. The Mann-Whitney Test was used for comparison of median values. The correlation between the number of EC cells and urinary 6-HMS excretion was estimated by the determination of Pearson's correlation coefficient and linear regression equation. The differences between the results was regarded as significant at a  $P$  value 0.05-0.001. Statistica 9.0 (StatSoft, Inc., United States) and MS Excel 2007 (Microsoft Co, United States) were used for statistical analysis.

## RESULTS

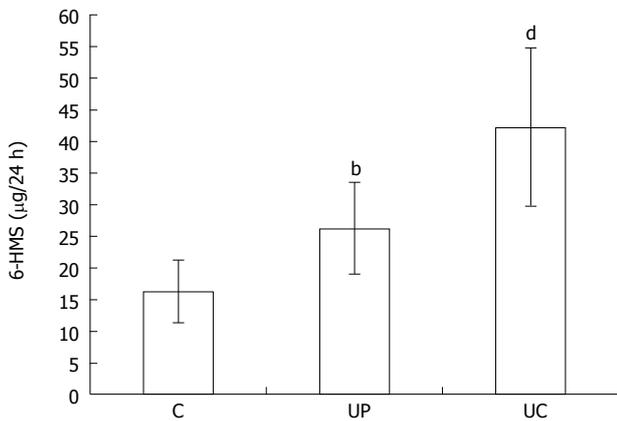
The mean number of colonic EC cells in 10 fields of view in healthy subjects (group I) was  $132.40 \pm 31.26$ . However, in patients with ulcerative colitis, the number was twice as high:  $225.40 \pm 37.35$  ( $P < 0.001$ ) in the rectal mucosa (group II) and  $225.24 \pm 40.50$  ( $P < 0.001$ ) in patients with pancolitis (group III) (Figure 1).

Similar differences were related to HIOMT expression:  $1.04 \pm 0.36$  in healthy subjects,  $1.56 \pm 0.56$  ( $P < 0.01$ ) in proctitis and  $2.00 \pm 0.35$  ( $P < 0.001$ ) in pancolitis (Figure 2).

Urinary 6-HMS excretion in healthy subjects was  $16.32 \pm 4.95$  and it was lower than in patients with proc-



**Figure 2** Expression of hydroxyindole-O-methyltransferase in colonic mucosa in healthy subjects (C), patients with ulcerative proctitis and ulcerative colitis. Healthy subjects [control group (C);  $n = 30$ ]; Patients with acute phase of ulcerative proctitis (UP;  $n = 30$ ); Patients with acute phase of ulcerative colitis (UC;  $n = 30$ ); Differences between group C and UP  $^bP < 0.01$ ; Differences between group C and UC  $^dP < 0.01$ . HIOMT: Hydroxyindole-O-methyltransferase.



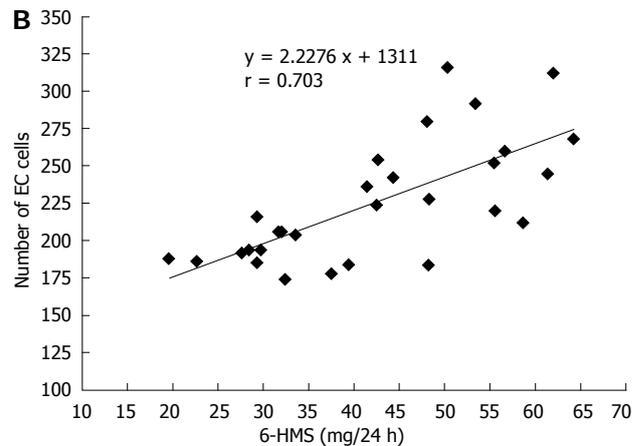
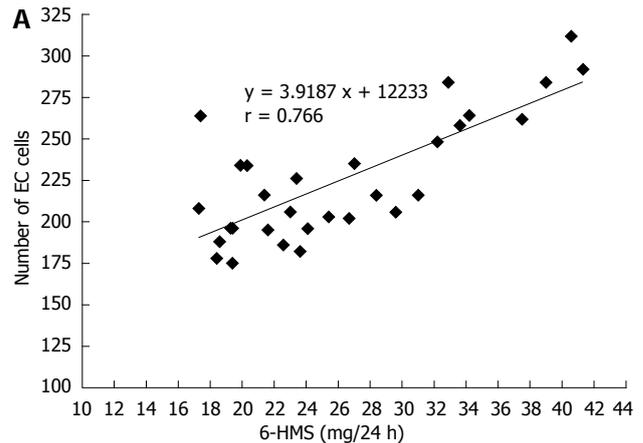
**Figure 3** Urinary excretion of 6-sulfatoxymelatonin in healthy subjects (C) and patients with ulcerative proctitis and ulcerative colitis. The number of enterochromaffin cells was counted in 10 fields in each biopate at  $\times 200$  magnification; Healthy subjects [control group (C);  $n = 30$ ]; Patients with acute phase of ulcerative proctitis (UP;  $n = 30$ ); Patients with acute phase of ulcerative colitis (UC;  $n = 30$ ); Differences between group C and UP  $^bP < 0.01$ ; Differences between group C and UC  $^dP < 0.01$ . 6-HMS: 6-sulfatoxymelatonin.

titis -  $26.30 \pm 7.29$  ( $P < 0.01$ ) and with pancolitis -  $42.30 \pm 12.56$  ( $P < 0.001$ ; Figure 3).

Urinary 6-HMS excretion was found to be dependent on the number of EC cells in all groups: In healthy subjects,  $r = 0.855$ , and in patients with proctitis,  $r = 0.766$  (Figure 4A). The number of EC cells was found to have a particularly strong positive correlation with urinary 6-HMS excretion in patients with pancolitis  $r = 0.703$  (Figure 4B).

## DISCUSSION

The obtained results confirm earlier observations, which indicate that EC cell proliferation is present in the active phase of ulcerative colitis, regardless of the location of



**Figure 4** Correlation between the number of enterochromaffin cells and excretion of 6-sulfatoxymelatonin in patients. A: With ulcerative proctitis (group II); B: with ulcerative colitis (group III). The number of enterochromaffin cells was counted in 10 fields in each biopate at  $\times 200$  magnification; y: Linear regression equation; r: Pearson's correlation coefficient; 6-HMS: 6-sulfatoxymelatonin; EC: Enterochromaffin cells.

inflammatory changes in colonic mucosa<sup>[21-23]</sup>. Additional observations found in this group of patients are firstly, an increase of HIOMT expression in the colon and secondly, a high level of urinary 6-HMS excretion. All these observations indicate increased activity of the melatonergic system in patients with ulcerative colitis. The increase of urinary 6-HMS excretion, in particular, is recognized to be the correct indicator of total melatonin production and content in the whole organism.

This pool of melatonin, which includes both the intestinal and pineal components, which represent nearly the whole amount, is metabolized in the liver. Nevertheless, the positive correlation between the number of EC cells and level of urinary 6-HMS excretion indicates an increase of melatonin production mainly in the colon, as confirmed by the high HIOMT expression, compared to healthy subjects. However, it should be emphasized that melatonin catabolism both in the liver and in the colon can be modified by other factors; an excessive increase of pro-inflammatory cytokines in the colonic wall can disturb metabolism of this hormone.

Furthermore, due to the “consumption” of melato-

nin in the reaction with oxygen free radicals at the infection site, and its metabolism in the cells of the immune system, other metabolites such as 2-hydroxymelatonin, 3-hydroxymelatonin can be formed<sup>[28,29]</sup>. In turn, amino salicylates and immunosuppressive drugs applied in the treatment of ulcerative colitis exert a hepatotoxic effect and can also impair the melatonin metabolism in hepatocytes.

Due to these reasons, and probably also to the profound destruction of the colonic mucosa, decreased urinary 6-HMS excretion was observed in patients with severe ulcerative colitis. In less severe forms of the disease, these values were higher<sup>[30]</sup>. Therefore, it should be acknowledged that inflammatory processes in the colon are accompanied by increased secretion of enteral melatonin. This hormone plays a crucial enteroprotective role and its increase in ulcerative colitis is an important defense mechanism.

However, these beneficial changes in melatonin homeostasis are not sufficient to inhibit the inflammatory process and to obtain spontaneous remission of the disease. Remission requires the administration of many medications, including anti-inflammatory ones and immunosuppressants, because the pathogenesis of ulcerative colitis is complex and conditioned by numerous pro-inflammatory factors.

Serotonin is one such factor. It is secreted by the same EC cells and it is the precursor of melatonin. EC cell proliferation can, at the same time, lead to increase of serotonin secretion. The balance between serotonin and melatonin depends on the expression of enzymes regulating their synthesis and catabolism. Previous studies have not given unequivocal results. Carpuso *et al.*<sup>[31]</sup> and Magro *et al.*<sup>[32]</sup> found decreased serotonin concentration in colonic mucosa in patients with ulcerative colitis. However, in experimental animals with ulcerative colitis, a significant increase was observed both of EC cells and serotonin concentration in the colonic mucosa<sup>[33,34]</sup>.

Regardless of the factors which contribute to the maintenance of the inflammatory process, the increase of melatonin secretion is a beneficial reaction. In our earlier studies, exogenous melatonin was also found to exert a positive effect on maintaining the remission of ulcerative colitis<sup>[35]</sup>, which is the main goal of pharmacotherapy in this disease.

In conclusion, EC proliferation, HIOMT expression and increased urine excretion of 6-HMS is seen in the acute phases of ulcerative proctitis and ulcerative colitis. Consequently, the increase of enteral melatonin secretion is a beneficial response in antiinflammatory and defense mechanisms.

## COMMENTS

### Background

Melatonin is synthesized mainly by pinealocytes and by enterochromaffin cells in the gastrointestinal tract. This indoleamine displays endocrine and paracrine properties which account for enteroprotective action. Particular, melatonin and its metabolites are powerful antioxidants.

### Research frontiers

It is known that melatonin deficiency plays an important role in the pathogenesis of certain gastrointestinal diseases, such as gastroesophageal diseases, duodenal ulcer and functional disorders, dyspepsia, irritable bowel syndrome and others. Melatonin homeostasis in inflammatory bowel diseases is not determined and the results of some researches are not unequivocal.

### Innovations and breakthroughs

It is believed to be the first study of report an association between the number of enterochromaffin cells, expression of hydroxyindole-O-methyltransferase (HIOMT) and urine excretion of 6-sulfatoxymelatonin (6-HMS) in patients with ulcerative colitis.

### Applications

Evaluation of 24-h urinary excretion of 6-sulfatoxymelatonin may be a useful method for the estimation of melatonin secretion and intensity of inflammatory process in colon mucosa in patients with acute phase of ulcerative colitis. During non active phase of this disease secretion of melatonin is probably decreased and the supplementation of melatonin may be useful in complex treatment to maintain the remission.

### Terminology

Melatonin is secreted by enterochromaffin cells (EC) in the gastrointestinal tract under the effect of HIOMT but the mechanism regulating its release have not been recognized thoroughly. In the gastrointestinal tract melatonin fulfils important enteroprotective role *via* paraendocrine activity. Melatonin from the gut is transported *via* portal vein to the liver where is metabolized mainly to 6-HMS. The amounts of the urinary 6-HMS excretion are recognized indices of the synthesis and metabolism of this hormone.

### Peer review

The authors evaluated the melatonin synthetic pathway in EC in the colonic mucosa in patients with ulcerative proctitis and ulcerative colitis. This is novel paper reporting an important role of melatonin in the injured gastrointestinal tract. The role showed that during acute phase of this disease there was a proliferation of EC cells accompanied with elevated expression of HIOMT and urinary excretion of 6-HMS. The authors concluded that the response of melatonin synthesis may account for a beneficial response against the inflammatory process.

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**P- Reviewers** Acuna-Castroviejo D, Shirazi A  
**S- Editor** Huang XZ **L- Editor** A **E- Editor** Zhang DN



## Diagnostic value of endothelial markers and HHV-8 staining in gastrointestinal Kaposi sarcoma and its difference in endoscopic tumor staging

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Supported by A Grant from the National Center for Global Health and Medicine (21-101)

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Received: February 16, 2013 Revised: April 4, 2013

Accepted: April 9, 2013

Published online: June 21, 2013

nohistochemical (IHC) staining in gastrointestinal Kaposi's sarcoma (GI-KS) in relation to endoscopic tumor staging.

**METHODS:** Biopsy samples ( $n = 133$ ) from 41 human immunodeficiency virus-infected patients were reviewed. GI-KS was defined as histologically negative for other GI diseases and as a positive clinical response to KS therapy. The receiver operating characteristic area under the curve (ROC-AUC) was compared in relation to lesion size, GI location, and macroscopic appearances on endoscopy.

**RESULTS:** GI-KS was confirmed in 84 lesions (81.6%). Other endoscopic findings were polyps ( $n = 9$ ), inflammation ( $n = 4$ ), malignant lymphoma ( $n = 4$ ), and condyloma ( $n = 2$ ), which mimicked GI-KS on endoscopy. ROC-AUC of HE, D2-40, blood vessel markers, and HHV-8 showed results of 0.83, 0.89, 0.80, and 0.82, respectively. For IHC staining, the ROC-AUC of D2-40 was significantly higher ( $P < 0.05$ ) than that of HE staining only. In the analysis of endoscopic appearance, the ROC-AUC of HE and IHC showed a tendency toward an increase in tumor staging (*e.g.*, small to large, patches, and polypoid to SMT appearance). D2-40 was significantly ( $P < 0.05$ ) advantageous in the upper GI tract and for polypoid appearance compared with HE staining.

**CONCLUSION:** The diagnostic value of endothelial markers and HHV-8 staining was found to be high, and its accuracy tended to increase with endoscopic tumor staging. D2-40 will be useful for complementing HE staining in the diagnosis of GI-KS, especially in the upper GI tract and for polypoid appearance.

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### Abstract

**AIM:** To clarify the diagnostic values of hematoxylin and eosin (HE), D2-40, CD31, CD34, and HHV-8 immu-

**Key words:** Gastrointestinal Kaposi's sarcoma; Hematoxylin and eosin; CD31; CD34; D2-40; Human herpesvirus-8

**Core tip:** Diagnosis of gastrointestinal Kaposi sarcoma (GI-KS) is important because treatment specifics depend on the extent of the disease. Endoscopic biopsy is a definitive diagnostic method for GI-KS, but its diagnostic accuracy has not been fully studied. In the current study, receiver operating characteristic area under the curve of hematoxylin and eosin (HE) staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 was found to be high ( $> 0.80$ ), and its accuracy tended to increase with endoscopic tumor staging. D2-40 will be useful for complementing HE staining in the diagnosis of GI-KS, especially in the upper GI tract and for polypoid appearance.

Nagata N, Igari T, Shimbo T, Sekine K, Akiyama J, Hamada Y, Yazaki H, Ohmagari N, Teruya K, Oka S, Uemura N. Diagnostic value of endothelial markers and HHV-8 staining in gastrointestinal Kaposi sarcoma and its difference in endoscopic tumor staging. *World J Gastroenterol* 2013; 19(23): 3608-3614 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3608.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3608>

## INTRODUCTION

Kaposi sarcoma (KS) is a rare cancer that was highly prevalent in the early stages of the acquired immune deficiency syndrome (AIDS) endemic<sup>[1]</sup>. Although the rate of KS has shown a marked reduction since the introduction of highly active antiretroviral therapy (HAART)<sup>[1-3]</sup>, KS remains the most common malignancy in patients with AIDS<sup>[4]</sup>.

KS primarily involves the skin but can also involve the viscera<sup>[5-7]</sup>. Because the need for treatment and choice of treatment depend on visceral involvement<sup>[1-3,8-11]</sup>, diagnosis of the gastrointestinal (GI) tract, a common site of visceral involvement<sup>[11-13]</sup>, is important. Definitive diagnosis of GI-KS requires endoscopic biopsy<sup>[6,7,14-16]</sup>, but GI-KS often presents on endoscopy with submucosal or small protruded appearance<sup>[17,18]</sup>, which can lead to false-negative biopsy results<sup>[14-16]</sup>.

Recently, immunohistochemical (IHC) staining with D2-40, CD31, CD34, and HHV-8 has been reported as useful for distinguishing cutaneous KS from other diseases<sup>[19-28]</sup>. However, no IHC studies have reported on the utility of KS diagnosis in the GI tract. In addition, it is not known how well such staining methods provide additive effects compared with HE staining alone.

With regard to the diagnosis of cutaneous KS, there may be subtle differences in the staining patterns for endothelial markers between different histologic stages (patch, plaque, and nodular) of KS<sup>[19,21,22,25]</sup>. In the GI tract, KS has various macroscopic presentations<sup>[13-15,17,18,29]</sup> and KS may affect any part of the GI tract<sup>[7,13,14,17,18]</sup>. However, the effect of IHC-positive staining in the diagnosis of GI-KS on the basis of lesion appearance has not been fully investigated.

The purpose of this study was to clarify the diagnostic value of IHC staining in the diagnosis of GI-KS and to assess the difference in accuracy between HE and IHC

staining in relation to endoscopic tumor staging.

## MATERIALS AND METHODS

### Subjects

We retrospectively reviewed histologic slides from 103 consecutive lesions for which IHC staining was performed between 2006 and 2012 at the National Center for Global Health and Medicine (NCGM). Lesions were obtained from 41 human immunodeficiency virus (HIV)-infected patients who had not received anti-KS therapy. The institutional review board at NCGM approved this study.

### Clinical factors

Sexual behavior was classified subjects into two groups: men who have sex with men (MSM); and heterosexual. CD4<sup>+</sup> cell counts and HIV-RNA viral load (VL) determined by real-time quantitative polymerase chain reaction (PCR) were reviewed within 1 mo of endoscopy. A positive result for real-time HIV-RNA was defined as  $\geq 40$  copies/mL. History of HAART was collected from medical records prior to endoscopy. GI symptoms were assessed by the physician who interviewed each patient. Those without GI symptoms and negative screening endoscopy were considered to be symptom-free.

### Diagnosis of GI-KS

Confirmed GI-KS lesions were defined as those that fulfilled with following criteria. (1) Histologically negative biopsy for other GI diseases; (2) A positive response to KS therapy (HAART or systemic therapy of liposomal anthracycline); and (3) partial or complete resolution was confirmed on follow-up endoscopy after KS therapy (Figure 1). We usually perform endoscopy after 1 mo, 2 mo, or 6 mo of KS therapy to evaluate GI-KS regression.

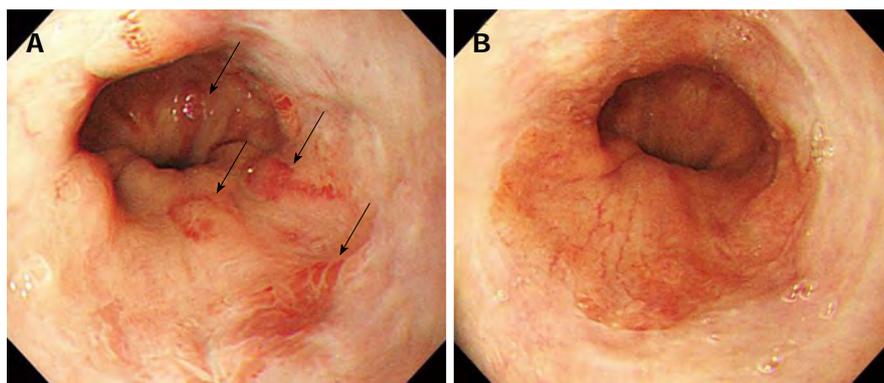
All 103 lesions were suspected to be GI-KS on endoscopy, as they exhibited properties such as reddish with patches, polypoid appearance, submucosal tumor (SMT)-like lesions, and ulcerative SMT, as previously reported<sup>[13-15,17,18,29]</sup>. Therefore, IHC staining in addition to HE staining was performed.

### Endoscopic assessment

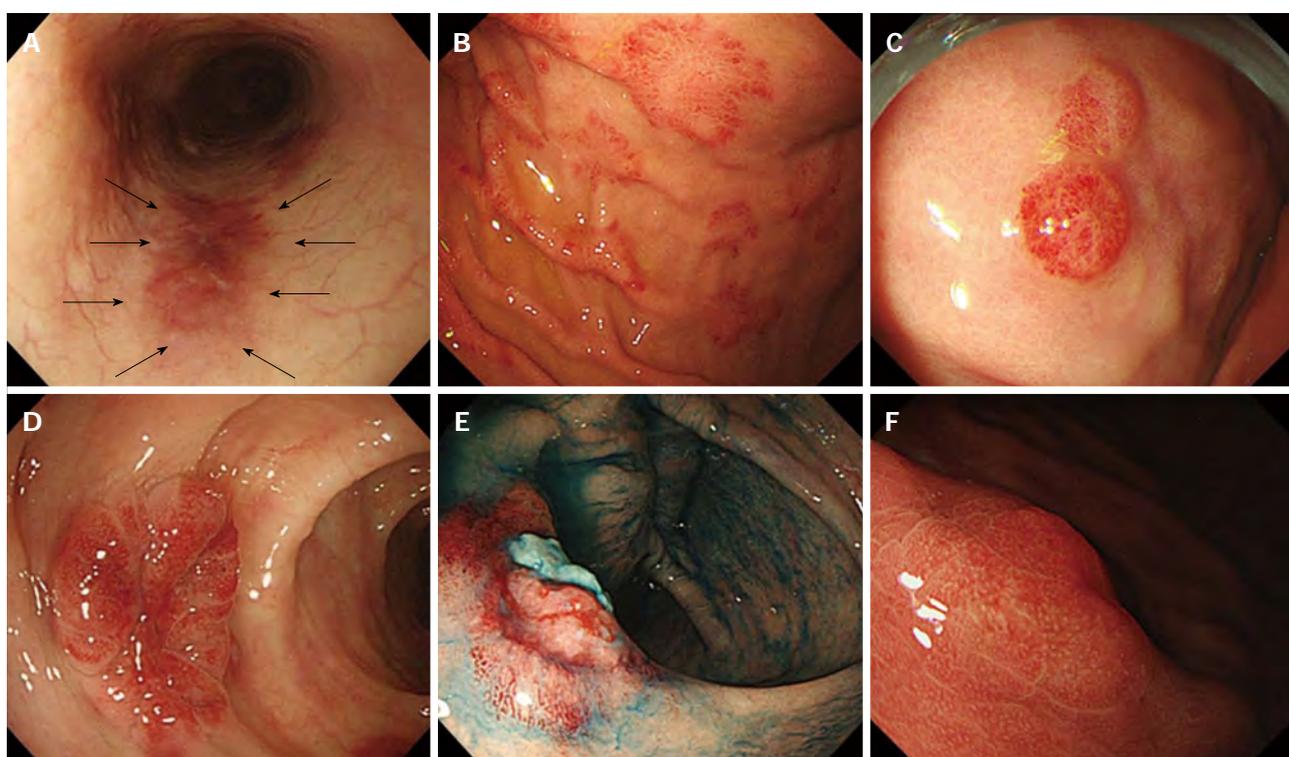
Endoscopic images were taken using a high-resolution scope (model GFH260, CFH260AI; Olympus Optical, Tokyo, Japan) in all patients. We performed a biopsy using biopsy forceps (FB-240U, FB230-K, Olympus Co., Tokyo, Japan).

Size ( $< 10$  mm or  $\geq 10$  mm), GI location, and macroscopic findings were assessed endoscopically. Locations of GI involvement were classified as upper GI (esophagus, stomach, and duodenum) and lower GI (ileum, colon, and rectum).

Macroscopic findings were evaluated as the presence of reddish mucosa with patches (Figure 2A), polypoid lesions (Figure 2B), submucosal tumor (SMT) (Figure 2C), and ulcerative SMT (Figure 2D), as previously reported<sup>[13-15,17,18,29]</sup>. Ulceration was defined endoscopically as a distinct, visible crater  $> 5$  mm in diameter with a slough base.



**Figure 1** Confirmation of clinical response on follow-up endoscopy before and after Kaposi's sarcoma therapy. A: Gastrointestinal Kaposi sarcoma (arrows) in the esophagogastric junction before Kaposi's sarcoma (KS) therapy; B: After four months of KS therapy with liposomal anthracycline.



**Figure 2** Gastrointestinal Kaposi sarcoma and mimicking lesions on endoscopy. A: Kaposi sarcoma, dark reddish patch (arrows) in the esophagus; B: Kaposi sarcoma, multiple patch appearance in the stomach; C: Kaposi sarcoma, small (< 10 mm) and polypoid appearance in the stomach; D: Kaposi sarcoma, submucosal tumor (SMT) appearance with large size ( $\geq 10$  mm) in the sigmoid colon; E: Kaposi sarcoma, submucosal tumor (SMT) appearance with ulceration in the ileo-cecal valve with indigo-carmin dye; F: Hyperplastic polyps mimicking Kaposi sarcoma with small size (< 10 mm) in the stomach.

### Histological assessment

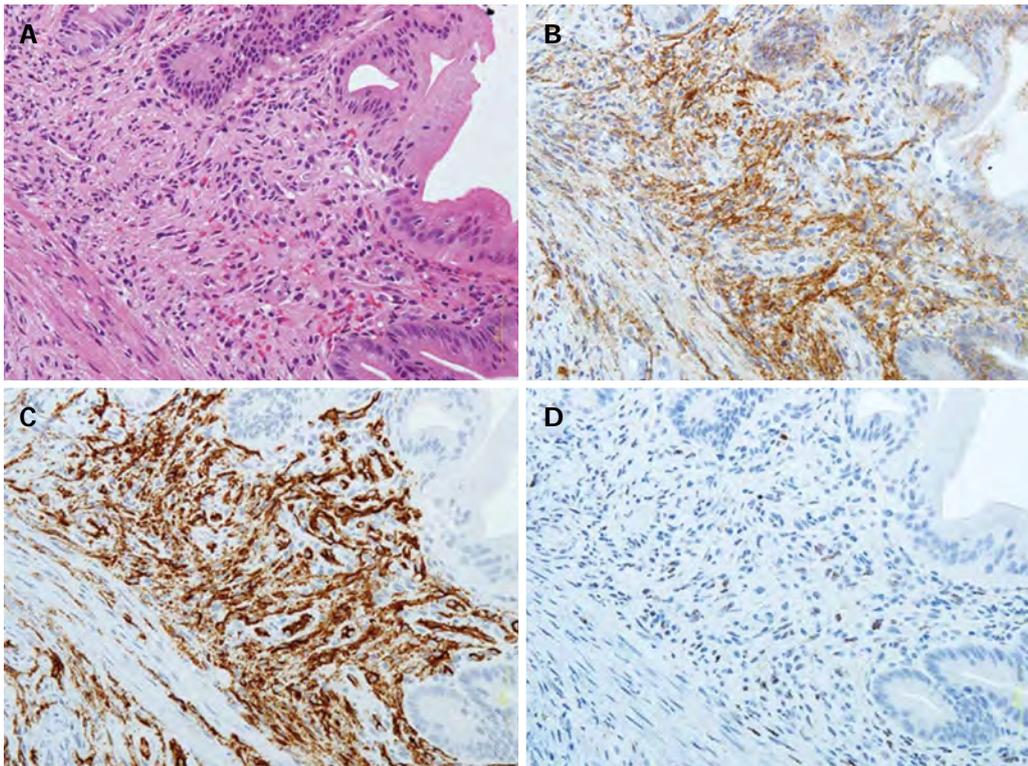
The presence of proliferating spindle cells with vascular channels filled with blood cells (Figure 3A) from biopsy specimens was evaluated with HE staining by an investigator blinded to IHC staining results. IHC staining for the lymphatic vessel endothelial cell marker D2-40 (Dako North America, Carpinteria, CA) (Figure 3B) and the blood vessel endothelial cell markers CD31 (Dako North America) or CD34 (Dako North America) (Figure 3C), and the use of the mouse monoclonal antibody against HHV-8 LNA-1 (Novocastra Laboratories Ltd, Newcastle upon Tyne, United Kingdom) (Figure 3D), were also evaluated on formalin-fixed, paraffin-embedded tissue sections as previously reported<sup>[19-28]</sup>. IHC slides were

evaluated at  $\times 200$  and  $\times 400$  magnification by expert GI pathologists.

### Statistical analysis

To elucidate the accuracy of HE and IHC staining for the diagnosis of GI-KS, the sensitivity, specificity, positive and negative likelihood ratio (LR+ and LR-, respectively), and area under the receiver operating characteristic curve (ROC-AUC) were calculated and estimated with a 95%CI.

The difference of the ROC-AUC of the four specific stains (HE, D2-10, vessel markers, and HHV-8) was compared. Subgroup analysis was performed to identify differences in four specific stains according to gross ap-



**Figure 3** Pathological features of gastrointestinal Kaposi sarcoma. A: Spindle cell proliferation in the submucosa on hematoxylin and eosin (HE) staining; B: Vascular gaps are lined with endothelial cells when staining with D2-40; C: Vascular gaps are lined with endothelial cells when staining with CD34; D: Some endothelial cells are positive for human herpesvirus 8.

**Table 1** Definitive diagnosis of gastrointestinal lesion from endoscopic biopsy samples ( $n = 103$ )  $n$  (%)

| Diagnosis                                 | No.       |
|---|-----------|
| GI-KS                                     | 84        |
| Upper GI tract                            | 57 (67.9) |
| Esophagus                                 | 7 (8.3)   |
| Stomach                                   | 38 (45.2) |
| Duodenum                                  | 12 (14.3) |
| Lower GI tract                            | 27 (32.1) |
| Cecum                                     | 8 (9.5)   |
| Ascending colon                           | 1 (1.2)   |
| Transverse colon                          | 2 (2.4)   |
| Descending colon                          | 1 (1.2)   |
| Sigmoid colon                             | 7 (8.3)   |
| Rectum                                    | 8 (9.5)   |
| Non-KS lesion                             | 19        |
| Hyperplastic polyp                        | 8         |
| Fundic gland polyps                       | 1         |
| <i>Helicobacter</i> -associated gastritis | 1         |
| Malignant lymphoma                        | 4         |
| Anorectal condyloma                       | 2         |
| Non-specific colitis                      | 3         |

GI-KS: Gastrointestinal Kaposi sarcoma.

pearances, and the ROC-AUC was compared in each group. ROC-AUC differences between HE staining and specific IHC staining in each groups were also compared.

Values of  $P < 0.05$  were considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, College Station, TX).

## RESULTS

### Baseline clinical characteristics

All 41 HIV-infected patients were male and the HIV infection route was MSM in all cases. The median CD4 cell count (interquartile range; IQR) was 77 (33, 157) cells/mL and the median HIV viral load (IQR) was 48500 (< 40, 150000) copies/mL. There were 18 (43.9%) patients with a history of HAART. GI symptoms were noted in 10 patients (24.4%). No notable gastrointestinal bleeding or perforation, either spontaneously or after endoscopic biopsy, was noted.

Table 1 provides details on the definitive diagnosis of GI lesions. Of the 103 lesions, 84 (81.6%) were confirmed as GI-KS while the remainder were other GI lesions (19) consisted of hyperplastic polyps (8), fundic gland polyps (1), *Helicobacter*-associated gastritis (1), malignant lymphoma (4), anorectal condyloma (2), and non-specific colitis (3).

### Diagnostic value of specific staining for the diagnosis of GI-KS

Sensitivity, specificity, LR+, LR-, and ROC-AUC of specific staining for the diagnosis of GI-KS are shown in Table 2. The ROC-AUC values of four specific stains (HE, D2-40, blood vessel marker, and HHV-8) were significantly different ( $P < 0.01$ ) in the diagnosis of GI-KS (Table 2). The ROC-AUC of D2-40 staining was only significantly higher ( $P < 0.05$ ) than that of HE staining (Table 2).

**Table 2** Diagnostic value of endoscopic biopsy in gastrointestinal Kaposi sarcoma (*n* = 103)

| KS/non-KS (84/19)          | Sensitivity, % (95%CI) | Specificity, % (95%CI) | Positive LR (95%CI)           | Negative LR (95%CI)           | ROC area <sup>1</sup> (95%CI) |
|----------------------------|------------------------|------------------------|-------------------------------|-------------------------------|-------------------------------|
| HE (59/1)                  | 70.2 (59.3-79.7)       | 94.7 (74.0-99.9)       | 9.33 (1.99-43.8) <sup>2</sup> | 0.32 (0.23-0.46) <sup>2</sup> | 0.83 (0.75-0.90)              |
| D2-40 (65/0)               | 77.4 (67.0-85.8)       | 100 (82.4-100)         | 30.8 (1.99-477) <sup>2</sup>  | 0.24 (0.16-0.35) <sup>2</sup> | 0.89 (0.84-0.93) <sup>a</sup> |
| Blood vessel marker (68/4) | 81 (70.9- 88.7)        | 78.9 (54.4- 93.9)      | 3.85 (1.6-9.24)               | 0.24 (0.15-0.40)              | 0.80 (0.70-0.90)              |
| HHV-8 (53/0)               | 63.1 (51.9-73.4)       | 100 (82.4-100)         | 25.2 (1.62-391) <sup>2</sup>  | 0.38 (0.29-0.51) <sup>2</sup> | 0.82 (0.76-0.87)              |

LR: Likelihood ratio; HE: Hematoxylin and eosin; HHV: Human herpesvirus. <sup>1</sup>Receiver operating characteristic (ROC) area is significantly (*P* < 0.01) different in this category; <sup>2</sup>LR estimated using the substitution formula. A value of 0.5 was added to all cell frequencies before calculation; <sup>a</sup>*P* < 0.05 for comparisons of lesions by HE staining.

**Table 3** Diagnostic values of gastrointestinal Kaposi sarcoma in relation to size, location, and macroscopic appearances on endoscopy (*n* = 103)

| Subgroup               | Specific stain      | No. of lesions (KS/non-KS) | ROC-AUC (95%CI)               | <i>P</i> value <sup>1</sup> |
|------------------------|---------------------|----------------------------|-------------------------------|-----------------------------|
| Size                   | Size < 10 mm        | 26/7                       |                               |                             |
|                        | HE                  | 14/0                       | 0.77 (0.67-0.87)              |                             |
|                        | D2-40               | 16/0                       | 0.81 (0.71-0.90)              |                             |
|                        | Blood vessel marker | 18/4                       | 0.56 (0.34-0.78)              |                             |
|                        | HHV-8               | 10/0                       | 0.69 (0.60-0.79)              | < 0.05                      |
|                        | Size > 10 mm        | 58/12                      |                               |                             |
|                        | HE                  | 45/1                       | 0.85 (0.75-0.94)              |                             |
|                        | D2-40               | 49/0                       | 0.92 (0.88-0.97)              |                             |
|                        | Blood vessel marker | 50/0                       | 0.93 (0.89-0.98)              |                             |
|                        | HHV-8               | 43/0                       | 0.93 (0.89-0.98)              | < 0.01                      |
| Location               | Upper GI tract      | 57/9                       |                               |                             |
|                        | HE                  | 36/0                       | 0.82 (0.75-0.88)              |                             |
|                        | D2-40               | 42/0                       | 0.87 (0.81-0.93) <sup>a</sup> |                             |
|                        | Blood vessel marker | 44/4                       | 0.66 (0.48-0.84)              |                             |
|                        | HHV-8               | 36/0                       | 0.82 (0.75-0.88)              | < 0.01                      |
|                        | Lower GI tract      | 27/10                      |                               |                             |
|                        | HE                  | 23/1                       | 0.88 (0.76-1.00)              |                             |
|                        | D2-40               | 23/0                       | 0.93 (0.86-0.99)              |                             |
|                        | Blood vessel marker | 24/0                       | 0.94 (0.88-1.00)              |                             |
|                        | HHV-8               | 17/0                       | 0.82 (0.72-0.91)              | < 0.01                      |
| Macroscopic appearance | Patches             | 23/4                       |                               |                             |
|                        | HE                  | 12/1                       | 0.64 (0.37-0.90)              |                             |
|                        | D2-40               | 14/0                       | 0.80 (0.70-0.91)              |                             |
|                        | Blood vessel marker | 15/0                       | 0.83 (0.73-0.93)              |                             |
|                        | HHV-8               | 8/0                        | 0.67 (0.57-0.77)              | < 0.01                      |
|                        | Polypoid            | 9/3                        |                               |                             |
|                        | HE                  | 5/0                        | 0.78 (0.61-0.95)              |                             |
|                        | D2-40               | 8/0                        | 0.94 (0.84-1.00) <sup>a</sup> |                             |
|                        | Blood vessel marker | 8/3                        | 0.44 (0.34-0.55) <sup>a</sup> |                             |
|                        | HHV-8               | 4/0                        | 0.72 (0.55-0.89)              | < 0.01                      |
| SMT                    | 37/7                |                            |                               |                             |
| HE                     | 32/0                | 0.93 (0.88-0.99)           |                               |                             |
| D2-40                  | 33/0                | 0.95 (0.90-0.10)           |                               |                             |
| Blood vessel marker    | 33/1                | 0.88 (0.73-1.00)           |                               |                             |
| HHV-8                  | 30/0                | 0.91 (0.84-0.97)           | 0.15                          |                             |
| SMT with ulcer         | 15/5                |                            |                               |                             |
| HE                     | 10/0                | 0.83 (0.71-0.96)           |                               |                             |
| D2-40                  | 10/0                | 0.83 (0.71-0.96)           |                               |                             |
| Blood vessel marker    | 12/0                | 0.90 (0.80-1.00)           |                               |                             |
| HHV-8                  | 11/0                | 0.87 (0.75-0.98)           | 0.34                          |                             |

<sup>1</sup>*P* values of receiver operating characteristic (ROC) area in each category were compared. <sup>a</sup>*P* < 0.05 for the comparison with lesions by hematoxylin and eosin (HE) staining. GI-KS: Gastrointestinal Kaposi sarcoma; ROC-AUC: ROC area under the curve; SMT: Submucosal tumor.

### Diagnostic value of GI-KS according to size, location, and macroscopic appearance

The ROC-AUC of four specific stains showed a tendency toward an increase in tumor staging on endoscopy (*e.g.*, small to large, flat, protruded, and SMT appearance) (Table 3). The ROC-AUC of blood vessel marker in polypoid appearance was extremely low compared with other lesions (Table 3).

The ROC-AUC of four specific stains was significantly different in size, GI tract location, appearance of patches, and polypoid lesion for the diagnosis of GI-KS (Table 3). No significant differences were noted in the ROC-AUC of four specific stains for SMT lesions (*P* = 0.15) or ulcerative SMT lesions (*P* = 0.34) (Table 3).

### Comparison of the ROC-AUC between HE staining and specific staining

The ROC-AUC of the D2-40 stain was higher than that of the HE stain for lesions < 10 mm, lesions ≥ 10 mm, upper GI tract, lower GI tract, patches, polypoids, and SMT (Table 3). Of these, upper GI tract and polypoid appearance were statistically significant (*P* < 0.05). The ROC-AUC of blood vessel marker or HHV-8 stain was higher than that of HE staining for lesions ≥ 10 mm, patches, and ulcerative SMT (Table 3), with no statistical significance (*P* > 0.05).

## DISCUSSION

Previous IHC studies have shown the utility of differential diagnosis between cutaneous KS and vascular tumors such as hemangioma, lymphangioma, hemangioendothelioma, and angiosarcoma<sup>[19-28]</sup>. However, development of vascular tumor in the GI tract is extremely rare<sup>[30]</sup>. Therefore, differential diagnosis for GI-KS can be different for cutaneous and GI tract sites. In the present study, lesions that were difficult to distinguish from GI-KS are inflammation-associated protruded lesions with reddish color. The reason for this is that GI-KS can appear as a strong reddish mucosa and vary from flat maculopapular or polypoid masses to SMT, ulceration, or bulky tumor masses on endoscopy<sup>[14,17,18,29,31]</sup>.

Previous studies investigated only GI-KS cases, and

only sensitivity can be elucidated<sup>[14-16]</sup>. In the current study, the ROC-AUC values of the four IHC stains and HE stain were > 0.8, demonstrating that all had good diagnostic accuracy. However, it is not feasible in clinical practice to diagnose KS using all stains. Based on the results of this study, we conclude that D2-40 is the only stain capable of complementing HE staining.

We found that the ROC-AUC of four specific stains tended to increase with endoscopic tumor staging (*e.g.*, small to large, flat, protruded, and SMT). Previous studies, particularly those on cutaneous KS, have also shown that diagnostic accuracy varies according to tumor staging<sup>[19,21,22,25]</sup>. Although it is not feasible to apply staging classification of cutaneous KS to the evaluation of the macroscopic appearance of GI-KS, it is important--based on their results and our findings--to take tumor appearance and staging into consideration for the pathological diagnosis of KS.

We further performed subgroup analysis of four stains to reveal differences in diagnostic accuracy. No significant differences were noted in the ROC-AUC of four specific stains in SMT lesions ( $P = 0.15$ ) and ulcerative SMT lesions ( $P = 0.34$ ), indicating that HE staining alone is sufficient for diagnosing lesions with SMT appearance. Although we attempted to find lesions that can be better diagnosed with the addition of other IHC stains, polypoid lesions, and location of upper GI tract attained significant ROC-AUC ( $P < 0.05$ ) scores with D2-40. The ROC-AUC of D2-40 was always > 0.8, regardless of the size, location, or macroscopic appearance of lesions, indicating its utility as an additional staining modality.

One of the characteristic findings of this study is that the ROC-AUC of the blood vessel marker for polypoid appearance was extremely low compared with other lesions. This was due to the presence of hyperplastic polyps, meaning that CD34 staining produces positive results due to vessel proliferation. This can result in higher false-positive cases ( $n = 38$ ) and lower diagnostic accuracy of KS.

There are several limitations of the present study. First, we assessed IHC staining as positive or negative instead of using a scoring system; a semi-quantitative system might provide more accurate or available results in clinical practice. Second, positive vessel marker staining was defined as CD31- or CD34-positive because CD31 or CD34 was used by each pathologist. However, because 80% (82/103) of the lesions were examined using CD34, and because CD34 is reportedly a more accurate marker than CD31<sup>[25]</sup>, we believe the results of the vessel marker staining in the present study are reliable.

In conclusion, endoscopic biopsy for diagnosing GI-KS can be performed safely. The diagnostic accuracy of HE staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 was found to be high. Among these, D2-40 had the highest accuracy. The diagnostic accuracy of four specific stains tended to increase with endoscopic tumor staging. In particular, polypoid lesions and those in the upper GI tract respond well to HE staining complemented by D2-40 staining.

## ACKNOWLEDGMENTS

We wish to thank Hisae Kawashiro, Clinical Research Coordinator, for assistance with data collection.

## COMMENTS

### Background

Diagnosis of Kaposi sarcoma (KS) involving the gastrointestinal (GI) tract is important because treatment specifics depend on the extent of the disease. Definitive diagnosis of GI-KS requires endoscopic biopsy with hematoxylin and eosin (HE) or immunohistochemical (IHC) staining. IHC staining for the differential diagnosis of cutaneous disease has been extensively studied, but the diagnostic value of GI-KS remains unknown.

### Research frontiers

GI-KS often presents various endoscopic appearances, which can lead to false-negative biopsy results. Furthermore, the difference in accuracy of IHC staining in relation to endoscopic appearances has not been fully investigated. In the current study, the authors demonstrate the diagnostic value of IHC staining for GI-KS and to assess the difference in accuracy between HE and IHC staining in relation to endoscopic tumor staging.

### Innovations and breakthroughs

Previous reports have highlighted the accuracy of IHC for diagnosing cutaneous KS. This is the first study to report that the receiver operating characteristic area under the curve (ROC-AUC) of HE staining, lymphatic and blood vessel endothelial cell markers, and HHV-8 for diagnosing GI-KS was found to be high (> 0.80), and its accuracy tended to increase with endoscopic tumor staging. In particular, polypoid lesions and those in the upper GI tract respond well to HE staining complemented by D2-40 staining.

### Applications

In the current study, the ROC-AUC values of the four IHC stains and HE stain were good, but it is not feasible in clinical practice to diagnose KS using all stains. Based on the results of this study, the authors conclude that D2-40 is the only stain capable of complementing HE staining.

### Terminology

The ROC is a diagnostic testing modality that presents its results as a plot of sensitivity vs 1-specificity (false-positive rate). The ROC-AUC indicates the probability of a measure or predicted risk being higher for patients with disease than for those without disease.

### Peer review

This is an excellent paper describing novel findings of IHC in diagnosing GI-KS.

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## Gastric precancerous lesions are associated with gene variants in *Helicobacter pylori*-susceptible ethnic Malays

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Author contributions: Maran S, Lee YY, Xu S, Majid NA and Zilfalil BA were involved in the design, analysis and writing of manuscript; Maran S performed the studies, with assistance from Rajab NS and Hasan N; Syed Abdul Aziz SH, Rajab N and Hasan N provided ideas to the study and manuscript.

Supported by Fundamental Research Grant Scheme (FRGS) 203/PPSP/6171121, 1001/PPSP/812016 and 1001/PPSP/8122022 of Universiti Sains Malaysia; The National Science Foundation of China grants, No. 30971577 and No. 31171218; the Shanghai Rising-Star Program, No. 11QA1407600; and the Science Foundation of the Chinese Academy of Sciences (CAS) (KSCX2-EW-Q-1-11; KSCX2-EW-R-01-05; KSCX2-EW-J-15-05)

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Received: February 18, 2013 Revised: April 2, 2013

Accepted: April 9, 2013

Published online: June 21, 2013

cancerous lesions in *Helicobacter pylori* (*H. pylori*)-susceptible ethnic Malays.

**METHODS:** Twenty-three Malay subjects with *H. pylori* infection and gastric precancerous lesions identified during endoscopy were included as "cases". Thirty-seven Malay subjects who were *H. pylori* negative and had no precancerous lesions were included as "controls". Venous blood was collected for genotyping with Affymetrix 50K Xba1 kit. Genotypes with call rates < 90% for autosomal single nucleotide polymorphisms (SNPs) were excluded. For each precancerous lesion, associated SNPs were identified from Manhattan plots, and only SNPs with a  $\chi^2$  *P* value < 0.05 and Hardy Weinberg Equilibrium *P* value > 0.5 was considered as significant markers.

**RESULTS:** Of the 23 *H. pylori*-positive subjects recruited, one sample was excluded from further analysis due to a low genotyping call rate. Of the 22 *H. pylori*-positive samples, atrophic gastritis only was present in 50.0%, complete intestinal metaplasia was present in 18.25%, both incomplete intestinal metaplasia and dysplasia was present in 22.7%, and dysplasia only was present in 9.1%. SNPs rs9315542 (*UFM1* gene), rs6878265 (*THBS4* gene), rs1042194 (*CYP2C19* gene) and rs10505799 (*MGST1* gene) were significantly associated with atrophic gastritis, complete intestinal metaplasia, incomplete metaplasia with foci of dysplasia and dysplasia, respectively. Allele frequencies in "cases" vs "controls" for rs9315542, rs6878265, rs1042194 and rs10505799 were 0.4 vs 0.06, 0.6 vs 0.01, 0.6 vs 0.01 and 0.5 vs 0.02, respectively.

**CONCLUSION:** Genetic variants possibly related to gastric precancerous lesions in ethnic Malays susceptible to *H. pylori* infection were identified for testing in subsequent trials.

### Abstract

**AIM:** To identify genes associated with gastric pre-

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**Key words:** Gastric precancerous lesions; Gene polymorphisms; Genome-wide association; *Helicobacter pylori*; Malays

**Core tip:** Gastric cancer and its precancerous lesions are exceptionally rare among ethnic Malays. Gene variants may be associated with precancerous lesions in *Helicobacter pylori*-susceptible Malays. Genome-wide association was performed to identify gene variants in Malays with a spectrum of gastric precancerous lesions. Results indicated that at different phases of the Correa cascade, different gene variants were manifest, but they followed a pattern of progression similar to their histological and clinical stages. It is possible that, in addition to histological staging, gene variant markers may serve to identify different phases of gastric cancer progression in the near future.

Maran S, Lee YY, Xu S, Rajab NS, Hasan N, Syed Abdul Aziz SH, Majid NA, Zilfalil BA. Gastric precancerous lesions are associated with gene variants in *Helicobacter pylori*-susceptible ethnic Malays. *World J Gastroenterol* 2013; 19(23): 3615-3622 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3615.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3615>

## INTRODUCTION

Gastric cancers are thought to arise from a cascade of histological changes or precancerous lesions (atrophic gastritis, intestinal metaplasia and dysplasia) before developing into full-blown malignancy<sup>[1]</sup>. In Japan, studies have shown that surveillance of these precancerous lesions is associated with increased detection of early gastric cancers and improved survival rates<sup>[2,3]</sup>.

These precancerous lesions are associated with *Helicobacter pylori* (*H. pylori*) infection acquired since childhood<sup>[4]</sup>. In populations with a high prevalence of *H. pylori* infection, including those in China and Japan, precancerous lesions can be detected in up to 80% of adults<sup>[5]</sup>. Eradication of *H. pylori* infection at this stage has not been shown to be effective in these high risk populations<sup>[6]</sup>.

Ethnic Malays residing in the north-eastern region of Peninsular Malaysia (state of Kelantan) have an exceptionally low prevalence of *H. pylori* infection<sup>[7,8]</sup>. Exact reasons for this low prevalence are unknown, but it could be a combination of unique environmental, host and strain virulence factors shaped by the population's evolutionary history<sup>[9-12]</sup>. Due to the extremely low acquisition of *H. pylori* infection, gastric cancer and its precancerous lesions are extremely rare in this population<sup>[13-15]</sup>.

In a survey of 234 subjects undergoing upper endoscopy in a tertiary hospital from the state of Kelantan, the reported rate of atrophic gastritis was 42.3% and intestinal metaplasia was present in 7.7% (14/234) of all biopsies, but was only present in 1.4% (2/146) of the ethnic Malays<sup>[15]</sup>. This low rate of gastric precancerous lesions

observed was a result of a low prevalence of *H. pylori* infection in the studied population of only 6.8%. As shown in a multivariable analysis, the risk of intestinal metaplasia and dysplasia was only significant in the presence of *H. pylori* infection<sup>[15]</sup>.

A minority of this Malay population is genetically susceptible to *H. pylori* infection, and *DCC* gene polymorphism has recently been found to be responsible<sup>[16]</sup>. An aberrant methylation of this tumor suppressor gene has been observed to occur in the course of gastric carcinogenesis<sup>[17]</sup>. As such, this population may also be genetically susceptible to the development of gastric precancerous lesions.

The current study aimed to determine the gene polymorphisms associated with gastric precancerous lesions in the Malay population from north-eastern region of Peninsular Malaysia using the genome-wide association approach.

## MATERIALS AND METHODS

### Study subjects

Only those ethnic Malay subjects (age range 20-80 years) whose gastrointestinal symptoms required upper endoscopy were screened for study eligibility. To avoid ascertainment bias, subjects had upper gastrointestinal symptoms (including dyspepsia and/or abdominal discomfort) and required upper endoscopy to exclude gastro-duodenal diseases before being included into the study.

All Malay subjects included in the study were born in the state of Kelantan, had resided within the region for at least 3 generations and were from different families but had similar socio-economic and socio-cultural backgrounds. Subjects positive for *H. pylori* infection according to a urease test and histology and with gastric precancerous lesions identified during endoscopy were categorized as "cases", while those negative for *H. pylori* infection and precancerous lesions were categorized as "controls". "Cases" and "controls" were matched for age and gender. Subjects satisfying the above inclusion criteria were recruited into the study. Exclusion criteria included an intake of antibiotics 3 mo prior to the upper endoscopy test, upper gastrointestinal bleeding, a positive family history of *H. pylori* infection and gastric cancer, a previous history of *H. pylori* infection and chronic psychiatric and medical conditions, including cancer. Informed consent was obtained from all subjects prior to their enrolment into the study.

Cases with *H. pylori* infection and positive for precancerous lesions were extremely limited in number due to an exceptionally low rate of *H. pylori* infection among ethnic Malays. Only 23 Malay subjects were eventually included as "cases". A larger sample size for the "controls" was sought to compensate for the low sample size in "cases". Furthermore, stringent criteria were set to ensure that only subjects of similar age, socio-economic and socio-cultural backgrounds were included in the study. From a total of 45 screened subjects, 37 Malay subjects

were recruited as “controls” with eight subjects being excluded as they did not meet the inclusion criteria, they did not give consent or blood samples were poor.

The study was approved by the Human Research and Ethics Committee of Universiti Sains Malaysia (USM).

### **Endoscopic diagnosis and histological definitions of precancerous lesions**

All upper endoscopies (model GIF-140 and GIF-160; Olympus Medical Systems, Tokyo, Japan) during this period were performed by one endoscopist with at least 5 years’ experience. If needed, patients were sedated accordingly. Subjects who did not stop proton pump inhibitors 2 wk before endoscopy, those who had received antibiotics prior to study, and patients who had upper gastrointestinal bleeding shortly before the study were excluded.

Endoscopic findings of gastritis and atrophy were recorded and classified based on established Sydney criteria<sup>[18]</sup> and Atrophy Club criteria<sup>[19]</sup>. Biopsies were taken using standard biopsy forceps at the antrum, incisura and body. A minimum of 2 to 4 biopsies (size between 2 to 4 mm) were taken in each sites and these gastric biopsies, preserved in formalin containers, were transported to the pathology laboratory on the same day.

Only one histopathologist was involved in reviewing all the slides. All biopsies were stained with routine hematoxylin and eosin (HE) stain followed by Alcian blue-periodic acid Schiff stain for the detection of intestinal metaplasia. The Warthin Starry stain would be used in sections where the *H. pylori* bacterium was not detected in the routine HE stain.

Chronic atrophic gastritis was identified based on the updated 1994 Sydney system<sup>[20]</sup> and Atrophy Club definitions<sup>[19]</sup>. Intestinal metaplasia was identified by replacing glandular epithelium with goblet cells<sup>[21]</sup>. Intestinal metaplasia was classified into complete or incomplete types. Complete type resembled the small intestinal phenotype with well-formed goblet cells while incomplete type resembled the colonic phenotype with irregular mucin droplets and absence of a brush border. Dysplasia was identified by epithelium disarray and increased nucleocytoplasmic ratio<sup>[22]</sup>.

For the purpose of the genotyping study, subjects were grouped as follows: atrophic gastritis only, complete intestinal metaplasia, incomplete metaplasia with foci of dysplasia, or dysplasia only.

### **Genomic DNA preparation**

All recruited subjects were called up by one of the investigators (SM) to have 1 mL of venous blood taken during the study day. Unlike conventional methods of DNA extraction, 1 mL of blood was sufficient for the commercially available kits. The blood was collected in an EDTA tube and was transported immediately to a facility (Human Genome Centre, USM, Kubang Kerian, Malaysia) to be stored at 4 °C. Subsequently, DNA for all recruited cases and controls was isolated using QIAamp DNA Blood

Mini Kit (QIAGEN, Hilden, Germany).

### **Genotyping with Affymetrix 50K Xba1**

The isolated DNA from all recruited cases ( $n = 23$ ) and controls ( $n = 37$ ) were processed and genotyped using Affymetrix 50k Xba1 array (Affymetrix, United States) following the instructions provided in the Affymetrix GeneChip Human Mapping 100K Assay Manual<sup>[23]</sup>. Genotypes with call rates  $< 90\%$  for autosomal single nucleotide polymorphisms (SNPs) were excluded. SNPs that had a minor allele frequency  $< 5\%$ , that failed to genotype in  $> 5\%$  of samples, or had a Hardy-Weinberg Equilibrium (HWE)  $P$ -value  $< 0.5$  were also excluded from the analysis.

### **Statistical analysis**

Genotype calling to assess the normalization of the SNPs was performed with the Bayesian Robust Linear Model with Mahalanobis distance classifier (BRLMM) algorithm from the Affymetrix<sup>®</sup> Genotyping Console<sup>™</sup> software version 4.0 (Affymetrix, United States). Quality control for genetic markers was assessed using the Genotype filtering tool in the SVS Golden Helix Bioinformatics Tools version 7.4 (Golden Helix Inc., Bozeman, MT, United States).

Association was evaluated for every single SNP in each gene with SVS Golden Helix Bioinformatics Tools. False Discovery Rate, and Bonferroni adjustments were used for multiple-testing corrections. A Manhattan plot for each phenotype was generated to determine SNPs with the highest significant value associated with that phenotype using SVS Golden Helix Bioinformatics Tools (version 7.4). A significant genomic threshold of  $3 \times 10^{-7}$  in Manhattan plots was set in this study and a  $\chi^2$   $P$  value for each SNP was calculated based on Fisher’s exact  $\chi^2$  test. For each type of precancerous lesion studied, of which a group of associated SNPs were identified from Manhattan plots, only a SNP with  $\chi^2$   $P$  value  $< 0.05$  and HWE  $P$  value  $> 0.5$  was considered as a significant marker.

## **RESULTS**

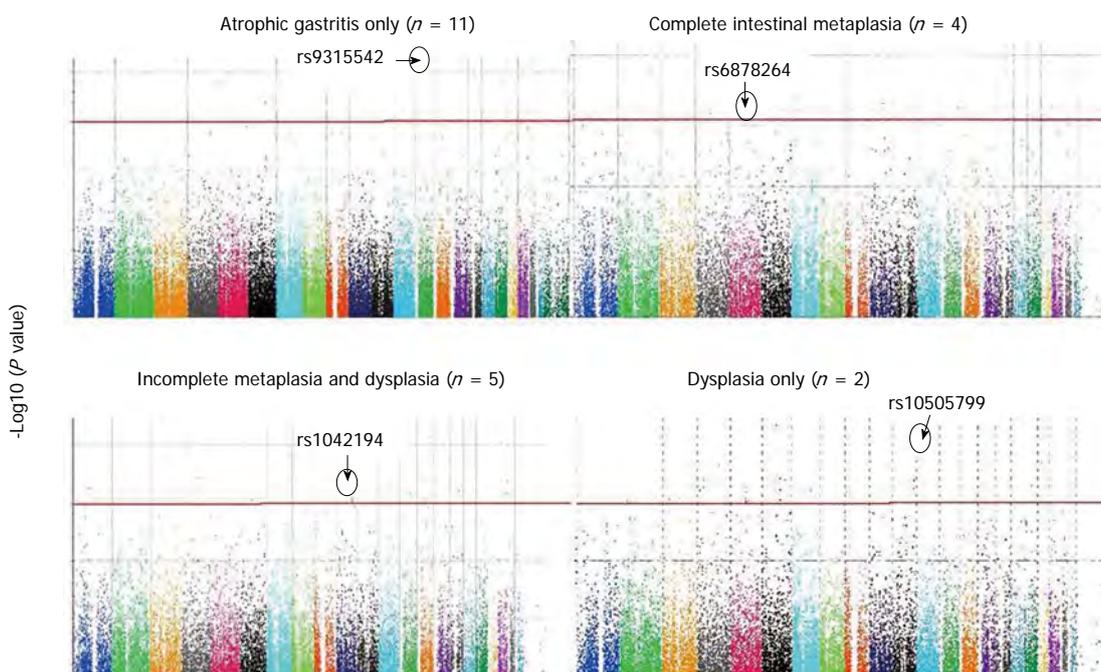
Of the 23 *H. pylori*-positive subjects recruited, one sample was excluded from further analysis due to a low genotyping call rate ( $< 90\%$ ). The mean age of the remaining 22 “cases” was  $56.5 \pm 16.5$  years, and was  $53.2 \pm 15.2$  years for the 37 “controls”. Cases were 54.5% (12/22) male compared with 50% (17/37) in “controls”. Of subjects positive for *H. pylori* infection (cases), atrophic gastritis only was present in 50.0% (11/22), complete intestinal metaplasia was present in 18.2% (4/22), both incomplete intestinal metaplasia and dysplasia was present in 22.7% (5/22) and dysplasia only was present in 9.1% (2/22). None of the gastric precancerous lesions were present in subjects negative for *H. pylori* infection (controls).

In 10 “cases” with atrophic gastritis only, compared with controls, 26 SNPs were above the significant ge-

**Table 1** Single nucleotide polymorphisms associated with atrophic gastritis among Malays with *Helicobacter pylori*

| rs ID      | Chromosome | Gene        | Minor allele | Allele frequency |         | $\chi^2$             | HWE     |
|------------|------------|-------------|--------------|------------------|---------|----------------------|---------|
|            |            |             |              | Cases            | Control | P-value <sup>1</sup> | P-value |
| rs2614074  | 8 p21.1    | <i>PNOC</i> | B            | 0.409            | 0.062   | 0.008                | 0.983   |
| rs10504944 | 8 q22.1    | <i>GDF6</i> | A            | 0.5              | 0       | 0.034                | 0.516   |
| rs9315542  | 13 q13.3   | <i>UFM1</i> | B            | 0.409            | 0.064   | 0.007                | 0.994   |
| rs4943552  | 13 q13.3   | <i>UFM1</i> | A            | 0.318            | 0.075   | 0.040                | 0.815   |
| rs489977   | 18 q12.3   | <i>KC6</i>  | A            | 0.181            | 0       | 0.064                | 0.752   |

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium. <sup>1</sup>All markers were run using the FAMHAP (Haplotype Association Analysis) program. The P value represents the simulated overall significance for the particular marker corrected for multiple testing and P < 0.05 was considered statistically significant.



**Figure 1** Manhattan plots for different gastric precancerous lesions (phenotype) in susceptible Malays with *Helicobacter pylori*. Red line indicates genomic threshold ( $3 \times 10^{-7}$ ) set to determine single nucleotide polymorphisms (SNPs) in Hardy-Weinberg Equilibrium associated with the studied phenotype. The most significant SNP, as determined by the  $\chi^2$  P value, for each phenotype is shown by an arrow.

genomic threshold. Five of the identified 26 SNPs were in HWE, of which rs9315542, located in chromosome 13 q13.3 (*UFM1* gene), was the most significant associated SNP ( $\chi^2$  P value = 0.007) (Table 1, Figure 1). The allele frequency for rs9315542 in cases *vs* controls was 0.4 *vs* 0.06.

In 4 “cases” with intestinal metaplasia only, compared with controls, 13 SNPs were above the genomic threshold and were in HWE, of which rs6878264, located in intron 4 of the *thrombospondin 4* (*THBS4*) gene, was the most significant associated SNP ( $\chi^2$  P value = 0.01) (Table 2, Figure 1). The allele frequency for rs6878264 in cases *vs* controls was 0.6 *vs* 0.01.

In 6 “cases” with both intestinal metaplasia and dysplasia, compared with controls, 17 SNPs were above the genomic threshold and in HWE, of which rs1042194, located in exon 8 of the *CYP2C19* gene, was the most significant associated SNP ( $\chi^2$  P value = 0.00536) (Table 3, Figure 1). The allele frequency for rs1042194 in cases *vs*

controls was 0.6 *vs* 0.01.

Finally, in 2 “cases” with dysplasia only, compared with controls, 2 SNPs were above the genomic threshold and in HWE, of which rs10505799, located in chromosome 12p12.3 (*MGST1* gene), was the most significant associated SNP ( $\chi^2$  P value = 0.006) (Table 4, Figure 1). The allele frequency for rs10505799 in cases *vs* controls was 0.5 *vs* 0.02.

## DISCUSSION

In this Malay population with an extremely low risk of *H. pylori* infection, gastric cancer and its precancerous lesions are very rare. However, in the current study of subjects susceptible to *H. pylori* infection and those who developed precancerous lesions, certain gene polymorphisms were found to be more commonly associated with precancerous lesions. Notwithstanding the low sample size, resulting from the extremely rare occurrence of gastric

**Table 2** Single nucleotide polymorphisms associated with intestinal metaplasia among Malays with *Helicobacter pylori*

| rs ID      | Chromosome | Gene     | Minor allele | Allele frequency |         | $\chi^2$<br>P-value | HWE<br>P-value |
|------------|------------|----------|--------------|------------------|---------|---------------------|----------------|
|            |            |          |              | Cases            | Control |                     |                |
| rs1166704  | 1p31.1     | NEXN     | B            | 0.375            | 0.031   | 0.0694              | 0.654          |
| rs2191508  | 2q32.3     | SLC39A10 | A            | 0.081            | 0.375   | 0.297               | 0.591          |
| rs1992736  | 3p24.3     | TBC1D5   | A            | 0.750            | 0.193   | 0.192               | 0.78           |
| rs10511297 | 3q13.13    | CD96     | A            | 0.375            | 0.030   | 0.171               | 0.659          |
| rs2615485  | 4q22.1     | DSPP     | A            | 0.750            | 0.136   | 0.190               | 0.625          |
| rs2434316  | 5q14.1     | THBS4    | A            | 0.750            | 0.257   | 0.118               | 0.743          |
| rs6878264  | 5q14.1     | THBS4    | B            | 0.625            | 0.010   | 0.010               | 1.000          |
| rs7800141  | 7p15.3     | DNAH11   | B            | 0.666            | 0.096   | 0.154               | 0.717          |
| rs4746259  | 10q22.2    | PPIALAG  | B            | 0.750            | 0.015   | 0.062               | 0.794          |
| rs9300471  | 13q32.2    | FARP1    | A            | 0.375            | 0.030   | 0.145               | 0.659          |
| rs1881344  | 16p13.2    | C16orf68 | A            | 0.375            | 0.030   | 0.078               | 0.659          |
| rs2253429  | 20p13      | SIRPB1   | B            | 0.375            | 0.030   | 0.0119              | 0.659          |
| rs2834681  | 21q22.12   | RUNX1    | B            | 0.250            | 0       | 0.0623              | 0.859          |

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

**Table 3** Single nucleotide polymorphisms associated with intestinal metaplasia and dysplasia among Malays with *Helicobacter pylori*

| rs ID      | Chromosome | Gene     | Minor allele | Allele frequency |         | $\chi^2$<br>P-value | HWE<br>P-value |
|------------|------------|----------|--------------|------------------|---------|---------------------|----------------|
|            |            |          |              | Cases            | Control |                     |                |
| rs10493872 | 1p21.3     | ABCD3    | A            | 0.375            | 0.062   | 0.00601             | 0.518          |
| rs10510792 | 3p14.3     | DNAH12   | A            | 0.25             | 0.030   | 0.341               | 0.728          |
| rs2889259  | 4p14       | KIAA1239 | A            | 0.375            | 0.015   | 0.0623              | 0.724          |
| rs6837437  | 4p14       | KIAA1239 | A            | 0.375            | 0.015   | 0.0623              | 0.728          |
| rs10498879 | 6q13       | RIMS1    | A            | 0.75             | 0.196   | 0.160               | 0.629          |
| rs4073894  | 7q22.1     | LHFPL3   | A            | 0.375            | 0.015   | 0.0623              | 0.728          |
| rs10487929 | 7q35       | CNTNAP2  | B            | 0.25             | 0.031   | 0.260               | 0.724          |
| rs10503727 | 8p21.2     | SLC25A37 | B            | 0.75             | 0.183   | 0.151               | 0.908          |
| rs2251417  | 10q21.3    | ANXA2P1  | A            | 0.375            | 0.045   | 0.579               | 0.591          |
| rs1042194  | 10q23.33   | CYP2C19  | B            | 0.625            | 0.011   | 0.00536             | 0.972          |
| rs10506855 | 12q21.31   | CCDC59   | B            | 0.375            | 0.046   | 0.0269              | 0.585          |
| rs10492652 | 13q22.1    | KLF12    | B            | 0.25             | 0.015   | 0.0623              | 0.797          |
| rs1565946  | 14q23.1    | SLC35F4  | A            | 0.625            | 0.156   | 0.0151              | 0.658          |
| rs10483683 | 14q23.1    | SLC35F4  | B            | 0.625            | 0.181   | 0.314               | 0.964          |
| rs10483837 | 14q24.2    | RGS6     | B            | 0.5              | 0.031   | 0.171               | 0.585          |
| rs245615   | 16q21      | CDH8     | A            | 0.5              | 0.140   | 0.183               | 0.844          |
| rs2058879  | 19q13.32   | IGFL2    | A            | 0.375            | 0.015   | 0.0623              | 0.728          |

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

precancerous lesions in this population, the current study, with the use of the genome-wide association approach, allowed identification of genetic markers that can be tested in a larger cohort in the near future<sup>[24]</sup>.

In the 50% of *H. pylori*-infected subjects with atrophic gastritis, the earliest lesion in the Correa cascade, rs9315542, located in chromosome 13 q13.3 (*UFM1* gene), was the identified marker. A recently identified expressed protein, ubiquitin-fold modifier 1 or *UFM1*, is a member of a large family of ubiquitin-like proteins or Ubls<sup>[25]</sup>. Ubiquitin, a small protein, is associated with the process of “ubiquitination”, a target of proteins for degradation by the proteasome. At the moment, the exact cellular functions of proteins modified by *UFM1* remain elusive. A recent report indicated that components of the *UFM1* conjugation pathway are highly expressed in the beta cells of the pancreas and some other protein secretory tissues<sup>[26]</sup>. In the same report, *UFM1* conjugate

prevented endoplasmic reticulum (ER) stress-induced apoptosis. While *UFM1* in gastric tissue has not been investigated, it is known that gastric mucosa secretes a number of peptides and hormones, including pepsinogen and ghrelin, whose levels are reduced in atrophic gastritis<sup>[27]</sup>. Speculatively, *UFM1* may be a marker of the secretory status of the gastric mucosa, similar to pepsinogen, and remains to be tested and validated.

Complete-type or type I intestinal metaplasia, considered as the benign version compared with the incomplete-type, was present in 18.2% of *H. pylori*-infected subjects. In these subjects, rs6878264 located in intron 4 of the *THBS4* gene, was the identified marker. THBS4 is a member of the THBS protein family, a glycoprotein in the extracellular matrix, which mediates cell-to-cell and cell-to-matrix interactions. Although the exact physiological functions of THBS4 are unknown, the published literature indicate that it promotes neurite outgrowth,

**Table 4** Single nucleotide polymorphisms associated with dysplasia among Malays with *Helicobacter pylori*

| rs ID      | Chromosome | Gene         | Minor allele | Allele frequency |         | $\chi^2$<br>P-value | HWE<br>P-value |
|------------|------------|--------------|--------------|------------------|---------|---------------------|----------------|
|            |            |              |              | Cases            | Control |                     |                |
| rs10505799 | 12p12.3    | <i>MGST1</i> | B            | 0.5              | 0.016   | 0.006               | 0.855          |
| rs10498391 | 14q21.2    | <i>FSCB</i>  | B            | 0.5              | 0       | 0.069               | 0.928          |

rs ID: The unique ID for the identified single nucleotide polymorphisms; HWE: Hardy-Weinberg Equilibrium.

stimulates proliferation of erythroid cells, skin fibroblasts and kidney epithelial cells, as well as myoblast adhesion and interaction with other extracellular matrix proteins<sup>[28-30]</sup>. Recently, *THBS4* has been found to be associated with gastric adenocarcinomas especially of the diffuse type<sup>[31]</sup>. While *H. pylori* infection is associated with atrophic gastritis and intestinal metaplasia, it is not commonly associated with diffuse-type gastric adenocarcinoma<sup>[32]</sup>. Complete-type intestinal metaplasia represents a reparative process of the epithelium following *H. pylori*-induced gastritis, and in this context, *THBS4* may act as an early proliferative marker but may have a more aggressive pro-oncogenic role in advanced stages. Again, this is speculative in the absence of any published studies, but it is a worthwhile marker for further studies.

Incomplete type intestinal metaplasia is more advanced compared with complete type, and therefore is more closely associated with dysplasia. In 22.7% of cases with both incomplete type intestinal metaplasia and dysplasia, rs1042194 located in exon 8 of *CYP2C19* gene was the identified marker. Cytochrome (CYP) P450 2C19, one of the isoforms of the CYP enzyme (phase I detoxification enzyme), plays an important role in metabolism of drugs and also detoxification of potential carcinogens<sup>[33]</sup>. Several studies indicated that *CYP2C19* gene polymorphism is associated with increased cancer susceptibility including hepatocellular carcinoma, and lung, esophageal and gastric cancer, especially in patients having a poor metabolizer (PM) genotype<sup>[34-36]</sup>. A study from Malaysia found that the PM genotype was uncommon among ethnic Malays (5.9%), compared with Chinese (19.1%) and Indians (10%)<sup>[37]</sup>. This may be one of the reasons for reduced susceptibility to gastric cancer and its precancerous lesions among ethnic Malays. The finding of *CYP2C19* in incomplete type intestinal metaplasia and dysplasia in a group of Malay subjects susceptible to *H. pylori* infection is therefore important and merits further study.

Dysplasia, a histological stage with high risk of malignant transformation, was present in only 9.1% or 2/23 subjects infected with *H. pylori*. Compared with controls, rs10505799 located in chromosome 12p12.3 (*MGST1* gene) was found to be the SNP marker associated with dysplasia. Microsomal glutathione S-transferase 1 (*MGST1*) is one of the glutathione S-transferase (GST) family of enzymes, and GSTs are phase II detoxification enzymes, which, similar to CYP enzymes, are involved in the detoxification of potential carcinogens<sup>[38,39]</sup>. Recently, *MGST1* gene polymorphism was found to be involved in

colorectal carcinogenesis in the Chinese population but there is no data as yet on gastric cancer<sup>[40]</sup>. However, since *MGST1* and *CYP2C19* are both carcinogen detoxification enzymes, with evidence supporting their involvement in gastrointestinal tract carcinogenesis, the role of *MGST1* in gastric precancerous lesions is likely to be valid. The limited number of cases with dysplasia in the current study means that the results need to be interpreted cautiously, but the potential of *MGST1* as a marker for dysplasia should not be disregarded.

There are a number of studies on gene polymorphisms associated with gastric precancerous lesions in high prevalence populations, but our study covered the entire spectrum of the Correa cascade in a population with an extremely low burden of gastric cancer and *H. pylori* infection. Development of gastric cancer is thought to involve multi-step carcinogenesis and follows a progressive pattern of pathological stages described by Correa. Our results indicated that, at different phases of the Correa cascade, different gene variants are manifest, but they follow a pattern of progression similar to their histological and clinical stages. During the stage of atrophic gastritis, *UFM1* expression reflects the secretory status of epithelium. With early development of intestinal metaplasia, *THBS4* acts as a proliferative marker but at more advanced stages, incomplete intestinal metaplasia and dysplasia involve polymorphisms of detoxification enzymes, *CYP2C19* and *MGST1*. Based on the current study, it is possible that, in addition to histological staging, gene variant markers may also serve to identify different phases of progression of gastric cancer in the near future. Recently, epigenetic silencing of *FOXD3* has been shown to be an early event in gastric carcinogenesis<sup>[41]</sup> and, together with genomic changes, it would allow a greater understanding of the pathogenesis of gastric cancer.

We acknowledge from the outset that the current study, based upon a genome-wide approach, was extremely limited in sample size, as gastric precancerous lesions are extremely rare among ethnic Malays from the north-eastern region of Peninsular Malaysia. In this respect, bioinformatics and statistical approaches were taken into consideration for a more reliable analysis of the data. To reduce false-positive results, a more stringent significance threshold of  $3 \times 10^{-7}$  was set for Manhattan plots in the current study. Only SNPs in HWE  $P$ -value  $> 0.5$  were selected to reduce occasionality. In addition to being long-term residents within the studied region, cases and controls were similar in age, socio-cultural and economic backgrounds. The current study only identified

SNPs associated with gastric precancerous lesions, and further validation studies are in progress to confirm their regulatory role in carcinogenesis.

In conclusion, we have shown that, compared with controls, susceptible ethnic Malays with *H. pylori* infection expressed different SNP markers at different spectrums of gastric precancerous lesions. These markers may allow efficient screening of precancerous lesions in larger cohorts of *H. pylori*-infected individuals.

## ACKNOWLEDGMENTS

Shuhua Xu is Max-Planck Independent Research Group Leader and member of CAS Youth Innovation Promotion Association. Shuhua Xu also gratefully acknowledges the support of the National Program for Top-notch Young Innovative Talents and the support of K.C. Wong Education Foundation, Hong Kong.

## COMMENTS

### Background

Gastric cancer and its precancerous lesions are exceptionally rare among ethnic Malays. Gene variants may be associated with precancerous lesions in *Helicobacter pylori* (*H. pylori*)-susceptible Malays.

### Research frontiers

In a case-control study, genome-wide association was performed to identify gene variants in the Malay population with a spectrum of *H. pylori*-associated gastric precancerous lesions.

### Innovations and breakthroughs

Results indicated that at different phases of the Correa cascade, different gene variants were manifest, but they followed a pattern of progression similar to the histological and clinical stages.

### Applications

It is possible that, in addition to histological staging, gene variant markers may also serve to identify different phases of progression of gastric cancer in the near future.

### Terminology

The genome-wide approach utilises microarray technology to identify thousands of single nucleotide polymorphisms (SNPs). Using novel bioinformatics and statistical approaches, the association between SNPs and the studied disease can be determined reliably.

### Peer review

Current study indicates that different gene variants exist that reflect different stages of progression during different spectrums of gastric carcinogenesis. These gene variants, appropriately confirmed in later studies, may be useful markers, in addition to histological staging, of gastric precancerous lesions.

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P- Reviewer Kozarek RA S- Editor Wen LL  
L- Editor Cant MR E- Editor Zhang DN



## XPC Lys939Gln polymorphism, smoking and risk of sporadic colorectal cancer among Malaysians

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Supported by Fundamental Research Grant Scheme (FRGS), No. 203/PPSP/6171112

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Received: September 26, 2012 Revised: December 24, 2012

Accepted: March 8, 2013

Published online: June 21, 2013

frequencies, variant allele C was associated with a significantly increased risk of CRC (OR = 1.375; 95%CI: 1.050-1.802;  $P = 0.020$ ). Moreover, the risk was markedly higher for those who were carriers of the Gln/Gln variant genotype and were also cigarette smokers (OR = 3.409; 95%CI: 1.061-10.949;  $P = 0.032$ ).

**CONCLUSION:** The XPC Gln/Gln genotype alone and in combination with smoking increases the risk of CRC among Malaysians.

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**Key words:** DNA repair; Xeroderma pigmentosum group C Lys939Gln polymorphism; Cigarette smoking; Colorectal cancer; Susceptibility risk

Ahmad Aizat AA, Siti Nurfatimah MS, Aminudin MM, Ankathil R. XPC Lys939Gln polymorphism, smoking and risk of sporadic colorectal cancer among Malaysians. *World J Gastroenterol* 2013; 19(23): 3623-3628 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3623.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3623>

### Abstract

**AIM:** To investigate the risk association of xeroderma pigmentosum group C (XPC) Lys939Gln polymorphism alone and in combination with cigarette smoking on colorectal cancer (CRC) predisposition.

**METHODS:** Peripheral blood samples of 510 study subjects (255 CRC patients, 255 controls) were collected. DNA was extracted and genotyping was performed using polymerase chain reaction-restriction fragment length polymorphism. The association between polymorphic genotype and CRC predisposition was determined using the OR and 95%CI.

**RESULTS:** The frequency of the homozygous variant (Gln/Gln) genotype was significantly higher in cases compared with controls (16.0% vs 10.2%,  $P = 0.049$ ). The Gln/Gln genotype of XPC showed a significantly higher association with the risk of CRC (OR = 1.884; 95%CI: 1.082-3.277;  $P = 0.025$ ). In the case of allele

### INTRODUCTION

Colorectal cancer (CRC) is a major public health problem worldwide, being the third most common cancer and the fourth most common cancer causing death<sup>[1]</sup>. In Malaysia, CRC has become the most common cancer among males and the second most common among females<sup>[2]</sup>. The development of CRC is a complex, multistep process involving interaction between environmental and genetic factors. Environmental factors such as dietary and lifestyle habits, smoking, alcohol consumption, and obesity interact with host's genetic factors, especially genetic variations, and may modulate the risk of CRC<sup>[3]</sup>. Genetic variations, such as single nucleotide polymorphisms (SNPs), may increase the sensitivity to environmental

carcinogens and may act as cancer predisposition factors. Environmentally sensitive genetic polymorphisms acting together with environmental factors are well documented candidates influencing cancer susceptibility.

DNA damage repair genes maintain the integrity of the genome against endogenous and exogenous factors. The xeroderma pigmentosum group C (*XPC*) gene is a DNA repair gene involved in the nucleotide excision repair (NER) mechanism which repairs bulky DNA lesions such as pyrimidine dimers, ultraviolet light-induced damage, photoproducts, intrastrand crosslinks, larger chemical adducts and other genotoxic agents<sup>[4]</sup>. Genetic variations in the *XPC* gene have been reported to modulate an individual's susceptibility to developing cancer<sup>[5,6]</sup>. The *XPC* Lys939Gln polymorphism, which leads to an amino acid change from lysine to glutamine at codon 939, is the most common SNP studied in the *XPC* gene and has been shown to be associated with increased risk of several cancers such as skin<sup>[7]</sup>, breast<sup>[8]</sup> and bladder cancers<sup>[9,10]</sup>.

However, there are only limited reports on the association of this polymorphism with CRC susceptibility. A case-control study was undertaken in order to investigate the association of this polymorphism alone and also in conjunction with cigarette smoking on the risk of CRC. This polymorphism is believed to alter the gene expression and modulate the DNA repair function of the protein product, as it is located at the coding sequence of the *XPC* gene. Thus, we hypothesized that *XPC* Lys939Gln polymorphism may have an effect on modulating the susceptibility to CRC, and cigarette smoking may further enhance the effect on CRC risk.

## MATERIALS AND METHODS

### Study subjects

The study was approved by the Research Review Board and Ethics Committee of Universiti Sains Malaysia, Kelantan and the Ministry of Health, Malaysia. For this hospital-based case control study, subjects were recruited from various hospitals in Malaysia, including Hospital Universiti Sains Malaysia, Hospital Raja Perempuan Zainab II and Hospital Sultanah Bahiyah, Kedah, Malaysia. Genotyping was carried out at the Human Genome Center, Universiti Sains Malaysia. Two hundred and fifty five sporadic CRC patients and 255 healthy normal controls were recruited as study subjects. Cases were histopathologically confirmed sporadic CRC patients, aged > 25 years, who did not have previous colon/rectal or other cancers. Cases with known (as indicated in the pathology reports) familial adenomatous polyposis, ulcerative colitis or Crohn's disease or any other previous malignancy were excluded. Controls were normal healthy individuals who were biologically unrelated to the study patients, aged > 25 years and had no history of cancer. Epidemiological data was collected from patients using a pre-structured questionnaire, which included sociodemographic status, physical status, dietary factors, occupation, tobacco/alcohol habits, previous illness, radiation exposure, *etc.*

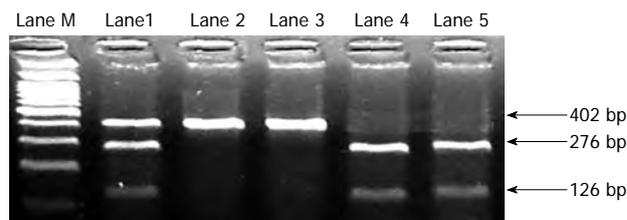


Figure 1 Gel picture showing the different categories of xeroderma pigmentosum group C Lys939Gln polymorphism genotype. Lane M: 100 bp DNA ladder; Lanes 2 and 3: Homozygous wildtype; Lane 1: Heterozygous; Lanes 4 and 5: Homozygous variant.

### Genotyping

Genotyping of *XPC* Lys939Gln polymorphism was carried out using polymerase chain reaction (PCR)-restriction fragment length polymorphism. Briefly, PCR primers for *XPC* Lys939Gln (F: 5'-GGCTTCCTGGTATCTGAT-TACT-3'R: 5'-CTCAGTTTGCCTTCTCAGCA-3') were used to generate a 402 bp product containing the polymorphic site. The PCR reactions were carried out in a 25  $\mu$ L volume of 1  $\times$  PCR Buffer, 2.0 mmol/L of MgCl<sub>2</sub>, 0.5 mmol/L dNTPs, 0.4 mmol/L of each primers and 1 U of AmpliTaq Gold Polymerase with a denaturation of 94  $^{\circ}$ C for 5 min, followed by 35 cycles at 94  $^{\circ}$ C for 30 s, 57  $^{\circ}$ C for 30 s, 72  $^{\circ}$ C for 30 s and finally 5 min at 72  $^{\circ}$ C. Following amplification, the PCR products were digested using *PvuII* restriction enzyme for 1 h at 37  $^{\circ}$ C and electrophoresed on 2% agarose gel. The homozygous wild-type genotype was identified by a single band at 402 bp level, the heterozygous genotype by 3 bands at 402, 276 and 126 bp levels and the homozygous variant by 2 bands at 276 and 126 bp levels (Figure 1).

### Statistical analysis

The sample size was calculated with power and sample size (PS) software version 2.1.31 using the uncorrected  $\chi^2$  test with 80% power and 95%CI. The difference in distribution of genotypes, gender and age between cases and controls were assessed using the  $\chi^2$  test. The ORs and 95%CI were calculated using binary logistic regression to evaluate the risk association. All statistical tests were two-sided, and statistical significance was determined as  $P < 0.05$ . SPSS v.18 (SPSS Inc., Chicago, IL, United States) was utilized for statistical analysis.

## RESULTS

The demographic characteristics of the study subjects are shown in Table 1. This case-control study recruited 510 study subjects, including 255 histopathologically confirmed sporadic CRC patients and 255 healthy normal controls. Of the 255 CRC patients, 139 (54.5%) were male and 116 (45.5%) were female. Of the 255 normal controls, 115 (45.1%) were male and 140 (54.9%) were female. The ages of the sporadic CRC patients ranged from 27 to 93 years (mean age: 53.17  $\pm$  7.07 years) and of the healthy normal controls ranged from 26 to 84

**Table 1** Demographic characteristics of the study population *n* (%)

| Variable          | Cases ( <i>n</i> = 255) | Controls ( <i>n</i> = 255) | <i>P</i> -value      |
|-------------------|-------------------------|----------------------------|----------------------|
| Gender            |                         |                            |                      |
| Male              | 139 (54.5)              | 115 (45.1)                 | 0.034 <sup>1</sup>   |
| Female            | 116 (45.5)              | 140 (54.9)                 |                      |
| Mean age (yr)     | 53.17 ± 7.07            | 46.47 ± 12.02              | 0.086                |
| Cigarette smoking |                         |                            |                      |
| Yes               | 110                     | 48                         | < 0.001 <sup>1</sup> |
| No                | 145                     | 207                        |                      |

<sup>1</sup>Statistically significant.

**Table 2** Genotype and allele frequencies of the xeroderma pigmentosum group C Lys939Gln polymorphism in study subjects *n* (%)

|                          | Cases ( <i>n</i> = 255) | Controls ( <i>n</i> = 255) | <i>P</i> -value    |
|--------------------------|-------------------------|----------------------------|--------------------|
| Genotype                 |                         |                            |                    |
| Homozygous wildtype (AA) | 108 (42.4)              | 129 (50.6)                 | 0.062              |
| Heterozygous (AC)        | 106 (41.6)              | 100 (39.2)                 | 0.588              |
| Homozygous variant (CC)  | 41 (16.0)               | 26 (10.2)                  | 0.049 <sup>1</sup> |
| Allele                   |                         |                            |                    |
| A                        | 322 (63.1)              | 358 (70.0)                 | 0.294              |
| C                        | 188 (36.9)              | 152 (30.0)                 |                    |

<sup>1</sup>Statistically significant.

years (mean age: 46.47 ± 12.02 years).

The frequencies of the homozygous wildtype (Lys/Lys), heterozygous (Lys/Gln) and homozygous variant (Gln/Gln) genotypes were 108 (42.4%), 106 (41.6%) and 41 (16.0%), respectively, among CRC cases. In controls, the frequencies were 129 (50.6%) for homozygous wildtype, 100 (39.2%) for heterozygous and 26 (10.2%) for homozygous variant genotypes. The frequency of the homozygous variant (CC) genotype was significantly higher in cases compared with the controls (*P* = 0.049). Table 2 shows the genotype and allele frequencies of *XPC* Lys939Gln polymorphism in cases and controls.

Binary logistic regression analysis was performed in order to find the risk association. Table 3 shows the association between the *XPC* Lys939Gln polymorphism and risk of CRC. It can be clearly seen that the homozygous variant genotype was significantly associated with increased risk of CRC (OR = 1.884; 95%CI: 1.082-3.277; *P* = 0.025). The variant allele C was found to be significantly associated with increased risk of CRC (OR = 1.375; 95%CI: 1.050-1.802; *P* = 0.020). Furthermore, the study subjects were stratified into smokers and non-smokers and the risk association was evaluated. Results showed that, for those with the homozygous variant genotype (CC) and who were smokers, the risk was significantly increased (OR = 3.409; 95%CI: 1.061-10.949; *P* = 0.032). In the non-smoking group, no significant association was observed between the *XPC* Lys939Gln polymorphism and the risk of CRC.

**Table 3** Xeroderma pigmentosum group C Lys939Gln polymorphism, smoking and colorectal cancer susceptibility risk

|              | Cases | Controls | OR (95%CI)                 | <i>P</i> -value    |
|--------------|-------|----------|----------------------------|--------------------|
| Genotype     |       |          |                            |                    |
| Lys/Lys (AA) | 108   | 129      | 1 (Reference) <sup>1</sup> |                    |
| Lys/Gln (AC) | 106   | 100      | 1.266 (0.871-1.841)        | 0.216              |
| Gln/Gln (CC) | 41    | 26       | 1.884 (1.082-3.277)        | 0.025 <sup>2</sup> |
| Allele       |       |          |                            |                    |
| A            | 322   | 358      | 1 (Reference) <sup>1</sup> | -                  |
| C            | 188   | 152      | 1.375 (1.050-1.802)        | 0.020 <sup>2</sup> |
| Non-smoker   |       |          |                            |                    |
| AA           | 64    | 104      | 1 (Reference) <sup>1</sup> |                    |
| AC           | 64    | 81       | 1.284 (0.817-2.018)        | 0.278              |
| CC           | 17    | 22       | 1.256 (0.620-2.542)        | 0.526              |
| Smoker       |       |          |                            |                    |
| AA           | 44    | 25       | 1 (Reference) <sup>1</sup> |                    |
| AC           | 42    | 19       | 1.256 (0.605-2.609)        | 0.541              |
| CC           | 24    | 4        | 3.409 (1.061-10.949)       | 0.032 <sup>2</sup> |

<sup>1</sup>Homozygous wildtype genotype served as the reference category;

<sup>2</sup>Statistically significant.

## DISCUSSION

To date, a variety of chemical carcinogens have been identified to cause DNA damage in humans. DNA damage repair genes are responsible for maintaining the integrity of the human genome through different pathways, locations and types of damage by base excision repair, NER, double strand break and mismatch repair pathways<sup>[4]</sup>. Genes involved in the NER pathway, such as *XPC*, commonly repair lesions induced by numerous exogenous agents such as those derived from food and smoking, including 2-amino-1-methyl-6-phenylimidazo-[4,5-β]-pyridine and benzo-[α]-pyrenediol-epoxide<sup>[11]</sup>.

The *XPC* gene, located on chromosome 3p25, contains 16 exons and 15 introns and encodes a 940 amino acid protein<sup>[12]</sup>. Several polymorphic variants in the *XPC* gene have been identified and *XPC* Lys939Gln is one of the three most common SNPs. This case-control study investigated the genotype and allele frequencies of the *XPC* Lys939Gln polymorphism, and the risk association with sporadic CRC susceptibility. Results from the present study showed that the frequency of the *XPC* homozygous variant genotype was significantly higher in cases compared with controls (16.0% *vs* 10.2%, *P* = 0.049). On evaluation of the risk association, the homozygous variant genotype (CC) was associated with significantly increased risk of CRC (OR = 3.409; *P* = 0.032). This result conforms with a study by Yasuda *et al*<sup>[13]</sup>, which demonstrated that a single amino acid alteration could be sufficient to compromise *XPC* function, thereby enhancing the risk of CRC development. Our results are in agreement with a few other reports by Qiu *et al*<sup>[5]</sup>, Zhang *et al*<sup>[6]</sup> and Gil *et al*<sup>[14]</sup> which revealed that *XPC* is one of the most important genes modulating an individual's risk of developing sporadic cancer.

Carcinogens consumed through dietary and lifestyle habits as well as from the environment play a major role

in damaging DNA. Cigarette smoking is one of the life-style factors that play an important role in the exposure of an individual to the carcinogens present in cigarette smoke. Carcinogens contained in tobacco smoke, such as polycyclic aromatic hydrocarbons, heterocyclic amines, nitrosamines and aromatic amines, are harmful to the human colon and rectum. Cigarette smoking has been reported to lead to the formation of DNA adducts and cause damage to the DNA in the human colon<sup>[15,16]</sup>. All these carcinogens can reach the colorectal mucosa, through direct ingestion or via the circulatory system, and has been demonstrated to induce bulky adducts in crypt cells and contribute to the formation of mutations in the colon<sup>[17,18]</sup>. In the present study, a combination of smoking and a variant genotype of *XPC* enhanced the risk of CRC 3-fold, indicating that smoking further enhanced the risk of CRC. Although non-smokers showed an OR of 1.256, this was not statistically significant.

The *XPC* gene encodes an important protein involved in the recognition of DNA damage in the NER pathway. This protein binds to HR23B protein to form XPC-HR23B that recognizes a DNA lesion and repairs the DNA damage along with other DNA repair proteins<sup>[19,20]</sup>. Polymorphism in the coding sequence of the *XPC* gene has been demonstrated to alter the gene expression and thereby modulate the DNA repair function<sup>[21]</sup>. The *XPC* Lys939Gln polymorphism is located in the coding sequence of the *XPC* gene. The nucleotide change from A to C leads to an amino acid change from lysine to glutamine in the coding sequence of the *XPC* gene and has been reported to lead to reduced repair capacity. This genetic variation has also been reported to result in reduced specificity of this gene in recognition and repair of the DNA damage as well as in protein expression, thus allowing more somatic DNA mutations or alterations to occur<sup>[21,22]</sup>.

The XPC protein plays a crucial role in repairing the DNA damage caused by tobacco smoke. Individuals with the *XPC* variant genotype may possess deficient DNA repair capability. Accordingly, the XPC protein product may be less efficient in repairing the DNA lesions induced by tobacco smoke, and thereby could enhance the susceptibility, favoring the development of CRC.

Previous studies focusing on the role of the *XPC* variant in the modulation of an individual's risk of developing CRC are scarce, and reported results are inconsistent. Berndt *et al.*<sup>[22]</sup>, in an American population, found an OR of 1.19 for the variant genotype's association with risk of CRC, but the result was statistically insignificant ( $P = 0.50$ ). In contrast, a study conducted by Hansen *et al.*<sup>[23]</sup>, in a Polish population, observed no significant risk association between this polymorphism and CRC susceptibility. However, when the variant genotype in conjunction with red meat consumption was evaluated, the risk was reported to be significantly higher (OR = 3.7) in those who carried the homozygous variant (CC) genotype and consumed 100 g red meat per day.

A recent study by Wu *et al.*<sup>[24]</sup> on 421 CRC patients and

845 controls, showed that the homozygous variant (CC) genotype was significantly associated with higher risk of CRC in a Chinese population (OR = 1.5; 95%CI: 1.0-2.2;  $P = 0.035$ ). Moreover, combination (AC + CC) genotypes in the homozygous wildtype model also showed a significant association with risk of CRC (OR = 1.3; 95%CI: 1.0-1.7;  $P = 0.039$ ). When the study subjects were stratified into tea drinkers and non-tea drinkers, the results showed that individuals who were non-tea drinkers and carried combination (AC + CC) genotypes, had increased risk of CRC (OR = 3.0; 95%CI: 1.9-4.7;  $P < 0.001$ ).

Apart from CRC, *XPC* polymorphism was reported to be associated with the risk of other cancers, such as head and neck<sup>[25]</sup>, lung<sup>[10]</sup>, breast<sup>[8]</sup> and bladder<sup>[26]</sup>. In addition, abnormal expression of the *XPC* gene has been shown in hepatocarcinogenesis<sup>[27]</sup>. The variant allele of the *XPC* gene is associated with lower DNA repair capacity (DRC). Several studies showed that the Lys939Gln polymorphism had an interaction with other SNPs located in the same gene, such as Ala499Val, PAT (-/+ ) and IVS11 C/A polymorphisms. According to Khan *et al.*<sup>[28]</sup>, the *XPC* Lys939Gln polymorphism is in linkage disequilibrium with the *XPC* PAT polymorphism as well as the C/A polymorphism at 25 positions of intron 11 of *XPC*. This causes exon 12 to be skipped and deleted, resulting in the loss of *XPC* cDNA function, thus reducing the DNA repair activity.

Researchers have shown that carriers of the C variant allele of the *XPC* Lys939Gln polymorphism exhibited significantly more DNA damage induced by BPDE<sup>[21,29,30]</sup>. Moreover, combinations of haplotype C + C (Lys939Gln, PAT +, Ala499Val) have been reported to cause poor DRC, thus increasing the risk of CRC. In contrast, haplotype T-A (Ala499Val-PAT-Lys939Gln) has been demonstrated to be associated with the least DNA damage<sup>[21,24]</sup>. Also, another study has shown that accumulation or the presence of alleles in the *XPC* gene associated with increased risk might reduce DRC and thus increase CRC susceptibility<sup>[31]</sup>.

The difference in results on the risk association between the present study and other previous studies might be explained by differences in genetic background of study subjects groups or populations, and also differences in exposure to environmental and lifestyle factors. Small sample sizes and/or inadequate control for certain confounder factors such as gender and age might also have contributed to the differing results and lack of association.

From the present study, it is reasonable to suggest that the *XPC* gene, especially the *XPC* variant genotype, may modulate the DRC of the host cell and thus play an important role in sporadic colorectal carcinogenesis. The risk could be much higher in individuals who possess the variant genotype and who are also cigarette smokers. The results also highlight the potential role of the NER pathway (especially of *XPC*) in modulation of an individual's risk of sporadic CRC. Further studies to explore the interaction of *XPC* Lys939Gln with other SNPs of

the XPC gene and with other genes involved in DNA repair pathways, either singly or in combination, and to examine the correlation with environmental factors such as alcohol consumption and dietary habits, as well as clinicopathological characteristics, would be beneficial in deriving more accurate risk predictive markers.

## ACKNOWLEDGMENTS

We would like to thank Dr. Muhammad Radzi Abu Hassan (Hospital Sultanah Bahiyah, Kedah), Prof Dr. BM Biswal, Dr. VMK Bhavaraju (Hospital Universiti Sains Malaysia), Dr. Ahmad Shanwani and Dr. Zaidi Zakaria (Hospital Raja Perempuan Zainab II, Kota Bharu) for their cooperation in patient recruitment.

## COMMENTS

### Background

Colorectal cancer (CRC) which is the most common cancer among males and the second most common among females in Malaysia, has become a major public problem. Environmental factors, such as dietary carcinogens and tobacco smoke, interacting with the host's genetic factors may modulate the risk of CRC. However, the genetic predisposition factors associated with colorectal carcinogenesis remain largely undetermined. Identification of the host's genetic predisposition factors may help in understanding the carcinogenic process. Thus, it was of interest to explore the contribution of single nucleotide polymorphisms (SNPs) in a DNA repair gene, alone and in combination with tobacco smoke, as a predisposition factor for CRC susceptibility.

### Research frontiers

Exposure to mutagens and carcinogens through diet, tobacco smoke, etc can cause varying degrees of DNA damage and can cause mutations in humans if left unrepaired. The xeroderma pigmentosum group C (XPC) gene is a DNA repair gene involved in the nucleotide excision repair (NER) pathway. The SNP, Lys939Gln, located at the coding region of the XPC gene has been associated with lower DNA repair capacity and has been shown to be associated with increased risk of cancers of the skin, breast and bladder. This study was designed to determine the frequencies and influence of the XPC Lys939Gln polymorphic genotype, alone and in combination with smoking, on sporadic CRC susceptibility risk in a Malaysian population.

### Innovations and breakthroughs

Only limited reports are available on the association of XPC Lys939Gln polymorphism with CRC susceptibility risk. To the best of our knowledge, this is the first report of an association between the genetic variant of XPC with CRC susceptibility risk in the Malaysian population. The observation that genetic variation of the XPC gene influences susceptibility to CRC implies the importance of the NER pathway in modulation of an individual's risk of CRC. This study provides support for the hypothesis that genetic variation in the XPC gene, acting together with environmental factors, contributes to CRC development and may be considered as a candidate marker for CRC predisposition risk in the Malaysian population.

### Applications

The present study observed that the genetic variation Lys939Gln of the XPC gene influences the risk of CRC in the Malaysian population. Further SNP mapping studies utilizing high throughput genotyping methods could facilitate the analysis of multiple polymorphisms within DNA repair genes. Genotyping of Malaysian CRC patients for polymorphism(s) in DNA repair genes will help in understanding the specific polymorphism(s), which act as predisposing genotype(s) in CRC susceptibility. In future, the study can be extended to a population level, to identify individuals with high risk predisposition genotypes. Appropriate surveillance programs could then be developed in order to reduce the morbidity and mortality of CRC at the population level.

### Peer review

This case-control study investigated the association between SNPs and cigarette smoking on sporadic CRC susceptibility risk in Malaysian population. The

genotype and allele frequencies of XPC Lys939Gln were investigated in 255 CRC patients and 255 healthy controls. Homozygous variant (CC) genotype frequency was found to be significantly higher in CRC cases compared to controls. When the risk association was evaluated singly, the homozygous variant XPC CC genotype was associated with an increased risk of CRC susceptibility. Moreover, when the risk was evaluated after stratifying the study subjects into smokers and non-smokers, the combination of smoking habits and variant genotype of XPC was found to enhance the CRC susceptibility risk to 3-fold times higher. These results prompt us to suggest that genetic variation Lys939Gln in XPC gene might modify the effect of smoking and contribute to sporadic colorectal cancer etiology.

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P- Reviewer Gusella M S- Editor Wen LL L- Editor Cant MR  
E- Editor Zhang DN



## Role of international criteria in the diagnosis of autoimmune hepatitis

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Received: June 16, 2012 Revised: November 1, 2012

Accepted: November 11, 2012

Published online: June 21, 2013

### Abstract

**AIM:** To study the clinical and laboratory characteristics of autoimmune hepatitis (AIH), and compare them with International Autoimmune Hepatitis Group (IAHG) criteria.

**METHODS:** Sixty consecutive patients with AIH attended the University Clinic at Tabriz University of Medical Sciences, Iran for a 12 mo period and were assessed in a case series study. Serological and biochemical evaluations were carried out in all patients. Autoantibodies, such as antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver-kidney microsomal antibody (ALKM-1) type 1, and perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) were evaluated in these patients. A liver biopsy was performed after diagnosis of the disease. Patients were evaluated in terms of their signs and symptoms, and laboratory results and the degree to which they corresponded with the diagnostic criteria of IAHG. In this study, both a comprehensive diagnostic scoring system and a simplified diagnostic scoring system were employed for AIH.

**RESULTS:** Sixty patients, 20 male, 40 female, mean age  $39.45 \pm 17.50$  years, participated in the study. Treatment began immediately after enrolment into the study. The percent distribution of the study population into definite and probable did not change after the treatment. The most common symptoms in descending order were fatigue (100%), icter (66.7%), abdominal discomfort (33.3%), abdominal distension (28.3%), dark urine (23.3%), edema (23.3%), hematemesis (20.0%), pruritus (20.0%), melena (11.7%) and pale stool (10.0%). At the physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and an abdominal mass were found in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of patients, respectively. Hypergammaglobulinemia was detected in 95.0% of cases. ALKM-1, P-ANCA, ANA and ASMA were positive in 71.4%, 66.7%, 42.4% and 19.4% of cases, respectively. Portal hypertensive gastropathy (45.0%), esophageal varices (41.7%) and cirrhosis (40.0%) were the most prevalent complications of AIH, and there was no evidence of primary sclerosing cholangitis, ulcerative colitis and overlap syndrome in these patients. According to IAHG criteria, 80.0% of cases had a definite diagnosis, 15.0% had a probable diagnosis and 5.0% had no AIH. The percent distribution of the study population into definite, probable and no AIH did not change after using the simplified diagnostic scoring system for AIH.

**CONCLUSION:** This research showed that the majority of cases in our study were appropriately diagnosed according to the IAHG criteria and simplified scoring system. Thus, these criteria are very useful.

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**Key words:** Autoimmune hepatitis; International criteria; Diagnosis; Clinical; Paraclinical

Abdollahi MR, Somi MH, Faraji E. Role of international criteria in the diagnosis of autoimmune hepatitis. *World J Gastroenterol*

2013; 19(23): 3629-3633 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i23/3629.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3629>

## INTRODUCTION

Autoimmune hepatitis (AIH) is a chronic inflammation of the liver, the cause of which is unknown. It is characterized by the presence of interface hepatitis on histological examination, hypergammaglobulinemia, and autoantibodies<sup>[1]</sup>. Autoimmune hepatitis occurs predominantly in women and affects all ages<sup>[2]</sup>. Autoimmune hepatitis afflicts 100000 to 200000 persons in the United States<sup>[3]</sup>, and accounts for 2.6% of transplant recipients in Europe<sup>[4]</sup> and 5.9% in the United States<sup>[5]</sup>. Among Northern European Caucasians, the mean annual incidence of AIH is 1.9 per 100000 population, and its point prevalence is 16.9 per 100000 population<sup>[6]</sup>. Three types of AIH have been proposed based on immunoserologic markers<sup>[7]</sup>. The International Autoimmune Hepatitis Group (IAHG) devised and subsequently revised scoring systems to aid in the diagnosis of AIH<sup>[8,9]</sup>. The revised 1999 criteria evaluated up to 12 patient variables to derive a score which identified individuals as “not AIH”, “probable AIH” or “definite AIH”<sup>[8]</sup>. Despite a high degree of sensitivity and specificity, these criteria have proven cumbersome in day-to-day clinical practice. Subsequently, the IAHG published simplified diagnostic criteria, evaluating just four parameters<sup>[10]</sup>. This study was designed to determine a standard and reliable method for early diagnosis and close follow-up by using the IAHG simplified diagnostic criteria and comparing clinical and laboratory characteristics in Iranian AIH patients.

## MATERIALS AND METHODS

All patients who had been diagnosed with autoimmune hepatitis and had been referred to the outpatient clinic of Tabriz University of Medical Sciences from 2010 to 2011 were evaluated for clinical and laboratory parameters and compared with the diagnostic criteria of IAHG. Patient evaluation started by recording their medical history, and performing a physical examination and complete blood count. Serological and biochemical evaluation included aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, serum  $\gamma$ -globulin, serum albumin, total and direct bilirubin, erythrocyte sedimentation rate, prothrombin time, serum creatinine, triglyceride, total cholesterol and fasting blood sugar. The researchers also evaluated the autoantibodies such as antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-liver-kidney microsomal antibody (ALKM-1) type 1 and perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) by indirect immunofluorescence. A titer of  $\geq 1:40$  was considered to be positive. Chronic viral hepatitis, Wilson's disease and hemochromatosis were also assessed using

hepatitis B core antibody, hepatitis B surface antibody, hepatitis C antibody, serum ceruloplasmin, urine copper, serum iron and total iron-binding capacity. Hepatitis B surface antigen was detected by enzyme-linked immunosorbent assay (Stat Fax Awareness Technology Inc., Palm City, FL, United States) and hepatitis C virus (HCV) antibody was analyzed using a third generation enzyme linked immunosorbent assay test (Ortho-Clinical Diagnostics, Amersham, United Kingdom).

A percutaneous liver biopsy was taken from all patients for histology after diagnosis of the disease. Specimens were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin, Masson's trichrome, and reticulin. All specimens were evaluated by a single pathologist. Liver biopsies were adequate if there were at least 6 portal tracts per high-power field. A modified Hepatitis Activity Index was used to score specimens, in which necroinflammation was graded from 0 to 18 and fibrosis from 0 to 6<sup>[11]</sup>.

An abdominal ultrasound examination was performed in all patients, and liver size and echogenicity, splenomegaly, gallstones and ascites were assessed. The exclusion criteria were having viral (hepatitis B and C), metabolic (Wilson's disease, hemochromatosis), or drug-induced liver disease or overlap syndrome. Continuous variables are expressed as mean  $\pm$  SD. Statistical analysis was carried out using SPSS, version 16.0 (SPSS Inc., Chicago, IL, United States). The study protocol was approved by the Ethics Committee of the Liver and Gastrointestinal Diseases Research Center of Tabriz University of Medical Sciences, and informed consent was procured from all patients before enrolment in the study.

## RESULTS

Sixty patients with AIH were evaluated, of whom 40 (66.7%) were female. The mean age was  $39.45 \pm 17.50$  years (range, 19-75 years). Twelve patients had a familial history of liver disease (20.0%) and 8 patients had a familial history of AIH (13.3%). Four patients (6.7%) were diagnosed with other simultaneous autoimmune diseases. None of the patients had a history of hepatotoxic drug use. Patient characteristics are described in Table 1.

The most common symptoms in descending order were fatigue (100%), icterus (66.7%), abdominal discomfort (33.3%), abdominal distension (28.3%), dark urine (23.3%), edema (23.3%), hematemesis (20.0%), pruritus (20.0%), melena (11.7%) and pale stools (10.0%). At the time of physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and abdominal mass were found in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of the patients, respectively. Portal hypertensive gastropathy (45.0%), esophageal varices (41.7%) and hepatic cirrhosis (40.0%) were the most common complications in the patients.

The proportion of patients seropositive for ALKM1, P-ANCA, ANA and ASMA patients was 71.4%, 66.7%,

**Table 1 Characteristics of patients with autoimmune hepatitis**

| Parameter                    | n (%)     |
|------------------------------|-----------|
| Female                       | 40 (66.7) |
| Male                         | 20 (33.3) |
| Single                       | 22 (36.7) |
| Married                      | 38 (63.3) |
| Educated                     | 46 (76.7) |
| Uneducated                   | 14 (23.3) |
| Alcohol intake               | 3 (5)     |
| Smoking                      | 11 (18.3) |
| History of blood transfusion | 17 (28.3) |
| History of hospitalization   | 48 (80)   |

**Table 2 Serological, biochemical and histologic findings in patients with autoimmune hepatitis**

| Parameter                                    | Result          | Range     |
|--|-----------------|-----------|
| AST, IU/L                                    | 127.8 ± 108.6   | 17-812    |
| ALT, IU/L                                    | 146.0 ± 98.4    | 14-916    |
| Total bilirubin, mg/dL                       | 2.7 ± 2.2       | 0.5-8.6   |
| Direct bilirubin, mg/dL                      | 1.3 ± 1.2       | 0.05-4.3  |
| Alkaline phosphatase, IU/L                   | 499.9 ± 386.2   | 71-997    |
| White blood cell, No./mm <sup>3</sup>        | 6052.4 ± 2461.5 | 800-13500 |
| Hemoglobin, g/dL                             | 12.1 ± 3.0      | 7-17      |
| ESR  | 40.0 ± 28.7     | 3-95      |
| Platelet count (× 1000), No./mm <sup>3</sup> | 139.2 ± 91.4    | 36.4-464  |
| Prothrombin time                             | 15.8 ± 3.3      | 12.5-31   |
| Creatinine, mg/dL                            | 0.8 ± 0.3       | 0.5-2.1   |
| FBS, mg/dL                                   | 106.9 ± 48.6    | 68-302    |
| Triglyceride, mg/dL                          | 163.2 ± 109.5   | 50-506    |
| Total cholesterol, mg/dL                     | 208.1 ± 106.3   | 47-472    |
| Albumin, g/dL                                | 3.8 ± 0.7       | 2.1-5.1   |
| Grade  | 5.5 ± 2.7       |           |
| Stage  | 3.2 ± 1.5       |           |

All values are mean ± SD. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ESR: Erythrocyte sedimentation rate; FBS: Fasting blood sugar.

42.4% and 19.4% respectively. Simultaneous seropositivity for ASMA/ANA occurred in 6.7%, ANA/P-ANCA in 6.7%, ANA/ALKM1 in 5.0%, and ALKM1/ASMA and/or ANA in 3.3%. Hepatitis C virus antibody was assessed in all patients, and all were negative. There was no evidence of primary sclerosing cholangitis, ulcerative colitis or overlap syndrome in these patients.

Liver biopsy and histological assays were performed in all the patients, and all had interface hepatitis. Forty-five cases of pathology reports were descriptive and the rest showed autoimmune hepatitis. The researchers found portal fibrotic expansion (stage 1-2) in 15 patients (33.3%), bridging fibrosis (stage 3-4) in 17 patients (37.8%) and cirrhosis (stage 5-6) in 13 patients (28.8%).

Blood proteins (electrophoresis analysis) were assessed in patients whose mean level of α1 protein was 3.0 ± 1.6 g/dL (0-7.1 g/dL). The mean level of α2 protein was 10.0 ± 3.2 g/dL (0.3-16.6 g/dL). The mean level of β protein was 11.4 ± 4.4 g/dL (0.5-17.0 g/dL). The mean level of γ protein was 25.8 ± 11.4 g/dL (3.7-44.1 g/dL). Hypoalbuminemia was found in 20 (33.3%) patients. Further serological and biochemical data are shown in Table 2.

**Table 3 Autoimmune hepatitis diagnosis using simplified score and revised International Autoimmune Hepatitis Group criteria**

| AIH score      | Probable AIH | Definite AIH | No AIH    |
|----------------|--------------|--------------|-----------|
| Revised IAHG   |              |              |           |
| Pre-treatment  | 17.7 ± 1.7   | 12.8 ± 1.4   | 7.7 ± 1.5 |
| Post-treatment | 19.7 ± 1.4   | 14.8 ± 1.4   | 9.7 ± 1.5 |
| Simplified     | 7.5 ± 0.5    | 5.6 ± 0.5    | 3.3 ± 0.6 |

Data are mean scores ± SD. AIH: Autoimmune hepatitis; IAHG: International Autoimmune Hepatitis Group.

Liver and bile duct ultrasonic imaging was performed in all patients and increased echogenicity in liver was found in 36 (60.0%). Splenomegaly was found in 32 patients (53.3%). Increased liver size (hepatomegaly) was found in 12 patients (20.0%), decreased liver size in 20 (33.3%) and normal liver size in 28 (46.7%). A gallstone was found in 11 patients (18.3%) a dilated bile duct in 9 (15.0%), and ascites in 13 (21.7%).

There was a good outcome during the 1-year follow-up in 58 patients (96.7%), one patient (1.7%) had no response to treatment and one patient with complications died while the research was being conducted. The 1-year mortality rate was 1.7%.

According to the revised IAHG criteria, 80.0% of cases had a definite diagnosis of AIH, 15.0% of cases had a probable diagnosis and 5.0% of cases had no AIH. Using the simplified diagnostic scoring system for AIH, the percent distribution of the study population into definite, probable and no AIH did not change. The mean scores and standard deviations for both the revised IAHG and simplified scoring criteria are presented in Table 3.

## DISCUSSION

The researchers surveyed 60 patients with AIH. The most common symptoms were fatigue, icterus, abdominal discomfort, abdominal distension, dark urine, edema, hematemesis, pruritus, melena and pale stools. At the physical examination, splenomegaly, ascites, hepatomegaly, epigastric tenderness and abdominal mass were discerned in 50.0%, 16.7%, 13.3%, 5.0% and 3.3% of the patients, respectively.

Koay *et al*<sup>121</sup> had performed a similar study in Taiwan, and the most common clinical findings of AIH were fatigue, icterus and loss of appetite. Another study conducted by Choudhuri *et al*<sup>131</sup> in India reported icterus (55.2%), edema (44.7%), fatigue (44.7%), encephalopathy (23.6%), pruritus (23.6%), abdominal pain (23.6%), fever (21.0%), arthritis (18.4%), hepatomegaly (44.7%), splenomegaly (34.2%) and ascites (34.2%) in their clinical findings. In a study by Gupta *et al*<sup>141</sup> in India (2001), the most common manifestations were fatigue, icterus and loss of appetite.

Variable findings can be seen in the studies reported above. Nevertheless, there is a similarity between the

general findings of the present study and those studies. The characteristic laboratory findings in the patients in the present study were an increase in bilirubin and abnormalities in liver enzyme levels. The pathologic found interface hepatitis in all patients, and 71.4%, 66.7%, 42.4% and 19.4% seropositivity for ALKM1, P-ANCA, ANA and ASMA, respectively.

Koay *et al.*<sup>[12]</sup> reported abnormalities in liver tests and increased bilirubin. Patients were 98.0% positive for ANA. Zhao *et al.*<sup>[15]</sup> in China reported interface hepatitis in all their AIH cases. In a study by Choudhuri *et al.*<sup>[13]</sup>, reported positivity for ANA in 39.4%, ASMA in 63.1% and P-ANCA in 2.6%. Johnson *et al.*<sup>[9]</sup> reported ANA or ASMA in 70.0%-80.0% and ALKM1 in 3.0%-4.0%. In a study by Nezu *et al.*<sup>[16]</sup> in Japan, 34.0% of patients were positive for ANA. In another study in Japan by Omagari *et al.*<sup>[17]</sup>, 34.0% of patients were positive for ANA. In another study by Terjung *et al.*<sup>[18]</sup> in 175 patients, 81.0% were positive for P-ANCA. Pavić *et al.*<sup>[19]</sup> in Serbia had reported ANA seropositivity in 15.0%-60.0%, ASMA in 34.0%-60.0% and ALKM1 in 0-6.0%. In a study by Adams *et al.*<sup>[20]</sup> in the United States, 20.0% of patients were positive for ANA and 3.0% for ASMA.

In summary, the range of antibody positivity in the above-mentioned studies were: ANA 15.0%-98.0%, ASMA 3.0%-80.0%, ALKM1 0%-6.0% and P-ANCA 2.6%-81.0%. The ranges in the present study are mostly in the reported ranges. Although technical differences in measurement of antibodies in different centers can produce some variability in the results, these wide differences may be a sign of racial differences in patients with AIH. This issue requires more controlled studies. Also, the present study had a small sample of patients and larger studies are required in future to examine the applicability of antibody measurements.

In the present study, 95.0% of patients were diagnosed using the diagnostic criteria of the IAHG. Several studies which had been conducted around the world that insisted on using these criteria, including Heurgué *et al.*<sup>[21]</sup> in Italy, Koay *et al.*<sup>[12]</sup> in Taiwan (China), Lee *et al.*<sup>[22]</sup> in Southern Korea, Michalska *et al.*<sup>[23]</sup> in Poland, Primo *et al.*<sup>[24]</sup> in Spain, Zhao *et al.*<sup>[15]</sup> in China, McFarlane *et al.*<sup>[25]</sup> in England and Yatsuji *et al.*<sup>[26]</sup> in Japan.

In conclusion, this research showed that the majority of cases in the present study were diagnosed according to the criteria of IAHG and the simplified scoring system. There were some deficiencies in the assessment of autoantibodies; therefore, recommendations are made for controlled studies to diagnose the probable causes of these deficiencies. The results of this study conform with reports in the literature. Further studies should be carried out in similar centers.

## COMMENTS

### Background

Autoimmune hepatitis (AIH) is an unresolving inflammation of liver, the cause of which is unknown. It is characterized by the presence of interface hepatitis on histological examination, hypergammaglobulinemia, and autoantibodies.

### Research frontiers

Despite a high degree of sensitivity and specificity, the diagnostic criteria have proven cumbersome in day-to-day clinical practice. Subsequently, the International Autoimmune Hepatitis Group (IAHG) have published simplified diagnostic criteria, evaluating just four parameters.

### Innovations and breakthroughs

This study was designed to determine a standard and reliable method for early diagnosis and close follow-up by using IAHG criteria and simplified diagnostic criteria and comparing clinical and laboratory characteristics in Iranian patients.

### Applications

This research showed that the majority of cases in the present study were diagnosed according to the criteria of IAHG and simplified scoring system. There were some deficiencies in autoantibodies assessment; therefore, recommendations are made for controlled studies to diagnose the probable causes of these deficiencies.

### Peer review

The authors collected data for one of the largest AIH cohort in the Middle East and compared laboratory and histologic findings with the international AIH Score. Using the score they found a definite AIH in 80% of their patients and a probable AIH in 15%. So the authors showed that the score is useful and can be used in patients of the Middle East without limitations. Altogether the achievement of the study is to collect such a cohort in a single center. The study was well performed.

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P- Reviewer Weigand K S- Editor Gou SX  
L- Editor Cant MR E- Editor Zhang DN



## Focal autoimmune pancreatitis: Radiological characteristics help to distinguish from pancreatic cancer

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Supported by National Nature Science Foundation of China No. 30970801; National Nature Science Foundation of China, No. 81170435; the China Post-doctoral Science Foundation, No. 20100480545; and the Shanghai Leading Talent Team Construction Special Funds, No. 2011-036

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Received: February 2, 2013 Revised: April 12, 2013

Accepted: May 18, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To identify the radiological characteristics of focal autoimmune pancreatitis (f-AIP) useful for differentiation from pancreatic cancer (PC).

**METHODS:** Magnetic resonance imaging (MRI) and triple-phase computed tomography (CT) scans of 79 patients (19 with f-AIP, 30 with PC, and 30 with a normal pancreas) were evaluated retrospectively. A radiologist measured the CT attenuation of the pancreatic parenchyma, the f-AIP and PC lesions in triple phases. The mean CT attenuation values of the f-AIP lesions were compared with those of PC, and the mean CT attenuation values of pancreatic parenchyma in the three groups were compared. The diagnostic performance of

CT attenuation changes from arterial phase to hepatic phase in the differentiation between f-AIP and PC was evaluated using receiver operating characteristic (ROC) curve analysis. We also investigated the incidence of previously reported radiological findings for differentiation between f-AIP and PC.

**RESULTS:** The mean CT attenuation values of f-AIP lesions in enhanced phases were significantly higher than those of PC (arterial phase:  $60 \pm 7$  vs  $48 \pm 10$ ,  $P < 0.05$ ; pancreatic phase:  $85 \pm 6$  vs  $63 \pm 15$ ,  $P < 0.05$ ; hepatic phase:  $95 \pm 7$  vs  $63 \pm 13$ ,  $P < 0.05$ ). The mean CT attenuation values of f-AIP lesions were significantly lower than those of uninvolved pancreas and normal pancreas in the arterial and pancreatic phase of CT ( $P < 0.001$ ,  $P < 0.001$ ), with no significant difference at the hepatic phase or unenhanced scanning ( $P = 0.4$ ,  $P = 0.1$ ). When the attenuation value increase was equal or more than 28 HU this was considered diagnostic for f-AIP, and a sensitivity of 87.5%, specificity of 100% and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved. Five findings were more frequently observed in f-AIP patients: (1) sausage-shaped enlargement; (2) delayed homogeneous enhancement; (3) hypoattenuating capsule-like rim; (4) irregular narrowing of the main pancreatic duct (MPD) and/or stricture of the common bile duct (CBD); and (5) MPD upstream dilation  $\leq 5$  mm.

**CONCLUSION:** Analysis of a combination of CT and MRI findings could improve the diagnostic accuracy of differentiating f-AIP from PC.

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**Key words:** Focal autoimmune pancreatitis; Pancreatic cancer; Computer tomography; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography

**Core tip:** At present, focal autoimmune pancreatitis (f-

AIP) is still very difficult to differentiate from pancreatic cancer (PC). In this study, we compared the incidence of radiological features, investigated the differences in the triple-phase enhancement pattern of f-AIP and PC, and found that the combination analysis contributed to improve the diagnostic accuracy of f-AIP thus avoiding unnecessary surgery.

Sun GF, Zuo CJ, Shao CW, Wang JH, Zhang J. Focal autoimmune pancreatitis: Radiological characteristics help to distinguish from pancreatic cancer. *World J Gastroenterol* 2013; 19(23): 3634-3641 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3634.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3634>

## INTRODUCTION

Autoimmune pancreatitis (AIP) is a rare form of immune-mediated chronic pancreatitis (CP) due to an autoimmune mechanism and is characterized by a marked infiltration of lymphocytes and plasma cells in pancreatic tissue (lymphoplasmacytic sclerosing pancreatitis) and was first described in 1961<sup>[1-5]</sup>. It can be classified into two radiological types: the diffuse form (the most frequent, 70% of cases) and the focal form (30% of cases), which is characterized by focal swelling of the pancreas, localized narrowing of the main pancreatic duct (MPD) with an irregular wall on imaging modalities<sup>[6-8]</sup>. Focal AIP (f-AIP) can be due to a mass formation or swollen pancreas located in one or two segments of the gland. AIP has a variety of manifestations, obstructive jaundice occurs in 76% and weight loss in 35% of patients, usually in combination with pancreatic enlargement, especially with focal pancreatic enlargement on imaging, making it very difficult to differentiate from pancreatic cancer<sup>[7,9,10]</sup>.

Treatment for AIP and pancreatic cancer (PC) are completely different. Autoimmune pancreatitis is a benign disease and steroid therapy can rapidly resolve symptoms; for PC, however, surgical resection is preferred, and if necessary, PC patients might receive combined radiotherapy or chemotherapy. However, about 3%-5% of patients undergoing pancreatic resection for presumed PC in fact have AIP<sup>[11]</sup>. Kamisawa *et al*<sup>[12]</sup> reported that 7 of 37 (18.9%) AIP patients had surgery because they were misdiagnosed as having PC or bile duct cancer. In particular, it is very difficult to differentiate between f-AIP and PC. Chang *et al*<sup>[13]</sup> reported that 8 of 26 (31.8%) AIP patients had f-AIP who were frequently treated with surgery because differentiating f-AIP from PC was so difficult. It was reported that approximately 2.2%-35.2% of f-AIP patients had undergone surgery due to a presumed diagnosis of PC<sup>[12,14-16]</sup>.

Here, we report the radiological features of 19 cases of f-AIP presenting as PC to: (1) increase the awareness and recognition of f-AIP; and (2) improve the diagnostic accuracy and avoid unnecessary surgery due to misdiagnosis.

## MATERIALS AND METHODS

### Patient sample

This study was approved by the hospital ethical committees. Consecutive patients with focal autoimmune pancreatitis (f-AIP,  $n = 19$ ) were recruited between November 2009 and October 2012. Patients with AIP were diagnosed according to the Asian Diagnostic Criteria for Autoimmune Pancreatitis (2008)<sup>[17]</sup>. A diagnosis of AIP was established when the criterion of (1) narrowing of the MPD with enlargement of the pancreas, as determined by a review of an imaging study, is met together with the criterion of (2) an increase in serum markers (g-globulin, IgG, or IgG4) and/or the criterion of (3) pathology. In this study, the diagnosis of AIP was established by the presence of criteria 1 and 2 in 4 patients; criteria 1 and 3 in 3 patients; and criteria 1, 2, and 3 in 12 patients.

The f-AIP group included 19 patients (14 men and 5 women; age range, 41-75 years; mean age, 54 years) with 21 lesions who had undergone contrast-enhanced computed tomography (CE-CT) ( $n = 19$ ), CE magnetic resonance imaging (MRI) ( $n = 11$ ), and magnetic resonance cholangiopancreatography (MRCP) ( $n = 16$ ). One patient had 3 lesions which were located at the pancreatic head, body and tail.

Identification of patients with focal pancreatic carcinoma was performed by reviewing patient records between March 2012 and December 2012 obtained from the hospital's pathology database. All 30 patients (21 men, 9 women; age range, 43-79 years; mean age, 58.2 years) with pathologically (histopathological examination of the surgically resected or biopsied tumor specimen) confirmed pancreatic ductal carcinoma ( $n = 30$ ) were included in this study. All 30 patients had undergone CE-CT, 26 had undergone MRCP, and 12 had undergone CE MRI using extracellular MR contrast agents (Gd-DTPA).

Thirty patients (20 men, 10 women; age range, 41-68 years; mean age, 53 years) with normal pancreas were also recruited. They were confirmed by reviewing the radiological images and followed-up for more than 6 months. All 30 patients had undergone CE-CT.

### CE-CT

In this study, all 79 patients underwent the triple-phase pancreatic CT protocol (Brilliance 16; PHILIPS Medical System) which included an unenhanced scan followed by triple-phase contrast-enhanced scans of the abdomen. The tube voltage was 120 kV, the tube current was 250 mA, and the rotation period was 0.75 s. A total of 90 mL of IV contrast material (iohexol, Omnipaque 300, GE Healthcare) containing 300 mg/mL iodine was intravenously administered as a bolus *via* a power injector. The injection rate was 3.5 mL/s. Images were obtained during the arterial, pancreatic and hepatic phases at 20, 40 and 80 s, respectively, after contrast medium injection. The median slice thickness for contrast-enhanced images was 3 mm.

### **MRI and MRCP**

MR imaging and MR cholangiopancreatography were performed using a 1.5-T MR imaging system (AVANTO; SIEMENS Medical Systems) and a pre-contrast coil. Pre-contrast T1-weighted MR imaging [repetition time msec/echo time msec, 150 (R) 200/2.1, 4.2] with and without fat saturation and respiratory-triggered T2-weighted MR imaging (5000-8000/80-135) were performed, followed by dynamic fat-suppressed T1-weighted MR imaging (150-200/2.1) after administration of a gadolinium-based contrast agent. MRCP was performed with a single-shot fast spin-echo thick-slab technique (25000-30000/800-1000).

### **Imaging analysis**

Three board-certified abdominal radiologists (with 9, 10 and 15 years of experience) reviewed all CT and MRI images retrospectively using a picture archiving and communication system (PACS) work station (General Electric Medical System). During analysis of the CT and MRI findings, all cases were randomly intermixed. The radiologists were blinded to the patients' clinical data, official reports, radiological examinations on other dates, and histopathological findings. Decisions were made by consensus.

For each patient, the radiologists were asked to make judgments on the following signs: (1) focal pancreatic enlargement and the location of the lesion; (2) capsule-like rim of the lesion; (3) localized irregular narrowing of MPD; (4) stricture of the distal common bile duct; and (5) other associated findings such as calcification, peripancreatic lymphadenopathy and vascular invasion.

When the lesion was confirmed, CT attenuation values of the f-AIP, PC, the apparently unaffected pancreatic parenchyma, and the normal pancreas were measured by one radiologist using a workstation (Advantage Version 4.2, GE Healthcare). The CT attenuation values were measured using unenhanced images and images obtained from arterial, pancreatic and hepatic phases after contrast administration. Following the placement of a region of interest (ROI) in each segment of the pancreas (head, body and tail), CT attenuation values of the pancreatic parenchyma were measured. The mean value of the three segments was used as the CT attenuation value of the pancreatic parenchyma. In f-AIP and PC patients, ROIs were placed both above the lesion and in the unaffected segments of the pancreas. The largest possible spherical ROI was marked, ruling out the pancreatic duct and partial volume averaging from the extrapancreatic structures. The smallest ROI was approximately 3 mm in diameter when the pancreas was atrophic. Delayed enhancement of the AIP and PC lesions was defined as the change in CT attenuation of the lesion between the arterial phase and the hepatic phase. CT attenuation values of the liver and spleen were similarly measured on unenhanced images (when available) and on images obtained in the arterial, pancreatic and hepatic phases.

The MRCP images were reviewed to identify changes

in the common bile duct (CBD) and MPD. The MPD and CBD observations were classified into 1 of 3 categories: as displaying (1) normal appearance; (2) stenosis; and (3) complete obstruction (nonvisualization of the obstructed segment). The MPD upstream diameter was evaluated by a review of MRCP images. The MPD upstream diameter and presence of distal pancreatic atrophy were not evaluated in patients with lesions in the pancreatic tail, while stenosis of CBD was not evaluated for patients with lesions in the pancreatic head.

### **Statistical analysis**

Statistical analysis was performed using the Fisher's exact test to compare the frequencies of imaging findings. The inter-reader agreement was evaluated by measurement of the kappa value. The mean CT attenuation value of the lesion in patients with f-AIP was compared with that of PC. Similarly, The mean CT attenuation values of unaffected segmental pancreas in f-AIP were compared with those of PC and normal pancreas. A comparison of the mean CT attenuation values of other organs (liver and spleen) was also performed in the three groups of patients.

Statistical analyses were performed with nonparametric tests due to the nongaussian distribution of the data and smaller sample sizes involved in some comparisons of interest. Wilcoxon's rank sum test was used to compare the CT attenuation values. When comparing two groups, the KruskalWallis test was performed before Wilcoxon's rank sum test. Fisher's exact test was applied to compare frequencies of delayed enhancement of the masses and focal enlarged segments. The diagnostic performance of attenuation value increase between the arterial and hepatic phase in the differentiation between f-AIP and PC was evaluated using receiver operating characteristic (ROC) analysis. From the ROC curves, the appropriate cutoff values were determined by selecting the point at which the Yoden index (sum of sensitivity + specificity - 1) was largest. All tests were two sided, and  $P < 0.05$  was considered statistically significant. Statistical analysis was performed with SPSS software (version 18.0, SPSS).

## **RESULTS**

The imaging characteristics of f-AIP and PC identified by reviewing the CE-CT and MRI/MRCP results are summarized in Tables 1 and 2.

### **Focal pancreatic enlargement**

Of the 49 f-AIP and PC cases, the affected segments of pancreas differed in the extent of enlargement. Of the f-AIP cases, the affected sites of the pancreas were the head in 5 patients, the body in 5 patients, and the tail in 9 patients. Of the PC cases, these values were 16, 6 and 8, respectively. The sausage-shaped enlargement of the affected segments of the pancreas was observed in 11 patients with f-AIP, but not in PC cases. Atrophy of

**Table 1 Comparison of imaging findings**

| Imaging features                 | Data of assessable patients |                  | Kappa | P       |
|----------------------------------|-----------------------------|------------------|-------|---------|
|                                  | f-AIP<br>(n = 19)           | f-PC<br>(n = 30) |       |         |
| Sausage-shaped enlargement       | 0.58 (11/19)                | 0.03 (1/30)      | 0.58  | < 0.001 |
| Delayed homogeneous enhancement  | 1.00 (19/19)                | 0.1 (3/30)       | 0.88  | < 0.001 |
| Hypoattenuating capsule-like rim | 0.63 (12/19)                | 0.03 (1/30)      | 0.64  | < 0.001 |
| Distal pancreatic atrophy MPD    | 0.30 (3/10)                 | 0.95 (20/22)     | -0.55 | < 0.001 |
| Normal                           | 0 (0/19)                    | 0 (0/30)         |       | NS      |
| Irregular narrowing              | 0.58 (11/19)                | 0.06 (2/30)      | 0.05  | NS      |
| Complete obstruction             | 0.42 (8/19)                 | 0.93 (28/30)     | -0.06 | NS      |
| CBD                              |                             |                  |       |         |
| Normal                           | 0.53 (10/19)                | 0.47 (14/30)     | -0.36 | 0.027   |
| Stenosis <sup>1</sup>            | 0.29 (4/14)                 | 0 (0/14)         | 0.3   | 0.022   |
| Complete obstruction             | 0 (3/19)                    | 0.53 (16/30)     | -0.38 | < 0.01  |
| MPD upstream dilation ≤ 5 mm     | 1.00 (10/10)                | 0.14 (5/22)      | 0.66  | < 0.001 |
| Affected location of pancreas    |                             |                  |       |         |
| Head                             | 0.26 (5/19)                 | 0.53 (16/30)     | -0.08 | NS      |
| Body                             | 0.26 (5/19)                 | 0.2 (6/30)       | -0.27 | NS      |
| Tail                             | 0.47 (9/19)                 | 0.27 (8/30)      | 0.11  | NS      |
| Other findings                   |                             |                  |       |         |
| Vascular invasion                | 0 (0/19)                    | 0.13 (4/30)      | -0.17 | NS      |
| Pancreatic lymph node            | 0.16 (3/19)                 | 0.87 (26/30)     | -0.65 | < 0.001 |
| Calcification                    | 0 (1/19)                    | 0 (0/30)         |       | NS      |

Data are percentages and numbers in parentheses refer to numbers of lesions. <sup>1</sup>The stenosis of common bile duct (CBD) was not evaluated for focal autoimmune pancreatitis (f-AIP) and pancreatic cancer (PC) patients with lesions in the pancreatic head. When the Kappa value was negative, the corresponding item was not suitable for differentiation. f-AIP: Focal autoimmune pancreatitis; MPD: Main pancreatic duct; NS: Not significant.

the pancreas was observed in 3 f-AIP and 20 PC cases whose lesions were located in the head and body.

### Density or signal abnormalities

CE-CT scans showed hypoattenuated or isoattenuated lesions in the involved segments of the pancreas in 4 and 15 f-AIP cases on precontrast scans, respectively. In all f-AIP patients, the affected pancreas appeared uniformly enlarged with the absence of pancreatic clefts and with a sharp outline (Figures 1-3). AIP and PC lesions all showed decreased enhancement on arterial phase and delayed enhancement on pancreatic and hepatic phases, however, the degree of delayed enhancement in f-AIP was greater than that in PC (Figures 2 and 4, Table 2). MRI imaging showed that the lesion was isointense or slightly hypointense in T1WI and slightly hyperintense in T2WI (Figure 3A and B). The MR enhancement patterns were similar to those of CE-CT.

### CBD and MPD Abnormalities

Of the AIP lesions ( $n = 21$ ), irregular narrowing of the MPD was observed in 13 lesions (Figure 3E), and the remaining 8 lesions did not display this MPD abnormality. Bile duct dilatation was observed in 5 cases which included 4 patients who had lesions at the pancreatic head

and 1 at the tail. The range of dilation was 9-14 mm (mean, 11 mm). The CBD showed a beak-like stricture (Figure 1C). In 3 patients with lesions in the pancreatic head, the upstream MPD was slightly dilated (less than 5 mm), distal pancreatic atrophy was not observed in 14 patients with lesions in the pancreatic body and tail.

Of the PC lesions ( $n = 30$ ), no irregular narrowing of the MPD was observed. Bile duct dilatation was observed in all 8 patients with lesions located in the pancreatic head, and the distal CBD showed an abrupt interruption sign.

### Capsule-like rim

Capsule-like rims were observed in f-AIP cases ( $n = 11$ ), which were shown continuously or discontinuously as hypodense peripancreatic strands on the precontrast CT and delayed enhanced on the delayed phase of dynamic CT (Figure 2). On MR imaging, the capsule-like rim appeared as a T1WI isointense and T2WI hypointense area surrounding the pancreas (Figure 3A and B).

### Other associated findings

Other associated findings included calcification (f-AIP,  $n = 1$ ; PC,  $n = 0$ ), peripancreatic lymphadenopathy (f-AIP,  $n = 3$ ; PC,  $n = 26$ ) and vascular invasion (f-AIP,  $n = 0$ ; PC,  $n = 4$ ).

### Comparison of CT attenuation values

The results of the mean CT attenuation values of the f-AIP lesions and uninvolved segments in f-AIP patients and normal pancreas are shown in Table 2. The mean CT attenuation values of f-AIP lesions in enhanced phases were significantly higher than those of PC ( $P < 0.05$ ,  $P < 0.05$ ,  $P < 0.05$ ) (Figure 5). The mean CT attenuation values of the f-AIP lesions were significantly lower than those of uninvolved pancreas and normal pancreas in the arterial and pancreatic phase of CT ( $P < 0.001$ ,  $P < 0.001$ ), however, there were no significant differences in the hepatic phase or unenhanced scanning ( $P = 0.4$ ,  $P = 0.1$ ). The mean CT attenuation values of normal and unaffected pancreatic parenchyma in the three groups showed no significant differences in the arterial, pancreatic, and hepatic phases ( $P = 0.1$ ,  $P = 0.8$ ,  $P = 0.2$ ). The mean CT attenuation values of the liver and spleen were not significantly different between the three groups. When the attenuation value increase was equal or more than 28 HU this was considered diagnostic for f-AIP, and a sensitivity of 87.5%, specificity of 100% and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved (Figure 6).

## DISCUSSION

Although the diagnosis of AIP has improved due to a growing awareness of the condition and proposed diagnostic criteria<sup>[18]</sup>, there is no practical strategy to differentiate PC from f-AIP. One must distinguish between the two disorders to prevent unnecessary surgery or delayed



Figure 1 A 55-year-old woman with focal autoimmune pancreatitis of the pancreatic head. A, B: Contrast-enhanced computed tomography images obtained during enhanced phases showing enhanced thickening of the common bile duct (CBD) wall (A, black arrow), narrowing of the main pancreatic duct (MPD) and distal CBD (B, black arrows); C: Bile duct dilation and irregular narrowing of the MPD in the pancreatic head can be observed on the magnetic resonance cholangiopancreatography image (white arrows).



Figure 2 A 59-year-old man with focal autoimmune pancreatitis of the pancreatic tail. Contrast-enhanced computed tomography image obtained during the arterial phase (A, white arrow) showing that a hypoattenuating capsule-like rim can be observed around the pancreatic tail, which manifested delayed enhancement during the pancreatic phase (B, white arrow) and hepatic phase (C, white arrow). Attenuation values of the autoimmune pancreatitis lesion were 36, 51, 76 and 89 HU during the pre-contrast and enhanced phases, respectively.

| Table 2 Mean computed tomography attenuation values |    |                     |                      |                     |                       |                    |
|---|----|---------------------|----------------------|---------------------|-----------------------|--------------------|
| Group   | n  | Condition           | Unenhanced scan (HU) | Arterial phase (HU) | Pancreatic phase (HU) | Hepatic phase (HU) |
| f-AIP   | 19 | Lesions             | 36 ± 4               | 60 ± 7              | 85 ± 6                | 95 ± 7             |
|   |    | Uninvolved pancreas | 42 ± 5               | 83 ± 10             | 105 ± 14              | 95 ± 7             |
|   |    | Liver               | 57 ± 4               | 68 ± 8              | 119 ± 15              | 100 ± 10           |
|   |    | Spleen              | 48 ± 3               | 111 ± 15            | 129 ± 15              | 96 ± 9             |
| PC  | 30 | Lesion              | 34 ± 5               | 48 ± 10             | 63 ± 15               | 63 ± 13            |
|   |    | Uninvolved pancreas | 39 ± 6               | 76 ± 14             | 103 ± 15              | 87 ± 12            |
|   |    | Liver               | 58 ± 6               | 69 ± 9              | 108 ± 17              | 102 ± 10           |
|   |    | Spleen              | 46 ± 4               | 102 ± 19            | 141 ± 18              | 101 ± 7            |
| Normal  | 30 | Pancreas            | 44 ± 7               | 87 ± 12             | 105 ± 20              | 93 ± 10            |
|   |    | Liver               | 57 ± 7               | 65 ± 8              | 110 ± 18              | 99 ± 13            |
|   |    | Spleen              | 47 ± 4               | 107 ± 17            | 133 ± 23              | 99 ± 11            |

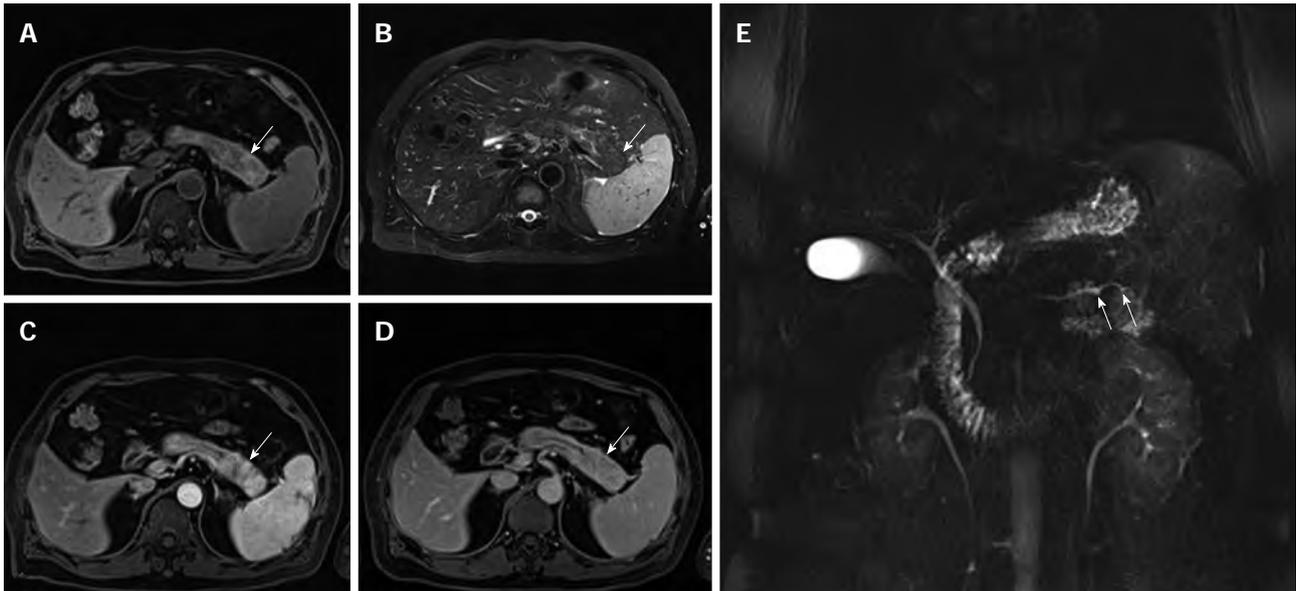
Data are computed tomography attenuation values (mean ± SD). f-AIP: Focal autoimmune pancreatitis; PC: pancreatic cancer.

initiation of corticosteroid therapy. A review of the CE-CT and MRI data indicated five imaging features of AIP: (1) delayed homogeneous enhancement; (2) hypoattenuating capsule-like rim; (3) the absence of distal pancreatic atrophy; (4) irregular narrowing of the MPD; and (5) stenosis of the CBD in patients with lesions in the body or/and tail. The analysis also indicated that those imaging features could be used to differentiate AIP from PC with high accuracy.

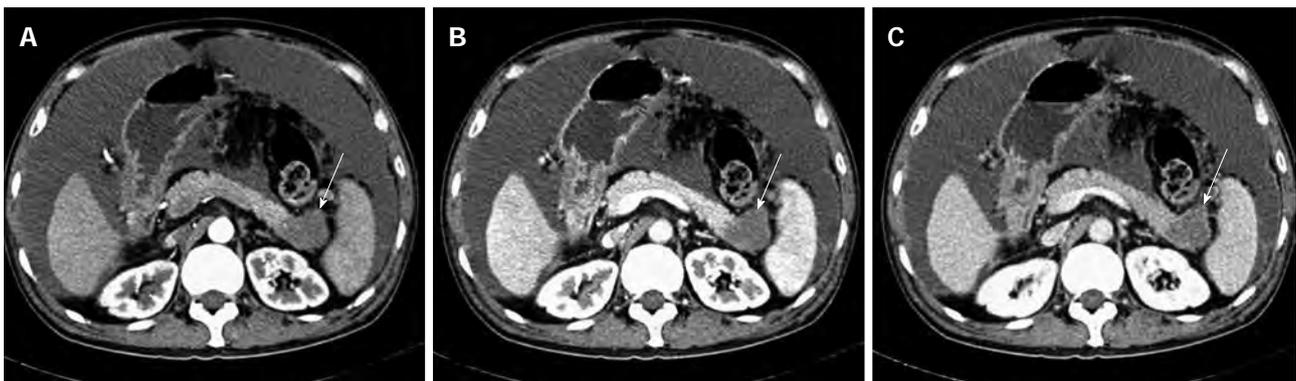
Some studies have reported that the head of the pan-

creas is involved in most AIP cases<sup>[19,20]</sup>, which is consistent with the study by Woo Ik Chang who showed that the affected site was the pancreatic head in 5 (62.5%) of 8 patients<sup>[13]</sup>. However, in our study, the affected site was the pancreatic head in only 4 patients (21.1%). This difference may be due to the following reasons: (1) the pancreatic head may not be the most commonly involved site in f-AIP; and (2) the number of patients studied may have been too small.

In our study, we identified the homogeneous good



**Figure 3** A 53-year-old man with focal autoimmune pancreatitis of the pancreatic tail. A-D: The lesion showed heterogenous T1WI hypointensity and heterogenous T2WI hyperintensity, which was delayed enhanced. The capsule-like rim appearing as a T1WI iso- or slight hyperintensity and a T2WI hypointensity area surrounding the pancreas (white arrow), which were delayed and moderately enhanced; E: Magnetic resonance cholangiopancreatography image shows the irregular narrowing main pancreatic duct in the pancreatic tail (white arrows).



**Figure 4** A 54-year-old man with pancreatic cancer of the pancreatic tail. Triple-phase computed tomography images show irregular enlargement of the pancreatic tail. Enhancement of the lesion is decreased (A, 39 HU) during arterial phase, and slightly increased enhancement during pancreatic phase (B, 48 HU) and hepatic phase (C, 62 HU), respectively (white arrows).

enhancement during the delayed phases as useful in differentiating between f-AIP and PC. Similarly, in the study by Kamisawa<sup>[12]</sup>, delayed enhancement was observed in 17 of 17 (100%) AIP patients, while Wakabayashi *et al*<sup>[20]</sup> described delayed homogeneous enhancement in 9 of 9 (100%) AIP patients. In addition, Chang *et al*<sup>[13]</sup> described homogeneous enhancement during the hepatic phase in six of seven AIP patients. The consistency of delayed homogenous enhancement in this and previous studies shows one of the common imaging features of AIP, but the absence of a further quantitative analysis.

We found that the attenuation values of AIP were significantly higher than those of PC in the enhanced phases, lower than those of unaffected pancreatic parenchyma in the arterial and pancreatic phase, however, no significant differences were observed in the hepatic phase ( $95 \pm 7$  HU *vs*  $95 \pm 7$  HU, 75 s). Takahashi found

significantly higher CT attenuation values for AIP ( $90 \pm 19$  HU) than for pancreatic carcinoma ( $64 \pm 19$  HU) during the hepatic phase (60-70 s)<sup>[21]</sup>. They also found greater enhancement during the pancreatic phase ( $71 \pm 22$  HU *vs*  $59 \pm 20$  HU), although the difference between the two groups was not statistically significant<sup>[10]</sup>. The difference between the CT attenuation values obtained in this study and those by Takahashi may be related to differences in the timing of scan acquisition, contrast injection rates and CT scanners.

Irregular narrowing of the MPD is one of the most important features of AIP. In our study, 79% of AIP patients (15 of 19) showed segmental irregular narrowing of the MPD, while the number was 6% (2 of 30) in PC patients. Moreover, no or minimal upstream dilatation can also be helpful to differentiate AIP from PC<sup>[10,22]</sup>. In the current study, upstream MPD dilatation ( $\geq 5$  mm)

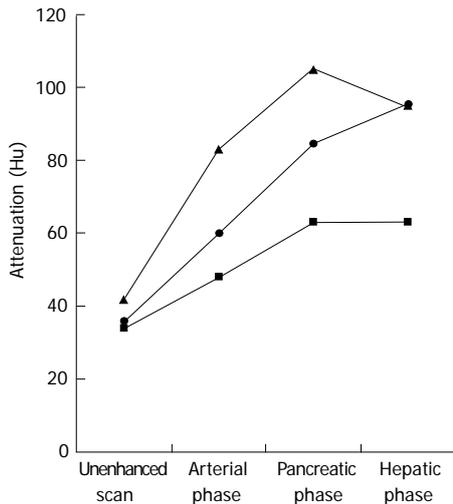


Figure 5 Graph shows mean computed tomography attenuation values of normal pancreatic parenchyma (triangle) and lesions in patients with focal autoimmune pancreatitis (circle) and pancreatic cancer (square) in relation to phase of contrast enhancement.

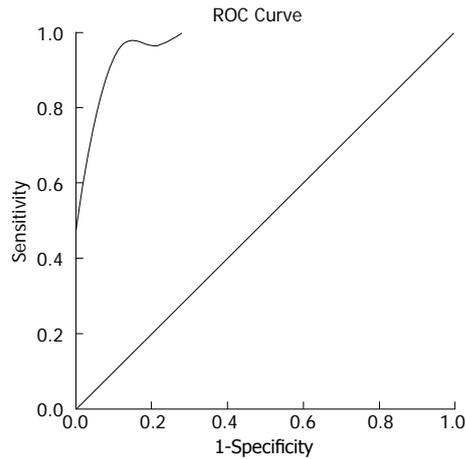


Figure 6 Receiver operating characteristic curve analysis result. Receiver operating characteristic (ROC) curve analysis revealed that when values were more than or equal 28 HU they were considered diagnostic for focal autoimmune pancreatitis, and a sensitivity of 87.5%, a specificity of 100%, and an area under the ROC curve of 0.974 (95%CI: 0.928-1.021) were achieved.

was observed in only 5% of patients (1 of 19), while the number was 86% in PC patients. Some studies reported that stricture of the distal CBD was often observed in AIP, and this occurred due to the combined effect of extrinsic compression by the inflamed pancreatic head and inflammatory changes in the CBD<sup>[10]</sup>. The f-AIP patients showed a smooth pattern, whereas the patients with PC showed an irregular pattern. In our study, CBD stricture of smooth pattern was seen in 8 of 19 patients, that is 4 of 5 patients with pancreatic head lesions and four with pancreatic tail lesions. Therefore, we suggest that the inflammatory changes in the CBD and AIP may be independent or have occurred one after another, and this might be useful for the diagnosis of f-AIP.

In the current study, the small number of patients may not represent all the characteristics of f-AIP according to radiological features only. Thus, we tried to identify radiological findings to diagnosis f-AIP, however, these findings were insufficient to exclude PC. Therefore, we recommend that IgG4 should be tested and histological findings should be obtained where possible to discriminate f-AIP from PC. We believe that future studies with more patients will clarify and validate the radiological features of f-AIP.

In conclusion, this study found that the analysis of a combination of imaging findings, either of delayed enhancement with more than 28 HU, or of three imaging findings (1) focal pancreatic enlargement with a capsule-like rim; (2) irregular narrowing of the MPD; and (3) stricture of the CBD in patients with lesions (not located in the pancreatic head) contribute to improve the diagnostic accuracy of f-AIP thus avoiding unnecessary surgery.

**COMMENTS**  
**Background**  
 Autoimmune pancreatitis (AIP), a rare form of chronic pancreatitis (CP), can

be classified as the diffuse form and the focal form. Although AIP is well-known among radiologists, focal AIP (f-AIP) is still very difficult to differentiate from pancreatic cancer (PC).

**Research frontiers**

f-AIP has a variety of manifestations and is very difficult to differentiate from PC. Improving the diagnostic accuracy can help avoid unnecessary surgery in f-AIP patients.

**Related publications**

The analysis of combined imaging with computed tomography and magnetic resonance to improve the diagnostic accuracy of differentiating f-AIP from PC has rarely been reported.

**Innovations and breakthroughs**

This is the first study to report that the triple-phase enhancement pattern of f-AIP is different from that of PC. Three imaging features are more frequently found in f-AIP, including focal pancreatic enlargement with a capsule-like rim, irregular narrowing of the main pancreatic duct and stricture of the common bile duct in patients with lesions not located in the pancreatic head. The combination of these findings could further improve the diagnostic accuracy of f-AIP and avoid unnecessary surgery.

**Applications**

The study results suggest that a combination of imaging findings will help improve the diagnostic accuracy of f-AIP and avoid unnecessary surgery.

**Peer review**

It is a good clinical study in which the authors analyzed the radiological characteristics of f-AIP. The results are interesting and suggest that the combination of triple-phase enhancement pattern and imaging features of f-AIP can help for the differential diagnosis of f-AIP from PC.

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P-Reviewer Shigeru BHK S-Editor Zhai HH

L-Editor Webster JR E-Editor Lu YJ



## Fast-track surgery could improve postoperative recovery in radical total gastrectomy patients

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Author contributions: Feng F, Ji G and Li JP contributed equally to this work; Feng F and Zhao QC designed the study and wrote the manuscript; Ji G, Li JP and Liu XN performed all the operations; Li XH and Shi H were mainly in charge of perioperative management of patients; Zhao ZW and Wu GS were mainly in charge of evaluating postoperative outcomes, discharge, follow-up and data analysis.

Supported by National Natural Scientific Foundation of China, No. 31100643

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Received: December 25, 2012 Revised: March 20, 2013

Accepted: April 27, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To assess the impact of fast-track surgery (FTS) on hospital stay, cost of hospitalization and complications after radical total gastrectomy.

**METHODS:** A randomized, controlled clinical trial was conducted from November 2011 to August 2012 in the Department of Digestive Surgery, Xijing Hospital of Digestive Diseases, the Fourth Military Medical University. A total of 122 gastric cancer patients who met the selection criteria were randomized into FTS and conventional care groups on the first day of hospitalization. All patients received elective standard D2 total gastrectomy. Clinical outcomes, including duration of flatus and defecation, white blood cell count, postoperative pain, duration of postoperative stay, cost of hospitalization and complications were recorded and evaluated.

Two specially trained doctors who were blinded to the treatment were in charge of evaluating postoperative outcomes, discharge and follow-up.

**RESULTS:** A total of 119 patients finished the study, including 60 patients in the conventional care group and 59 patients in the FTS group. Two patients were excluded from the FTS group due to withdrawal of consent. One patient was excluded from the conventional care group because of a non-resectable tumor. Compared with the conventional group, FTS shortened the duration of flatus ( $79.03 \pm 20.26$  h vs  $60.97 \pm 24.40$  h,  $P = 0.000$ ) and duration of defecation ( $93.03 \pm 27.95$  h vs  $68.00 \pm 25.42$  h,  $P = 0.000$ ), accelerated the decrease in white blood cell count [ $P < 0.05$  on postoperative day (POD) 3 and 4], alleviated pain in patients after surgery ( $P < 0.05$  on POD 1, 2 and 3), reduced complications ( $P < 0.05$ ), shortened the duration of postoperative stay ( $7.10 \pm 2.13$  d vs  $5.68 \pm 1.22$  d,  $P = 0.000$ ), reduced the cost of hospitalization ( $43783.25 \pm 8102.36$  RMB vs  $39597.62 \pm 7529.98$  RMB,  $P = 0.005$ ), and promoted recovery of patients.

**CONCLUSION:** FTS could be safely applied in radical total gastrectomy to accelerate clinical recovery of gastric cancer patients.

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**Key words:** Fast-track surgery; Gastric cancer; Radical total gastrectomy; Perioperative care; Outcomes

**Core tip:** Fast-track surgery (FTS) is a promising program for surgical patients, and has been applied in several surgical diseases. The value of FTS in radical distal gastrectomy has been demonstrated recently, but the safety and efficacy of FTS for radical total gastrectomy requires further evaluation. The present study showed that FTS was feasible for perioperative care in radical total gastrectomy. Compared with conventional care, FTS could shorten the duration of flatus and defeca-

tion, accelerate the decrease in white blood cell count, decrease postoperative complications, shorten the postoperative stay, reduce the cost of hospitalization, and promote postoperative recovery of patients.

Feng F, Ji G, Li JP, Li XH, Shi H, Zhao ZW, Wu GS, Liu XN, Zhao QC. Fast-track surgery could improve postoperative recovery in radical total gastrectomy patients. *World J Gastroenterol* 2013; 19(23): 3642-3648 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i23/3642.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3642>

## INTRODUCTION

Fast-track surgery (FTS) was initiated by the Danish surgeon H Kehlet in the field of elective colorectal surgery in the 1990s<sup>[1,2]</sup>, and has rapidly gained popularity around the world because of its significant benefits and safety<sup>[3]</sup>. The core elements of FTS include: epidural or regional anesthesia, perioperative fluid management, minimally invasive techniques, optimal pain control, early initiation of oral feeding and early mobilization<sup>[4]</sup>. The combination of these approaches has led to a significant reduction in complication rates, morbidity and mortality rates, duration of hospital stay and costs of hospitalization, and finally, greatly improved postoperative recovery<sup>[5-7]</sup>. In recent years, FTS has been applied in several surgical diseases, include radical prostatectomy<sup>[8]</sup>, cardiac surgery<sup>[9]</sup>, total knee replacement<sup>[10]</sup>, cesarean section<sup>[11]</sup>, coronary artery bypass grafting<sup>[12]</sup>, it has also been used for specific procedures in children<sup>[13]</sup> and the elderly<sup>[14]</sup>.

Gastric cancer is the fourth most common cancer worldwide but the second leading cause of cancer mortality<sup>[15]</sup>, and it is more common in men and in developing countries. Up to now, surgery has been the most common treatment. For radical gastrectomy, conventional elective gastric resection and perioperative care are associated with a morbidity of 20%-46%, a mortality of 0.8%-10%<sup>[16]</sup> and a postoperative hospital stay of 8-13 d<sup>[17]</sup>. The high rate of complications leads to prolonged duration of hospital stay and increased costs of hospitalization.

The value of FTS in radical distal gastrectomy has been demonstrated recently<sup>[18,19]</sup>, but the safety and efficacy of FTS in radical total gastrectomy still requires further evaluation. Therefore, we performed a slightly modified fast-track protocol in gastric cancer patients in our department. We evaluated the feasibility and safety of FTS in gastric cancer patients through a prospective, randomized comparative study.

## MATERIALS AND METHODS

### Patients

This study was performed in Xijing Hospital of Digestive Diseases affiliated to the Fourth Military Medical

University from November 2011 to August 2012. Selection criteria were: (1) diagnosis of gastric cancer based on clinical symptoms, imaging and pathology; (2) age between 18 and 75 years; (3) no preoperative radiotherapy or chemotherapy; (4) no distant metastasis; (5) no history of primary diabetes mellitus, bowel obstruction, severe cardiopulmonary diseases, and immune related diseases; (6) no pregnancy or breast feeding; (7) an American Society of Anesthesiologists (ASA) score of I or II; (8) undergoing elective standard D2 total gastrectomy; and (9) written informed consent was obtained from the patient and the family. Gastric cancer patients meeting the selection criteria were randomly divided into a FTS group and a conventional care group immediately after admission. The sample size of 122 patients (61 cases in each group) was calculated with an alpha level of 0.05 and 90% power for primary endpoints.

This study was approved by the Ethics Committee of Xijing Hospital. This study was registered under *chictr.org*, identifier number ChiCTR-TRC-11001440.

### Randomization and implementation

All the patients were clearly informed about the aims and details of the present study and signed consent forms. Random numbers were generated by computer. Eligible patients were randomly assigned in a 1:1 ratio. The specially trained investigator prepared allocation envelopes for the doctors of the enrolled patients. The investigator did not contact the patients throughout the clinical trial. The doctors and nurses administering the interventions and collecting the data had no role in the randomization process. Two specially trained doctors who were blinded to the treatment were in charge of evaluating postoperative outcomes, discharge and follow-up.

### Interventions

The patients were admitted to the hospital 1-2 d before surgery. A slightly modified fast-track protocol proposed by Kehlet *et al.*<sup>[20]</sup> was used in the present FTS group. Patients in the conventional surgery group received conventional perioperative care. Details of the interventions are listed in Table 1. Both groups were protocol-driven, with appropriate protocol details for patients, surgeons and nurses to ensure compliance.

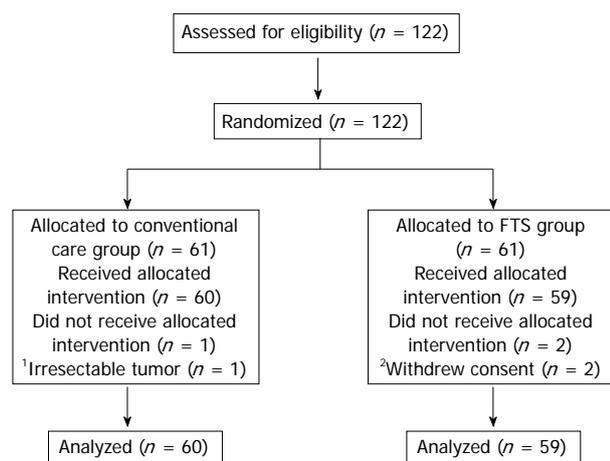
### Discharge criteria and readmission

Patients were considered dischargeable postoperatively if they met the following criteria: normal body temperature, pain controlled with oral analgesics, normal mobilization, no discomfort, normal oral diet, no parenteral nutrition, normal gastrointestinal function (normal flatus and defecation), Karnofsky Performance Status Scale score exceeding 80, and willing to go home.

After discharge, the patients were followed up by our specially trained surgeons through telephone within the first 24 h and once per week for 4 wk, and the patients could also contact us if they had any discomfort. The patients were readmitted if any of the following occurred:

**Table 1 Comparison of fast-track surgery and conventional perioperative intervention protocols**

| Perioperative intervention            | Conventional  | Fast-track surgery   |
|---------------------------------------|---|--|
| Diet before surgery                   | No intake of food and drink after supper the day before surgery   | Intake of 1000 mL 14% carbohydrate drink 12 h before and 350 mL 14% carbohydrate drink 3 h before surgery.   |
| Anesthesia                            | Tracheal intubation and general anesthesia  | Tracheal intubation and general anesthesia   |
| Thermal insulation during operation   | No thermal insulation, room temperature was maintained at 22 °C   | Thermal insulation of the body and extremities, body temperature was maintained at 36 °C   |
| Operation procedure                   | Standard laparotomy approach  | Standard laparotomy approach   |
| Placement of abdominal drainage       | Use of abdominal drainage tube  | No routine use of abdominal drainage tube  |
| Analgesia after operation             | Standard use of patient-controlled analgesic pump   | Infiltration of surgical wounds with ropivacaine at the end of surgery and 24 h after surgery. Oral intake of 200 mg celecoxib twice daily   |
| Mobilization after operation          | Mobilize out of bed on patients' own request  | Encourage patients to mobilize out of bed  |
| Diet after operation                  | Oral intake initiated after flatus (following a stepwise plan from water to other liquids to semi-fluids to normal food)  | Oral intake of 500-1000 mL glucose saline on the day of surgery. Intake of 2000-3000 mL liquid food containing 1000 kcal to 1200 kcal per day from the 1st day after surgery                   |
| Intravenous nutrition after operation | Infusion of glucose saline and amino acid injection <i>iv</i> on the day of surgery. Infusion of parenteral nutrition (25 kcal/kg of body weight) <i>iv</i> before oral intake. Appropriate level of <i>iv</i> fluid intake based on the volume of liquid intake and output, and physiological need | Infusion of parenteral nutrition <i>iv</i> if oral intake is not adequate. Appropriate level of <i>iv</i> fluid intake based on the volume of liquid intake and output, and physiological need |
| Removal of nasogastric tube           | Removal of nasogastric tube after flatus  | Removal of nasogastric tube within 24 h after surgery  |
| Removal of urine catheter             | Removal of urine catheter on the 3 <sup>rd</sup> or 4 <sup>th</sup> day after surgery   | Removal of urine catheter within 24 h after surgery  |
| Antibiotics                           | Standard use of antibiotics for 3 d after surgery   | Standard use of antibiotics before and once after surgery  |



**Figure 1** Flow diagram of the randomized control trial designed to compare the safety and efficacy of fast-track surgery and conventional care groups. <sup>1</sup>One patient had an irresectable tumor in the conventional care group; <sup>2</sup>Two patients withdrew consent in the fast-track surgery (FTS) group. All three patients were excluded from the analysis.

hyperpyrexia, abdominal pain, bowel obstruction, gastrointestinal hemorrhage, malnutrition, infection and poor healing of the wound.

### Data collection

The primary clinical endpoints were the duration of hospital stay and the cost of hospitalization. The second clinical endpoints were incidence of complications such as pneumonia, surgical site infection, abdominal infection, anastomotic leak, and bowel obstruction. We recorded preoperative data on age, sex, body mass index (BMI), nutritional risk screening (NRS) 2002 score, ASA

score, differentiation status, TNM classification, white blood cell (WBC) count, hemoglobin, albumin, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Surgical-related data such as operation time and blood loss were also recorded. Postoperative data such as timing of first flatus and defecation, duration of hospital stay, the cost of hospitalization and complications were recorded. WBC was measured from postoperative day (POD) 1 to POD 5. Pain intensity was evaluated from POD 1 to POD 5 using a visual analog scale (VAS).

### Statistical analysis

Data were processed using SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, United States). Numerical variables were expressed as the mean  $\pm$  SD unless otherwise stated. Differences between the two groups were tested using a two-tailed Student *t* test. Discrete variables were analyzed using the  $\chi^2$  test or Fisher's exact test. A *P* value < 0.05 was considered statistically significant.

## RESULTS

### Clinical characteristics

A total of 119 patients finished the study, including 60 patients in the conventional care group and 59 patients in the FTS group. Two patients were excluded from the FTS group after withdrawing consent. One patient was excluded from the conventional care group because of an irresectable tumor (Figure 1). The preoperative baseline characteristics of the two groups are compared in Table 2. There were no significant differences between the two groups in age, sex, BMI, NRS 2002 score, ASA score, differentiation status, TNM classification, WBC count, he-

**Table 2** Comparison of baseline characteristics of the two groups (mean  $\pm$  SD)

| Characteristics           | Conventional        | Fast-track surgery  | P value |
|---------------------------|---------------------|---------------------|---------|
| Age, yr                   | 55.79 $\pm$ 10.06   | 54.98 $\pm$ 11.35   | 0.682   |
| Sex                       |                     |                     | 0.689   |
| Male/female               | 44/16               | 41/18               |         |
| BMI                       | 21.01 $\pm$ 1.78    | 22.44 $\pm$ 3.51    | 0.061   |
| NRS 2002 score            | 0.81 $\pm$ 1.10     | 1.08 $\pm$ 1.41     | 0.424   |
| ASA score                 |                     |                     | 0.364   |
| I / II                    | 1/59                | 3/56                |         |
| Differentiation status    |                     |                     | 0.857   |
| Well differentiated       | 6                   | 4                   |         |
| Moderately differentiated | 20                  | 17                  |         |
| Poorly differentiated     | 34                  | 38                  |         |
| TNM classification        |                     |                     | 0.324   |
| I / II / III              | 8/31/2021           | 14/12/33            |         |
| White blood cell          | 6.20 $\pm$ 1.74     | 6.05 $\pm$ 2.08     | 0.671   |
| Hemoglobin, g/L           | 133.36 $\pm$ 22.03  | 130.65 $\pm$ 22.41  | 0.52    |
| Albumin, g/L              | 44.42 $\pm$ 4.89    | 42.83 $\pm$ 4.65    | 0.082   |
| ALT                       | 17.91 $\pm$ 11.35   | 21.29 $\pm$ 15.55   | 0.195   |
| AST                       | 21.84 $\pm$ 11.46   | 25.83 $\pm$ 17.00   | 0.151   |
| Operation time, min       | 242.38 $\pm$ 72.89  | 226.11 $\pm$ 65.87  | 0.214   |
| Blood loss, mL            | 221.17 $\pm$ 122.55 | 230.55 $\pm$ 171.82 | 0.735   |

BMI: Body mass index; ASA: American Society of Anesthesiologists; NRS: Nutritional risk screening; TNM: Tumor node metastases; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

**Table 3** Comparison postoperative pain intensity and white blood cell count between the two groups (mean  $\pm$  SD)

| Time                         | Conventional     | Fast-track surgery | P value |
|------------------------------|------------------|--------------------|---------|
| Postoperative pain intensity |                  |                    |         |
| POD 1                        | 5.41 $\pm$ 1.45  | 4.32 $\pm$ 1.65    | 0.000   |
| POD 2                        | 4.43 $\pm$ 1.54  | 3.39 $\pm$ 1.65    | 0.001   |
| POD 3                        | 3.63 $\pm$ 1.48  | 2.76 $\pm$ 1.36    | 0.002   |
| POD 4                        | 3.02 $\pm$ 1.45  | 2.51 $\pm$ 1.87    | 0.119   |
| POD 5                        | 2.21 $\pm$ 1.39  | 2.30 $\pm$ 1.56    | 0.789   |
| White blood cell count       |                  |                    |         |
| POD 1                        | 14.81 $\pm$ 5.34 | 14.55 $\pm$ 5.04   | 0.793   |
| POD 2                        | 15.36 $\pm$ 5.36 | 12.26 $\pm$ 4.78   | 0.002   |
| POD 3                        | 11.80 $\pm$ 4.80 | 9.35 $\pm$ 3.83    | 0.005   |
| POD 4                        | 8.56 $\pm$ 3.70  | 7.52 $\pm$ 3.57    | 0.223   |
| POD 5                        | 6.37 $\pm$ 2.34  | 6.91 $\pm$ 3.34    | 0.684   |

POD: Postoperative day.

hemoglobin, albumin, ALT, AST, operation time and blood loss (all  $P > 0.05$ ).

### Pain intensity

Pain intensity was evaluated from POD 1 to POD 5 in the two groups (Table 3). VAS analysis showed that pain intensity of patients in the FTS group was significantly lower than that of patients in the conventional care group on POD 1-3 ( $P < 0.05$ ).

### White blood cell count

The WBC counts of patients in the two groups were measured in the morning of POD 1 to POD 5 (Table 3). The WBC count in the conventional care group and FTS group were both elevated on POD 1. Although the WBC count in the conventional care group continued to rise on

**Table 4** Comparison clinical outcomes and postoperative complications between the two groups

|                              | Conventional           | Fast-track surgery     | P value |
|------------------------------|------------------------|------------------------|---------|
| Clinical outcomes            |                        |                        |         |
| First flatus, h              | 79.03 $\pm$ 20.26      | 60.97 $\pm$ 24.40      | 0.000   |
| First defecation, h          | 93.03 $\pm$ 27.95      | 68.00 $\pm$ 25.42      | 0.000   |
| Postoperative stay, d        | 7.10 $\pm$ 2.13        | 5.68 $\pm$ 1.22        | 0.000   |
| Cost of hospitalization, RMB | 43783.25 $\pm$ 8102.36 | 39597.62 $\pm$ 7529.98 | 0.005   |
| Postoperative complications  |                        |                        |         |
| Total cases                  | 17                     | 6                      | 0.019   |
| Pneumonia                    | 10                     | 5                      | 0.269   |
| Incision infection           | 3                      | 1                      | 0.619   |
| Urinary infection            | 1                      | 0                      | 1.000   |
| Abdominal infection          | 1                      | 0                      | 1.000   |
| Gastric retention            | 0                      | 0                      |         |
| Anastomotic leak             | 0                      | 0                      |         |
| Deep-vein thrombosis         | 0                      | 0                      |         |
| Ileus                        | 1                      | 0                      | 1.000   |
| Reoperation                  | 1                      | 0                      | 1.000   |
| Readmission                  | 0                      | 0                      |         |
| Mortality                    | 0                      | 0                      |         |

POD 2, the WBC count in the FTS group began to drop ( $P < 0.05$ ). The WBC count in the conventional care group began to drop on POD 3, but was significantly higher than in the FTS group ( $P < 0.05$ ).

### Outcomes

The outcomes were summarized in Table 4. Compared with the conventional care group, the patients in the FTS group showed significantly accelerated recovery of gastrointestinal function in terms of time to first flatus and first defecation ( $P < 0.05$ ). The duration of postoperative stay of the FTS group was significantly shorter than that of the conventional care group ( $P < 0.05$ ) and the cost of hospitalization was also significantly lower ( $P < 0.05$ ).

### Complications and readmissions

Table 4 summarizes the complications and readmissions in each group. The overall complication rate in the FTS group (10.17%) was significantly lower than in the conventional group (28.33%,  $P = 0.019$ ). In the conventional care group, 10 patients suffered from pneumonia, 3 patients suffered from incision infection, 1 patient experienced urinary infection, 1 patient experienced abdominal infection, and 1 patient underwent reoperation because of ileus. In the FTS group, 5 patients suffered from pneumonia and 1 experienced incision infection. All the patients were cured by surgery or conservative treatment.

## DISCUSSION

The aim of the present study was to evaluate the safety, efficacy and outcome of FTS protocol employed in the perioperative treatment of gastric cancer in comparison with conventional perioperative treatment. The data of the present study showed that the FTS protocol was feasible for perioperative care of gastric cancer patients who underwent radical total gastrectomy. Compared with

conventional care, FTS could shorten the duration of flatus and defecation, accelerate the decrease in WBC, decrease postoperative complications, shorten the duration of postoperative stay, reduce the cost of hospitalization, and eventually promote postoperative recovery of the patients.

Optimal pain control is very important. Pain can not only result in stress<sup>[21]</sup>, but also affects the mobilization of patients after surgery. Early mobility or activity is recognized as a critical step in fast-track care. Bed rest not only increases muscle loss and insulin resistance, but also decreases pulmonary function and supply of oxygen to tissues<sup>[22]</sup>. It has been reported that opioids may result in nausea, vomiting and fatigue that counteract the benefits of FTS<sup>[23]</sup>. Therefore, routine use of opioids was avoided in the FTS group. In our present study, the infiltration of surgical wounds with ropivacaine and oral intake of celecoxib were applied instead of a patient-controlled analgesia pump. Pain intensity was evaluated from POD 1 to POD 5 after surgery using the VAS. The results showed that VAS in the FTS group was significantly lower than that of conventional care group. This indicated that ropivacaine combined with celecoxib had a better analgesic effect than an analgesic pump, and the better analgesic effect in the FTS group ensured a longer duration of mobilization out of bed.

Conventionally, the duration of antibiotic use is 2-3 d after gastrectomy. In the present study, the antibiotics were only applied before and after surgery in the FTS group (Table 1). We noticed that even with shorter use of antibiotics in the FTS group, the WBC decreased earlier and faster than in the conventional postoperative care group.

Nasogastric tubes have been used traditionally for decompression after gastric surgery and remain a routine part of postoperative care in many centers. Nasogastric tubes are often left for several days until the first flatus after gastric resection. This is based on the rationale that this can prevent aspiration, and reduce the risk of intestinal obstruction and anastomotic leak in clinical practice. Previous studies have shown that the small intestine might return to normal enterocinesia 6 h after abdominal surgery<sup>[24]</sup>. Recent studies comparing nasogastric decompression *vs* no decompression demonstrated that a gastric tube may induce pulmonary complications after gastric cancer surgery<sup>[25,26]</sup> and prolong the time to first flatus with no difference in anastomotic leak rate<sup>[27]</sup>. Therefore, placement of a nasogastric tube is unnecessary. In our present study, a nasogastric tube was not routinely used in FTS group and was removed within 24 h after surgery.

Multiple studies have demonstrated that drains are unnecessary after gastrointestinal surgery<sup>[28]</sup>. The placement of abdominal drainage is prone to increased feelings of pain, intra-abdominal fluid collection, infection, internal organ injuries and risk of fistulas, resulting in delayed recovery<sup>[17]</sup>. Alvarez Usler *et al*<sup>[29]</sup> reported that operative morbidity and hospital stay were significantly higher in patients who underwent total gastrectomy with abdomi-

nal drains than that in patients without drains. However, we refrain from abolishing use of abdominal drains for total gastrectomy in China. Since all the patients received D2 total gastrectomy, the degree of lymph node dissection could lead to a higher risk of chyle leakage. Therefore, the use of drains after total gastrectomy continues to be an issue for debate in the development of FTS.

An early postoperative oral diet can hasten the return of gut function, protect gut mucosal barrier function, and enhance portal circulation<sup>[30]</sup>. Early enteral nutrition with dietary fiber can alleviate intestinal barrier dysfunction and decrease the incidence of bacterial translocation<sup>[31]</sup>. Although early enteral nutrition increases the incidence of vomiting and flatulence, a series of reports showed that it can reduce the risk of postoperative complications and mortality<sup>[32]</sup>, facilitate postoperative restoration without increasing the incidence of fistulas<sup>[33]</sup>, and be safely applied in gastrectomy<sup>[34]</sup>. In the present study, the majority patients in the FTS group well tolerated an early oral diet or enteral nutrition by jejunal feeding tube. We noticed that nausea and vomiting was rare, but abdominal distension did occur in some patients, the symptoms only lasted for a short time based on adequate mobilization out of bed and did not result in severe complications.

It is reported that the postoperative hospital stay of gastric patients could be decreased to 3.8 d in FTS group<sup>[35]</sup>. In the present study, the mean postoperative stay of patients in FTS group was 5 d, which was longer than that reported in the literature. We found that the traditional Chinese concepts of patients are the main obstacles. They believe that surgery could cause great damage to their bodies, and they could not recover in a short time. Thus, they worried about their safety after discharge from hospital. Therefore, preoperative patient instruction and education is crucial to the outcome of FTS<sup>[36]</sup>. It will let the patients fully understand the safety, efficacy and benefits of FTS, and guarantee the compliance of patients with medical and FTS protocols.

From the view of the doctors, concern about anastomotic leakage was the main reason which affected early discharge. A series of studies showed that the FTS protocol did not increase the incidence of anastomotic leakage<sup>[37]</sup>, and revealed that education of FTS concepts was also very critical for doctors. Compliance with the FTS protocol is the main factor influencing the outcome of FTS<sup>[38]</sup>. Thus, we established a study group made up of a researcher, surgeons, anesthesiologists and nurses. We periodically conducted meetings with all staff about the details of FTS, in order to ensure the quality of the study.

The limitation of our present study was the inadequate adherence to the FTS protocol. Epidural analgesia was critical for FTS. Intraoperative application and postoperative use of epidural analgesia could block sympathetic activation to outside stimulation, inhibit hormone secretions of the hypothalamic-pituitary-adrenal axis, and finally attenuate responses to stress<sup>[39]</sup>. In our present study, tracheal intubation and general anesthesia were applied in both groups, which may partially decrease the

efficacy of FTS.

The present study indicates that FTS could promote postoperative recovery, decrease the rate of complications, shorten the duration of hospital stay, and reduce the cost of hospitalization. Our data indicate that FTS is a safe and efficient perioperative management strategy in patients undergoing radical total gastrectomy. Along with the further understanding of stress and development of FTS perioperative care, FTS could probably be safely applied in critically ill patients and emergency surgery, and major operations such as tumor resection may become day procedures in the near future.

## COMMENTS

### Background

Fast-track surgery (FTS) is a promising comprehensive program for surgical patients in elective surgery. In recent years, FTS has been applied in several surgical diseases, include radical prostatectomy, cardiac surgery, total knee replacement, cesarean section, and coronary artery bypass grafting. It has also been used for specific procedures in children and elderly. The value of FTS in radical distal gastrectomy has been demonstrated recently, but the safety and efficacy of FTS in radical total gastrectomy still requires further evaluation.

### Research frontiers

The value of FTS in radical distal gastrectomy has been demonstrated recently. Chen *et al* evaluate the safety and effectiveness of fast-track surgery combined with laparoscopy-assisted radical distal gastrectomy for gastric cancer. They found that a combination of FTS and laparoscopy-assisted radical distal gastrectomy in gastric cancer is safe, feasible, and efficient and can improve nutritional status, lessen postoperative stress, and accelerate postoperative rehabilitation.

### Innovations and breakthroughs

The present study showed that the FTS protocol was feasible for perioperative care of gastric cancer patients. Compared with conventional care, FTS could shorten the duration of flatus and defecation, accelerate the decrease in white blood cell count, decrease postoperative complications, shorten the duration of postoperative stay, reduce the cost of hospitalization, and eventually promote postoperative recovery of patients.

### Applications

The data indicate that FTS is a safe and efficient perioperative management strategy in patients undergoing radical total gastrectomy. Along with further understanding of stress, and development of FTS perioperative care, FTS could probably be safely applied in critically ill patients and emergency surgery, and major operations such as tumor resection may become day procedures in the near future.

### Terminology

FTS: Fast-track surgery, initiated by the Danish surgeon H Kehlet in the field of elective colorectal surgery in the 1990s, is a promising comprehensive program for surgical patients in elective surgery; the visual analogue scale is a psychometric response scale which can be used in questionnaires. It is a measurement instrument for subjective characteristics that cannot be directly measured.

### Peer review

This was a good study in which the authors indicates that FTS could promote postoperative recovery, decrease rate of complications, shorten duration of hospital stay, and reduce the cost of hospitalization. However, the author should think about the reason of more pneumonia in conventional care group although it is not significant.

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**P- Reviewers** Gokhan K, Mario K **S- Editor** Zhai HH  
**L- Editor** Cant MR **E- Editor** Zhang DN



## Hepatocellular carcinoma: Clinical study of long-term survival and choice of treatment modalities

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Author contributions: Wu KT, Wang CC and Lu LG contributed equally to this work; Wu KT, Wang CC and Lu LG performed the majority of the study and wrote the manuscript; Zhang WD, Zhang FJ and Shi F were involved in editing the manuscript; Li CX contributed to data acquisition, analysis and interpretation; all authors were involved in revising and approving the final version for publication.

Supported by Guangdong Province Natural Science Fund, No. 10451008901006151

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Received: January 30, 2013 Revised: March 25, 2013

Accepted: April 9, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To analyze the prognostic factors of 5-year survival and 10-year survival in hepatocellular carcinoma (HCC) patients, and to explore the reasons for long-term survival and provide choice of treatment modalities for HCC patients.

**METHODS:** From January 1990 to October 2012, 8450 HCC patients were included in a prospective database compiled by the Information Center after hospital admission. Long-term surviving patients were included in a 10-year survival group (520 patients) and

a 5-year survival group (1516 patients) for analysis. The long-term survival of HCC patients was defined as the survival of 5 years or longer. Clinical and biologic variables were assessed using univariate and multivariate analyses. The survival of patients was evaluated by follow-up data.

**RESULTS:** The long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification. It was not found to be associated with tumor diameter, histological stage, and pretreatment level of serum  $\alpha$ -fetoprotein. The differences in clinical factors between the 5-year survival and the 10-year survival were found to be the number of lesions, liver cirrhosis, Child-Pugh classification, and time elapsed until first recurrence or metastasis. The survival period of different treatment modalities in the patients who survived for 5 years and 10 years showed significant differences: (in order of significance) surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA. The 10-year survival of HCC patients was not associated with the choice of treatment modality.

**CONCLUSION:** This retrospective study elucidated survival outcomes, prognostic factors affecting survival and treatment modalities in HCC patients.

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**Key words:** Hepatocellular carcinoma; Surgery; Radiofrequency ablation; Transcatheter arterial chemoembolization; Statistical analysis; Clinical study

**Core tip:** This manuscript was a retrospective analysis and it revealed that the long-term survival of hepatocellular carcinoma (HCC) patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification, while tumor diameter, histological stage, and pretreatment level of serum  $\alpha$ -fetoprotein

were not related. Conditions for long-term survival of HCC patients were: age over 50 years, no cirrhosis, a uninodular lesion, no vessel invasion, tumor-node-metastasis stage I or II, Child-Pugh classification Class A, and appropriate treatment. The best treatment modality for more than 10 years survival compared with 5 years survival were surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA.

Wu KT, Wang CC, Lu LG, Zhang WD, Zhang FJ, Shi F, Li CX. Hepatocellular carcinoma: Clinical study of long-term survival and choice of treatment modalities. *World J Gastroenterol* 2013; 19(23): 3649-3657 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3649.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3649>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. The prognostic factors of primary HCC have been studied by many researchers worldwide. Several reports conducted in the 1990s in both Eastern and Western centers have documented a 5-year overall survival rate of 26% to 44% after HCC resection<sup>[1-5]</sup>. Studies performed from 1975 to the present<sup>[6-15]</sup> showed that age, gender, metastasis, bilirubin levels, ascites, tumor thrombus of the portal vein, neoplasm staging, Child-Pugh classification, and serum levels of  $\gamma$ -glutamyltransferase (GGT), alkaline phosphatase, and lactate dehydrogenase may all have significant effects on the prognosis of HCC patients.

There are many different modes of HCC treatment and types of primary HCC. The effects of treatment and survival rate have seen only limited improvements<sup>[16-21]</sup>. Curative surgical therapies have shown the best long-term survival rates. However, most patients do not meet the selection criteria for these treatments. Surgical resection is not available to HCC patients with severe liver cirrhosis, multiple lesions located in different parts of the liver, or lesions located near a large vein or the junctions of large portal veins<sup>[22,23]</sup>. For this reason, effective, minimally invasive therapeutic options are essential to improving the prognosis in HCC patients. Existing minimally invasive local treatments for primary HCC include radiofrequency ablation (RFA), microwave ablation, cryoablation, transcatheter arterial chemoembolization (TACE), percutaneous ethanol injection therapy, and high-intensity focused ultrasound ablation. In recent years, preliminary clinical trials have suggested that targeted drugs may have certain curative effects in the treatment of HCC<sup>[24-26]</sup>.

This study discusses the factors relevant to duration of survival in these primary HCC patients. The method of treatment, the tumor's general prognostic factors, and the tumor's own characteristics were found to determine duration of survival. Therefore, the purpose of the pres-

ent study was to analyze and compare the prognostic factors between 5-year survival and 10-year survival groups, to explore the clinical reasons for long-term survival and the choice of treatment modalities for HCC patients.

## MATERIALS AND METHODS

### Patients

From January 1990 to October 2012, 8450 HCC patients from Sun Yat-Sen University Cancer Center, First Affiliated Hospital of Jinan University, and Guangdong General Hospital were included in a prospective database after hospital admission. Long-term surviving patients were those who survived more than 5 years and they were identified and included in a 10-year survival group (520 patients) and 5-year survival group (1516 patients) for analysis. The long-term survival of HCC patients was defined as survival of 5 years or longer. This study was approved by the internal review board and ethics committee of the hospital. Informed consent was not required because of the retrospective and anonymous nature of the study, although these data were derived from prospective statistics for inpatients.

The diagnosis of HCC was confirmed in all patients either by histopathological findings or by the appearance of a liver tumor with arterial hypervascularization on contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI) with a serum  $\alpha$ -fetoprotein (AFP) value exceeding 400 ng/mL. Liver cirrhosis was diagnosed by histological or clinical features and liver function was evaluated according to the Child-Pugh score. All patients were evaluated according to European Association for the Study of the Liver criteria up to 2005<sup>[27]</sup>, and to American Association for the Study of Liver Diseases criteria from January 2006<sup>[28]</sup>. We reviewed the patients' records for demographic parameters (age, sex), hepatitis B serology, Child-Pugh stage, AFP, tumor morphology and extension, including maximum diameter, tumor number, and vessel invasion determined by imaging studies (abdominal arteriography, CT during hepatic arteriography, and the portal phase of superior mesenteric arteriography), treatment modality, complications after treatment, hospital mortality, and time of recurrence. Patient characteristics of both survival groups are shown in Table 1.

### Treatment

**Surgical resection:** Surgery was performed with patients under general anesthesia using a right subcostal incision with a midline extension with the aid of intraoperative ultrasound. Anatomic resection was performed using a target resection margin of at least 1 cm. Pringle's maneuver was routinely used with a clamp time of 10 min and an unclamp time of 5 min. Suturing and fibrin glue were used to establish hemostasis on the surface of the liver<sup>[29]</sup>.

**TACE:** Chemoembolization involves the delivery of chemotherapeutic agents to liver tumors through the hepatic artery. Seldinger's method was used to insert a catheter

**Table 1** Baseline characteristics of the patients

| Characteristics                | 5-yr survival group                    | 10-yr survival group                   | P value |
|--------------------------------|--|--|---------|
| No. of patients                | 1516                                   | 520                                    |         |
| Mean age (yr)                  | 50.45 ± 12.32                          | 50.37 ± 11.59                          | 0.102   |
| Female/male patients           | 166/1350                               | 58/462                                 | 0.935   |
| No. of tumors                  |  |  | 0.000   |
| 1                              | 1285                                   | 484                                    |         |
| 2                              | 206                                    | 33                                     |         |
| ≥ 3                            | 25                                     | 2                                      |         |
| No. of treatments              |  |  | 0.000   |
| Surgery                        | 1100                                   | 410                                    |         |
| TACE                           | 2356                                   | 576                                    |         |
| RFA                            | 656                                    | 109                                    |         |
| Mean                           | 2.71 (4112/1516)                       | 2.11 (1095/520)                        |         |
| Microvascular invasion         | 7                                      | 3                                      |         |
| Histology                      |  |  | 0.381   |
| Well                           | 320                                    | 104                                    |         |
| Moderate                       | 580                                    | 184                                    |         |
| Poor                           | 382                                    | 144                                    |         |
| HBsAg                          |  |  | 0.104   |
| Positive                       | 948                                    | 342                                    |         |
| Negative                       | 568                                    | 178                                    |         |
| Cirrhosis                      |  |  | 0.000   |
| Absence                        | 1396 (91.1%)                           | 508 (97.7%)                            |         |
| Present                        | 120 (7.9%)                             | 12 (2.3%)                              |         |
| Position of lesion             |  |  | 0.246   |
| Left lobe                      | 87                                     | 126                                    |         |
| Right lobe                     | 1334                                   | 423                                    |         |
| Both lobes                     | 25                                     | 5                                      |         |
| Lesion diameter, <i>n</i> (cm) |  |  | 0.651   |
| ≤ 3                            | 214 (2.26 ± 0.62) <sup>1</sup>         | 82 (2.27 ± 0.69) <sup>1</sup>          |         |
| > 3-≤ 5                        | 404 (4.22 ± 0.60) <sup>1</sup>         | 143 (4.20 ± 0.61) <sup>1</sup>         |         |
| > 5- < 10                      | 560 (12.44 ± 1.98) <sup>1</sup>        | 190 (7.31 ± 1.28) <sup>1</sup>         |         |
| ≥ 10                           | 338 (7.24 ± 1.40) <sup>1</sup>         | 105 (11.94 ± 1.80) <sup>1</sup>        |         |
| Mean                           | 1516 (6.89 ± 3.69)                     | 520 (6.58 ± 3.44)                      |         |
| Child-Pugh classification      |  |  | 0.383   |
| Class A                        | 1466                                   | 505                                    |         |
| Class B                        | 50                                     | 15                                     |         |
| TNM stage                      |  |  | 0.109   |
| I                              | 387                                    | 90                                     |         |
| II                             | 1096                                   | 422                                    |         |
| III                            | 33                                     | 8                                      |         |
| α-fetoprotein, ng/mL           |  |  |         |
| ≤ 100                          | 712 (22.50 ± 23.59) <sup>1</sup>       | 261 (19.78 ± 17.93) <sup>1</sup>       | 0.528   |
| 100-400                        | 186 (257.92 ± 92.89) <sup>1</sup>      | 48 (204.91 ± 81.92) <sup>1</sup>       | 0.129   |
| ≥ 400                          | 618 (16661.32 ± 28889.57) <sup>1</sup> | 211 (15302.74 ± 23346.16) <sup>1</sup> | 0.853   |
| Liver function, mean ± SD      |  |  |         |
| Total bilirubin, mg/dL         | 19.65 ± 10.03                          | 21.04 ± 38.16                          | 0.080   |
| AST, U/L                       | 50.82 ± 26.46                          | 51.72 ± 37.24                          | 0.145   |
| ALT, U/L                       | 48.71 ± 27.35                          | 45.32 ± 30.95                          | 0.053   |
| γ-glutamyltransferase, U/L     | 71.54 ± 43.62                          | 64.42 ± 46.07                          | 0.069   |

<sup>1</sup>There is significant difference within each group. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HBsAg: Hepatitis B surface antigen.

through the femoral artery. Angiography of the celiac and superior mesenteric arteries was routinely performed to determine the tumor blood supply, distribution of hepatic arteries, and collateral circulation routes<sup>[30]</sup>. The tumor's primary artery was selected for catheter placement. Patients were given a standard drug regimen of emulsified THP (40-60 mg), DDP (20-60 mg) and lipiodol (5-40

mL) through the hepatic artery.

**Radiofrequency ablation:** The size and position of the tumors and the position and direction of the needle were confirmed by CT. RFA treatment was performed with patients under general anesthesia to prevent the patient from experiencing pain and to ensure immobilization. CT was used to guide the insertion of a radiofrequency electrode into each tumor. The diameter of the needle was adjusted for tumor size. The range of ablation was extended 0.5-1 cm into the non-cancerous tissue to ensure complete coverage<sup>[31]</sup>. Patients underwent enhanced CT scans 4 wk after TACE treatment to determine the distribution of lipiodol and the status of any remaining tumor. If living tumor tissue was found, RFA was repeated<sup>[32]</sup>.

### Follow-up and recurrence

Dual-phase spiral CT was performed 4 wk after treatment and every 2 mo thereafter for the next 2 years. Each of these follow-up visits included blood tests, including liver function tests and serum AFP tests. Residual viable tumor tissue was considered present upon the first CT assessment at 4 wk after treatment if enhancement areas were seen within the tumor at either the arterial or the portal venous phase. MRI was performed if CT results were unclear on whether residual viable tumor tissue was present. Additional treatment with RFA was given in these cases. If residual viable tumor was still present after repeated treatments, patients were given TACE<sup>[33]</sup>.

The level of serum AFP and CT scans were regularly assessed to determine tumor recurrence. Recurrence was defined as the presence of hypervascular or early wash-out tumors on dynamic CT, MRI or angiography, or by a diagnosis of HCC by a radiologist. In this way, the diagnosis of recurrence was based on typical imaging findings on CT or arteriography, and, if necessary, percutaneous fine-needle aspiration cytology. Treatment modalities for HCC included surgery alone, TACE alone, surgery-TACE, TACE-RFA and surgery-TACE-RFA.

### Statistical analysis

Patient characteristics are presented as mean ± SD.  $\chi^2$  tests and the Wilcoxon rank sum test were used to compare the characteristics of patients in the 5-year and 10-year survival groups. Overall survival was calculated by the Kaplan-Meier method from the beginning of treatment. Death from any cause was considered an event. The differences in survival between groups were compared by the generalized Wilcoxon's test. A stratified Cox's proportional hazards regression model was used for multivariate analysis of prognostic parameters identified by the survival analysis. SPSS statistical software, version SPSS 19.0 for Windows (IBM Corp., Armonk, NY, United States), was used. A *P* value < 0.05 was considered significant.

## RESULTS

### Patient characteristics and clinical features

As shown in Table 1, there were 520 patients in the 10-

**Table 2 Comparison of treatment modalities between 5-year survival group and 10-year survival group for hepatocellular carcinoma**

| Treatment modalities by tumor type   | 5-yr survival group       | 10-yr survival group      | P value between groups | P value between overall groups |
|--------------------------------------|---------------------------|---------------------------|------------------------|--------------------------------|
| No. of treatment modalities          |                           |                           |                        | 0.092                          |
| Surgery alone                        | 609                       | 235                       |                        |                                |
| TACE alone                           | 214                       | 80                        |                        |                                |
| Surgery + TACE                       | 458                       | 125                       |                        |                                |
| TACE + RFA                           | 85                        | 28                        |                        |                                |
| Surgery + TACE + RFA                 | 150                       | 52                        |                        |                                |
| Tumor size ≤ 5 cm (maximum diameter) |                           |                           |                        | 0.097                          |
| Surgery alone                        | 244 (3.53 ± 1.03)         | 100 (3.48 ± 1.06)         | 0.217                  |                                |
| TACE alone                           | 92 (2.59 ± 1.16)          | 38 (3.58 ± 1.16)          | 0.087                  |                                |
| Surgery + TACE                       | 133 (3.90 ± 1.02)         | 43 (3.47 ± 1.03)          | 0.192                  |                                |
| TACE + RFA                           | 72 (3.10 ± 1.16)          | 21 (2.93 ± 1.10)          | 0.824                  |                                |
| Surgery + TACE + RFA                 | 82 (3.88 ± 0.91)          | 26 (3.42 ± 0.98)          | 0.314                  |                                |
| Tumor size > 5 cm (maximum diameter) |                           |                           |                        | 0.106                          |
| Surgery alone                        | 365 (8.16 ± 2.27)         | 135 (8.532 ± 2.71)        | 0.130                  |                                |
| TACE alone                           | 122 (9.31 ± 2.94)         | 42 (9.36 ± 2.26)          | 0.925                  |                                |
| Surgery + TACE                       | 325 (9.59 ± 3.16)         | 82 (9.09 ± 2.50)          | 0.624                  |                                |
| TACE + RFA                           | 13 (11.68 ± 3.74)         | 7 (10.47 ± 5.59)          | 0.548                  |                                |
| Surgery + TACE + RFA                 | 68 (7.19 ± 3.02)          | 26 (10.65 ± 4.63)         | 0.131                  |                                |
| Uninodular HCC                       |                           |                           |                        | 0.039                          |
| Surgery alone                        | 531                       | 223                       |                        |                                |
| TACE alone                           | 178                       | 75                        |                        |                                |
| Surgery + TACE                       | 382                       | 113                       |                        |                                |
| TACE + RFA                           | 57                        | 24                        |                        |                                |
| Surgery + TACE + RFA                 | 100                       | 50                        |                        |                                |
| Multinodular HCC                     |                           |                           |                        | 0.445                          |
| Surgery alone                        | 78                        | 12                        |                        |                                |
| TACE alone                           | 36                        | 5                         |                        |                                |
| Surgery + TACE                       | 76                        | 12                        |                        |                                |
| TACE + RFA                           | 28                        | 4                         |                        |                                |
| Surgery + TACE + RFA                 | 50                        | 2                         |                        |                                |
| AFP > 400 ng/mL (AFP value)          |                           |                           |                        | 0.289                          |
| Surgery alone                        | 280 (16053.09 ± 27804.11) | 102 (18092.07 ± 26818.62) | 0.450                  |                                |
| TACE alone                           | 66 (15876.11 ± 30406.63)  | 28 (10984.83 ± 18402.28)  | 0.270                  |                                |
| Surgery + TACE                       | 188 (21164.79 ± 31964.15) | 47 (25114.26 ± 31807.02)  | 0.385                  |                                |
| TACE + RFA                           | 32 (742.50 ± 0.00)        | 12 (22597.20 ± 28745.64)  | 0.667                  |                                |
| Surgery + TACE + RFA                 | 56 (1884.33 ± 953.25)     | 19 (8789.88 ± 21862.41)   | 0.091                  |                                |
| AFP < 400 ng/mL (AFP value)          |                           |                           |                        | 0.065                          |
| Surgery alone                        | 329 (45.50 ± 71.08)       | 133 (56.35 ± 91.86)       | 0.853                  |                                |
| TACE alone                           | 148 (38.60 ± 71.56)       | 52 (38.09 ± 48.30)        | 0.355                  |                                |
| Surgery + TACE                       | 270 (66.35 ± 98.86)       | 78 (43.02 ± 74.45)        | 0.738                  |                                |
| TACE + RFA                           | 69 (133.34 ± 145.64)      | 16 (44.81 ± 43.02)        | 0.579                  |                                |
| Surgery + TACE + RFA                 | 94 (96.06 ± 138.35)       | 33 (15.43 ± 8.21)         | 0.064                  |                                |

AFP: α-fetoprotein; TACE: Transcatheter arterial chemoembolization; RFA: Radiofrequency ablation; HCC: Hepatocellular carcinoma.

year survival group. Single lesions were present in 93.1% and multiple lesions in 6.7%. Lesions located in only one lobe of the liver were present in 93.1%, and lesions located in two lobes were present in 0.9%. A portal vein tumor thrombus was present in 2.3%. Child-Pugh classification class A applied to 97.1% and class B to 2.9%. tumor-node-metastasis (TNM) stage I or II applied to 98.5% patients. Among the 1516 patients who survived for 5 years, 84.8% had one lesion, 13.6% had two lesions, 1.6% had three lesions, 98.6% had lesions located in one lobe of the liver, 1.4% had lesions located in the two lobes, 1.1% had vessel invasion, 96.7% had Child-Pugh classification class A, 3.3% had class B, and 97.8% had TNM stage I or II. In this way, the survival period of both groups showed some correlation with the number of tumor lesions and liver cirrhosis. No statistically significant differences were observed with respect to maximum

tumor diameter in either group. In the 5-year group, vessel invasion appeared in 1.1% of patients. In the 10-year group, vessel invasion appeared in 2.1% of patients. This suggests that patients with vessel invasion seldom survived for 5 years. In the 10-year survival group, 97.1% of patients were described as Child-Pugh classification class A and 2.9% as class B. TNM stage I and II was applied to 98.5% of patients. In the 5-year survival group, 96.7% of patients were described as Child-Pugh classification class A, 3.3% as class B, and 97.8% as TNM stage I or II. The survival periods of both groups may be related to Child-Pugh classification class A and TNM stage I or II.

**Treatment modalities**

As shown in Table 2, treatment modalities such as surgery alone, TACE alone, surgery-TACE, surgery-TACE-RFA, and TACE-RFA, showed no statistically significant

**Table 3 Comparison of treatment efficacy between the 5-year survival group and 10-year survival group for hepatocellular carcinoma**

| Characteristics  | 5-yr survival group       | P value | 10-yr survival group     | P value | P value between groups |
|--|---------------------------|---------|--------------------------|---------|------------------------|
| AFP the first time before and after treatment (ng/mL)    |                           |         |                          |         |                        |
| Surgery  |                           | 0.002   |                          | 0.000   |                        |
| Before surgery   | 1020 (4596.63 ± 17193.11) |         | 358 (7419.90 ± 18412.64) |         | 0.049                  |
| After surgery  | 1020 (1133.25 ± 4530.36)  |         | 358 (1255.09 ± 4337.24)  |         | 0.070                  |
| TACE   |                           | 0.039   |                          | 0.002   |                        |
| Before TACE  | 496 (4510.68 ± 15115.73)  |         | 162 (2827.99 ± 9506.04)  |         | 0.446                  |
| After TACE   | 496 (1732.58 ± 7631.56)   |         | 162 (502.21 ± 1526.13)   |         | 0.711                  |
| Liver function the first time before and after treatment |                           |         |                          |         |                        |
| Total bilirubin (mg/dL)                                  |                           | 0.721   |                          | 0.353   |                        |
| Before treatment   | 68.32 ± 58.98             |         | 19.63 ± 12.65            |         | 0.078                  |
| After treatment  | 67.22 ± 69.45             |         | 18.76 ± 11.56            |         | 0.120                  |
| AST (U/L)  |                           | 0.983   |                          | 0.730   |                        |
| Before treatment   | 51.99 ± 28.41             |         | 52.69 ± 40.86            |         | 0.137                  |
| After treatment  | 50.87 ± 27.50             |         | 52.42 ± 39.77            |         | 0.695                  |
| ALT (U/L)  |                           | 0.104   |                          | 0.262   |                        |
| Before treatment   | 52.46 ± 37.21             |         | 50.69 ± 53.10            |         | 0.291                  |
| After treatment  | 54.60 ± 42.26             |         | 51.16 ± 50.09            |         | 0.126                  |
| γ-glutamyltransferase (U/L)                              |                           | 0.366   |                          | 0.017   |                        |
| Before treatment   | 19.30 ± 8.09              |         | 69.85 ± 93.75            |         | 0.122                  |
| After treatment  | 19.53 ± 8.32              |         | 58.79 ± 67.08            |         | 0.670                  |
| Time to first tumor recurrence or metastasis (d)         |                           | 0.012   |                          | 0.045   |                        |
| Surgery alone  | 90 (1031.36 ± 662.61)     |         | 38 (3246.38 ± 2047.29)   |         | 0.003                  |
| TACE alone   | 32 (675.64 ± 412.31)      |         | 12 (1882.77 ± 1324.08)   |         | 0.015                  |
| Surgery + TACE   | 225 (1012.00 ± 818.86)    |         | 70 (2778.30 ± 6296.35)   |         | 0.007                  |
| Surgery + TACE + RFA                                     | 90 (798.41 ± 492.01)      |         | 52 (2553.20 ± 1746.48)   |         | 0.016                  |

RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

differences between the 5-year survival group and 10-year survival group, but there were significant differences in treatment modalities of uninodular lesions between the 5-year survival group and 10-year survival group. Surgery alone, TACE alone, surgery-TACE, TACE-RFA and surgery-TACE-RFA showed no statistical differences in the maximum diameter of the tumor for the two groups. The survival period had no relationship with tumor size. Similarly, the survival period had no relationship with the level of the serum AFP in patients treated with different modalities. The patients of the two groups showed statistically significant differences in survival period depending on the different treatment modalities, but only among patients with only one lesion. The patients of the groups showed statistically significant differences in survival period depending on liver cirrhosis. Survival period did not differ significantly across the 5-year survival and 10-year survival groups in patients multiple lesions. In short, different treatment modalities were found to have an effect on patients with only one lesion, and no effect on patients with multiple lesions.

### Effects of initial treatment

As shown in Table 3, serum levels of AFP and GGT differed significantly before and after the first treatment, but the levels of serum total bilirubin, aspartate aminotransferase, and alanine aminotransferase showed no statistically significant differences regardless of whether the first treatment was surgery or TACE and regardless of whether the patient survived for 5 or 10 years. The level of pre-surgery serum AFP showed statistically significant

differences between 5-year survival and 10-year survival groups. After the first surgery, the two groups showed no statistically significant differences from each other. The time of the first recurrence or metastasis showed significant differences between the 5-year and 10-year survival groups. Different treatments for each treatment group also showed statistically significant differences in the time of the first recurrence or metastasis: (in order of significance) surgery alone > surgery-TACE > TACE alone > surgery-TACE-RFA.

### Prognostic analysis

The influence of patient and tumor related factors on overall survival are shown in Table 4. Analysis of patients who survived for 5 years or longer showed that survival rates differed significantly with age, Child-Pugh classification, vessel invasion, liver cirrhosis, TNM stage, treatment method, and number of tumors. Statistical results also showed that, among patients who survived 5 years or longer, no significant differences could be attributed to sex, diameter of the largest tumor, or to pretreatment serum levels of AFP. Analysis of 10 years survival showed statistically significant differences in survival rates with respect to vessel invasion, number of tumors and Child-Pugh classification. No significant differences could be attributed to age, sex, tumor diameter, or pretreatment level of serum AFP. Multivariate analysis of patients who lived 5 years or longer showed survival rates to be related to age, vessel invasion, liver cirrhosis, TNM stage, Child-Pugh classification, treatment method, and number of tumors. Multivariate analysis of patients who survived

**Table 4 Comparison of univariate analysis for various prognostic factors between 5-year survival and 10-year survival groups for hepatocellular carcinoma**

| Factor                       | 5-yr survival group |               | 10-yr survival group |         |                |         |
|------------------------------|---------------------|---------------|----------------------|---------|----------------|---------|
|                              | Exp (B)             | 95%CI         | P value              | Exp (B) | 95%CI          | P value |
| Sex (men and women)          | 0.787               | (0.678-0.913) | 0.002                | 0.875   | (0.667-1.146)  | 0.332   |
| Age (yr) (< 50 and ≥ 50)     | 0.516               | (0.462-0.575) | 0.000                | 1.097   | (0.838-1.438)  | 0.500   |
| Diameter of largest tumor    | 0.971               | (0.921-1.023) | 0.274                | 0.961   | (0.879-1.051)  | 0.384   |
| Child-Pugh classification    | 0.070               | (0.060-0.082) | 0.000                | 0.559   | (0.334-0.938)  | 0.028   |
| No. of tumors                | 1.452               | (1.284-1.642) | 0.000                | 0.390   | (0.261-0.582)  | 0.000   |
| Pretreatment serum AFP level | 1.041               | (0.987-1.099) | 0.138                | 0.933   | (0.851-1.022)  | 0.137   |
| Cirrhosis                    | 0.832               | (0.775-0.893) | 0.000                | 10.539  | (8.164-13.606) | 0.000   |
| TNM stage                    | 0.739               | (0.662-0.862) | 0.000                | 0.911   | (0.739-1.124)  | 0.384   |
| Treatment methods            | 1.152               | (1.109-1.197) | 0.000                | 1.043   | (0.975-1.115)  | 0.220   |
| Microvascular invasion       | 1.038               | (0.494-2.181) | 0.922                | 1.233   | (0.396-3.838)  | 0.718   |

AFP:  $\alpha$ -fetoprotein; TNM: Tumor-node-metastasis.**Table 5 Comparison of multivariate analysis for various prognostic factors between 5-year survival and 10-year survival groups for hepatocellular carcinoma**

| Group                    | Sex   | Age   | Micro-vascular invasion | Pretreatment AFP level | Diameter of tumor | Treatment methods | TNM stage | Child-Pugh classification | No. of tumors | Cirrhosis |
|--------------------------|-------|-------|-------------------------|------------------------|-------------------|-------------------|-----------|---------------------------|---------------|-----------|
| 5-yr survival (P value)  | 0.344 | 0.004 | 0.033                   | 0.080                  | 0.211             | 0.000             | 0.001     | 0.000                     | 0.040         | 0.000     |
| 10-yr survival (P value) | 0.983 | 0.962 | 0.370                   | 0.783                  | 0.847             | 0.215             | 0.695     | 0.029                     | 0.025         | 0.000     |

AFP:  $\alpha$ -fetoprotein; TNM: Tumor-node-metastasis.

10 years or longer showed survival rates to be related to Child-Pugh classification, liver cirrhosis and to the number of tumors.

### Overall survival

Table 1 show that patients with only one lesion, 2 lesions, or 3 lesions showed statistically significant differences with respect to duration of survival period in both groups. Patients who had only one lesion survived significantly longer than other patients. Patients who had 2 lesions seldom survived 5 years. Patients with 3 lesions seldom survived 5 years. Patients who survived more than 5 years were associated with more influencing factors than those in the 10-year survival group. Factors associated with long-term survival among HCC patients included age over 50 years, single rather than multiple lesions, no vessel invasion, TNM stage I or II, Child-Pugh classification Class A, and only 1-3 rounds of treatment. Long-term survival of patients for HCC was found to be related to the number of lesions, liver cirrhosis and methods of treatment, not to tumor diameter or level of serum AFP.

Statistical results showed that, among patients who survived 10 years or longer, no significant differences could be obtained from the different treatment modalities ( $P = 0.202$ ). The survival period of different treatment modalities in the patients who survived between 5 years and 10 years showed statistically significant differences: (in order of significance) surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA (Figure 1).

Therefore, the 10-year survival of HCC patients was not associated with the choice of initial treatment modality. However, different treatment modalities had a significant effect on the survival of HCC patients who survived between 5 and 10 years (Table 5).

## DISCUSSION

There is a variety of treatment methods and models of HCC. Surgical resection is the preferred treatment for HCC. TACE, however, has broader indications. RFA, microwave ablation, cryoablation, radiation therapy, and high intensity focused ultrasound therapy have been widely used in clinical treatment<sup>[34-37]</sup>. When HCC is diagnosed early, the curative effects enjoyed by some HCC patients improve, as does the prognosis. However, improvements to the survival rate are very limited and the prognosis remains poor. By evaluating criteria to more accurately predict prognosis, patients at high risk of recurrence would be identified more easily and effective prevention and control measures could be implemented.

The results of this study show the 5-year and 10-year survival groups to have the following common features: lesions located in only one lobe of the liver, single lesions, no vessel invasion, no liver cirrhosis, TNM stage I or II, Child-Pugh classification class A, and 1 to 3 treatments. Long-term survival factors are assessed by using univariate and multivariate Cox proportional hazard regression analyses. Multivariate analysis indicated that survival for more than 10 years was associated with treatment modal-

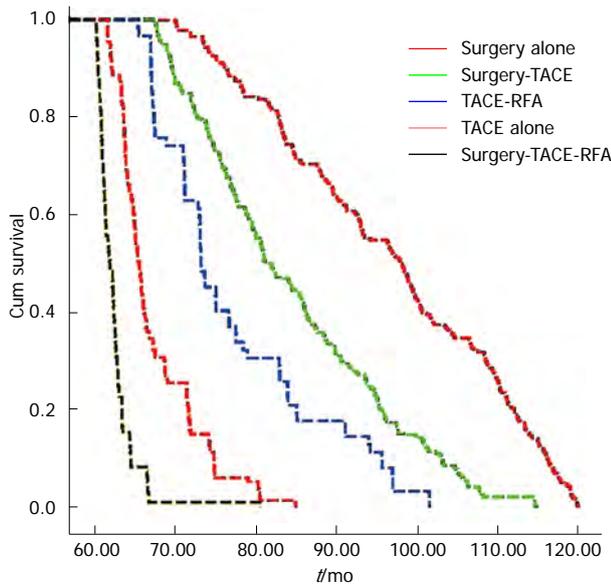


Figure 1 Kaplan-Meier curve shows overall survival rates of different treatment modalities in hepatocellular carcinoma patients who survived between 5 years and 10 years. Different treatment models showed statistically significant differences in the survival period: surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA.

ity, number of lesions, vessel invasion, age, and Child-Pugh classification. Survival for more than 5 years was associated with number of lesions, no liver cirrhosis and treatment modalities. The independent prognostic factors of both groups included method of treatment, liver cirrhosis and number of lesions. The diameter of the largest tumor and serum level of AFP were not associated with survival period in either group.

Patients with multiple lesions or lesions located in more than one lobe of the liver showed poorer prognosis and did not always survive 5 years. The treatment modalities showed that the most effective type of treatment was surgery. The three types of combination therapy were used in 412/520 (79.2%) of the patients who survived for 10 years or longer and in 1244/1516 (82.1%) of the patients who survived for 5 years or longer. The survival period for different treatment modalities in both groups also showed statistically significant differences: surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA. When a single lesion is present and that lesion can be removed by surgery, surgical resection should be the preferred method. Surgical resection is the preferred treatment for HCC. TACE should be performed in the treatment of the relapse or metastatic lesions after surgical resection.

The initial effects of treatment in both 10-year and 5-year survival groups showed serum levels of AFP and GGT to be significantly different before and after the first treatment regardless of whether this first treatment was surgery or TACE. The level of serum AFP showed statistically significant differences between 10-year group and 5-year survival groups before the first surgical operation, but no statistically significant differences between

the two groups were detected after the first surgical operation. This suggests that the serum level of AFP decreased quickly after surgical resection in the 10-year survival group, an ideal curative effect. With all five kinds of treatment, surgery alone, surgery-TACE, TACE-RFA, TACE alone, or surgery-TACE-RFA, the first relapse or metastasis showed statistically significant differences between the 5-year and 10-year survival groups. The first relapse or metastasis tended to occur later in the 10-year survival group than in the 5-year survival group. The time to tumor recurrence or metastasis was found to significantly affect the patients' survival periods.

The prognosis of HCC here showed heterogeneity caused by the interactions between many factors and by the interplay between the tumor and the rest of the body. Factors that affect prognosis have been found to be different, but this may be because the studies evaluating them had different goals and factors<sup>[38-40]</sup>. Lau *et al*<sup>[41]</sup> reported that the factors that affect the curative effect of mid-to-late HCC are tumor type, portal vein tumor thrombus, treatment method, and hepatic function. Yamamoto *et al*<sup>[42]</sup> found that portal vein tumor thrombus, tumor type, traces of iodized oil, and hepatic function all influence the survival and prognosis of the HCC patients.

This study evaluated patient age, gender, TNM stage, Child-Pugh classification, portal vein tumor thrombus, serum AFP level, number of tumor lesions, tumor diameter, and treatment method, all of which may have some relationship with prognosis. The independent prognostic survival factors of both the patients who survived more than 10 years and those who survived 5 years can be said to be related to treatment method and to the number of lesions. The number of lesions was found to be a common risk factor in the 10-year and 5-year survival groups. One possible reason for this may be that most of the intrahepatic tumor lesions had undergone intrahepatic metastasis. Multiple lesions tended to be caused by intrahepatic metastasis. Even when tumors were completely removed, subclinical tumor lesions remained in the liver and the tumor cells may have entered the bloodstream. The survival period showed obvious differences in patients who received different treatments. The treatments, in decreasing order of favorability, were as follows: surgery alone > surgery-TACE > TACE-RFA > TACE alone > surgery-TACE-RFA. The patients who survived more than 10 years were found to have more influencing factors than those in the 5-year group. The patients who were more than 50 years old, who had no portal vein tumor thrombus, who had Child-Pugh class A tumors, who had only one lesion, and who underwent appropriate treatment tended to live longer than other patients.

## ACKNOWLEDGMENTS

Thanks to all patients who took part in this clinical research. We thank all the members of Department of Medical Imaging and Interventional Radiology of Cancer Center for discussion and comments.

## COMMENTS

**Background**

Hepatocellular carcinoma (HCC) is one of the most common digestive malignancies cancer, which do serious threat to the people's life and health. Therefore, it has been studied by many scholars, but people still do not know what is the reason for the long-term survival and whether it has an optimal treatment.

**Research frontiers**

There are various prognosis factors for HCC, some researchers suggesting that age, sex and  $\alpha$ -fetoprotein (AFP) level play a critical role, while others suggest that the long-term survival of HCC is related to treatment modality or the biological nature of the tumor.

**Innovations and breakthroughs**

This manuscript reveals the long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification. It was not found to be associated with tumor diameter, histological stage, and pretreatment level of serum AFP. Conditions associated with long-term survival of HCC patients were: age over 50 years, no cirrhosis, uninodular lesion, no vessel invasion, tumor-node-metastasis stage I or II, Child-Pugh classification class A, and appropriate treatment. The clinical reasons for the differences between the 5-year survival and the 10-year survival were found to be the number of lesions, liver cirrhosis, Child-Pugh classification, and time elapsed until first recurrence or metastasis for HCC. The survival period of different treatment modalities in the patients who survived between 5 years and 10 years showed statistically significant differences: surgery alone > surgery-transcatheter arterial chemoembolization (TACE) > TACE-radiofrequency ablation (RFA) > TACE alone > surgery-TACE-RFA. The 10-year survival of HCC patients was not associated with the choice of treatment modality.

**Applications**

This study retrospectively studied HCC patients treated in the past 20 years, and the ultimate conclusion is that the long-term survival of HCC patients was associated with the number of lesions, liver cirrhosis and Child-Pugh classification, patients who survived for 5 and 10 years showed statistically significant differences in types of treatments. The 10-year survival of HCC patients was not associated with the choice of treatment modality. The results can provide information on the treatment choice of HCC patients.

**Terminology**

TACE is short for transhepatic arterial chemotherapy and embolization, used in the treatment of HCC patients especially for the terminal cancer patients; RFA is a treatment method for HCC patients which uses a RFA needle to send high radiofrequency waves to heat the tumor and cause cell degeneration and necrosis. It is widely used for small HCC tumor (diameter < 3 cm).

**Peer review**

This study was performed as a retrospective analysis of prognostic factors including individual therapy for patients with HCC who survived for 5 years or longer and 10 years or longer. A large population of more than 2000 patients was selected from over 8000 HCC patients, and thus this study is likely to give interesting and reliable data.

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P- Reviewer Watanabe M S- Editor Song XX  
L- Editor Cant MR E- Editor Li JY



## Analysis of long non-coding RNA expression profiles in gastric cancer

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Received: January 22, 2013 Revised: March 20, 2013

Accepted: May 9, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To investigate the expression patterns of long non-coding RNAs (lncRNAs) in gastric cancer.

**METHODS:** Two publicly available human exon arrays for gastric cancer and data for the corresponding normal tissue were downloaded from the Gene Expression Omnibus (GEO). We re-annotated the probes of the human exon arrays and retained the probes uniquely mapping to lncRNAs at the gene level. lncRNA expression profiles were generated by using robust multi-array average method in affymetrix power tools. The normalized data were then analyzed with a Bioconductor package linear models for microarray data and genes with adjusted *P*-values below 0.01 were considered differentially expressed. An independent data set was used to validate the results.

**RESULTS:** With the computational pipeline established to re-annotate over 6.5 million probes of the Affymetrix Human Exon 1.0 ST array, we identified 136053 probes

uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. By analyzing GSE27342 consisting of 80 paired gastric cancer and normal adjacent tissue samples, we identified 88 lncRNAs that were differentially expressed in gastric cancer, some of which have been reported to play a role in cancer, such as LINC00152, taurine upregulated 1, urothelial cancer associated 1, Pvt1 oncogene, small nucleolar RNA host gene 1 and LINC00261. In the validation data set GSE33335, 59% of these differentially expressed lncRNAs showed significant expression changes (adjusted *P*-value < 0.01) with the same direction.

**CONCLUSION:** We identified a set of lncRNAs differentially expressed in gastric cancer, providing useful information for discovery of new biomarkers and therapeutic targets in gastric cancer.

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**Key words:** Long non-coding RNA; Gastric cancer; Microarray analysis; Data mining

**Core tip:** Long non-coding RNAs (lncRNAs) have risen to prominence with important roles in a broad range of biological processes. lncRNA expression patterns and their biological functions in gastric cancer still remain unknown. We re-annotated the probes from an Affymetrix Human Exon 1.0 ST array and identified probes uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. We identified a set of lncRNAs that were differentially expressed in gastric cancer. In an independent data set, 59% of these differentially expressed lncRNAs showed significant expression changes with the same direction.

Cao WJ, Wu HL, He BS, Zhang YS, Zhang ZY. Analysis of long

non-coding RNA expression profiles in gastric cancer. *World J Gastroenterol* 2013; 19(23): 3658-3664 Available from: URL: <http://www.wjnet.com/1007-9327/full/v19/i23/3658.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3658>

## INTRODUCTION

Over the last decade, advances in genome-wide analysis of gene expression have revealed far more genomic transcription than previously anticipated, with the majority of the genome being transcribed into non-coding RNAs (ncRNAs)<sup>[1,2]</sup>. Much attention has focused on microRNAs (miRNAs), one class of small non-coding RNAs. MiRNAs are involved in specific regulation of both protein-coding and putatively non-coding genes by post-transcriptional silencing or infrequently by activation<sup>[3-5]</sup>.

More recently, long non-coding RNAs (lncRNAs), generally defined as having a size greater than 200 nucleotides, have risen to prominence with important roles in a broad range of biological processes. lncRNAs regulate gene expression at the level of post-transcriptional processing such as protein synthesis, RNA maturation, and transport. They also exert their effects in transcriptional gene silencing through the regulation of chromatin structure<sup>[6,7]</sup>. Dysregulation of lncRNAs is associated with many human diseases, including various types of cancers<sup>[8]</sup>. The well-studied lncRNA HOTAIR, for example, was found to be highly upregulated in both primary and metastatic breast tumors, and its expression level in primary tumors was a powerful predictor of eventual metastasis and death<sup>[9]</sup>. However, lncRNA expression patterns and their biological function in gastric cancer remain unknown.

In this study, we identified a set of lncRNAs that were differentially expressed in gastric cancer by analyzing publicly available data sets from the gene expression omnibus (GEO).

## MATERIALS AND METHODS

### Microarray data

Human exon arrays for gastric cancer and normal adjacent tissue were downloaded from the GEO. Two data sets were included: GSE27342 and GSE33335. GSE27342 consisted of 80 paired gastric cancer and normal adjacent tissue, including 4 stage I, 7 stage II, 54 stage III and 7 stage IV<sup>[10,11]</sup>. All samples were taken from three hospitals affiliated with Jilin University College of Medicine and Jilin Provincial Cancer Hospital, Changchun, China. GSE33335 consisted of 25 paired gastric cancer and normal adjacent tissue obtained from the tissue bank of Shanghai Biochip Center, Shanghai, China<sup>[12,13]</sup>. Three raw CEL files failed to be normalized and were excluded from our analysis, leaving 22 paired gastric cancer and normal adjacent tissue. GSE27342 was used as an experimental set to discover differentially expressed lncRNAs in gastric cancer while GSE33335 was used as a

validation set.

### Probe re-annotation pipeline

The sequences of protein-coding transcripts were retrieved from Ensembl release 67<sup>[14]</sup>, UCSC<sup>[15]</sup> and RefSeq release 54<sup>[16]</sup> in July 2012. Specifically, the protein-coding transcripts are a pool of transcripts with gene\_type as “protein\_coding” in Ensembl, transcripts with category as “coding” in UCSC and transcripts with an identifier beginning with NM\_ in RefSeq. The sequences of non-coding transcripts were compiled from Ensembl through Biomart. The probe sequences of the human exon array were downloaded from the Affymetrix website ([http://www.affymetrix.com/Auth/analysis/downloads/na25/wtexon/HuEx-1\\_0-st-v2.probe.tab.zip](http://www.affymetrix.com/Auth/analysis/downloads/na25/wtexon/HuEx-1_0-st-v2.probe.tab.zip)) and aligned to the sequences of protein-coding and non-coding transcripts using BLAST-2.2.26+<sup>[17]</sup>. The alignment results were then filtered by the following steps: (1) probes perfectly matched to a transcript were retained; (2) probes mapped to non-coding transcripts only were retained; (3) probes mapped to unique genes were retained; (4) probes mapped to known lncRNAs (genes annotated with processed\_transcript, lincRNA, antisense, non\_coding, sense\_intronic, ncrna\_host, sense\_overlapping and 3prime\_overlapping\_ncrna) were retained; and (5) genes with less than 3 probes were removed.

A new PGF file covering the re-annotated probe-lncRNA relationships was created. The official CLF file HuEx-1\_0-st-v2.r2.clf was downloaded from the Affymetrix website ([http://www.affymetrix.com/Auth/support/downloads/library\\_files/HuEx-1\\_0-st-v2.r2.zip](http://www.affymetrix.com/Auth/support/downloads/library_files/HuEx-1_0-st-v2.r2.zip)).

### Data analysis

Gene expression profiles were summarized by applying robust multi-array average (RMA)<sup>[18]</sup> normalization as implemented in affymetrix power tools (1.14.4 package apt-probeset-summarize), using the newly-created PGF file and the official CLF file.

The normalized data were analyzed with a Bioconductor package linear models for microarray data (LIMMA), a modified *t*-test incorporating the Benjamini-Hochberg multiple hypotheses correction technique<sup>[19]</sup>. Genes with adjusted *P*-values below 0.01 were considered differentially expressed. The heatmap of differentially expressed genes was generated using BRB-Array Tools Version 4.3.0 Beta 1 (<http://linus.nci.nih.gov/BRB-ArrayTools.html>)<sup>[20]</sup>.

## RESULTS

### Re-annotation of exon array probes

A computational pipeline was established to re-annotate over 6.5 million probes of the Affymetrix Human Exon 1.0 ST array (Figure 1). There were 315255 probes perfectly matched to non-coding RNAs but not to any protein-coding transcript. These probes were mapped from transcript level to gene level and 278918 probes matched to one gene were retained. Probes mapping to

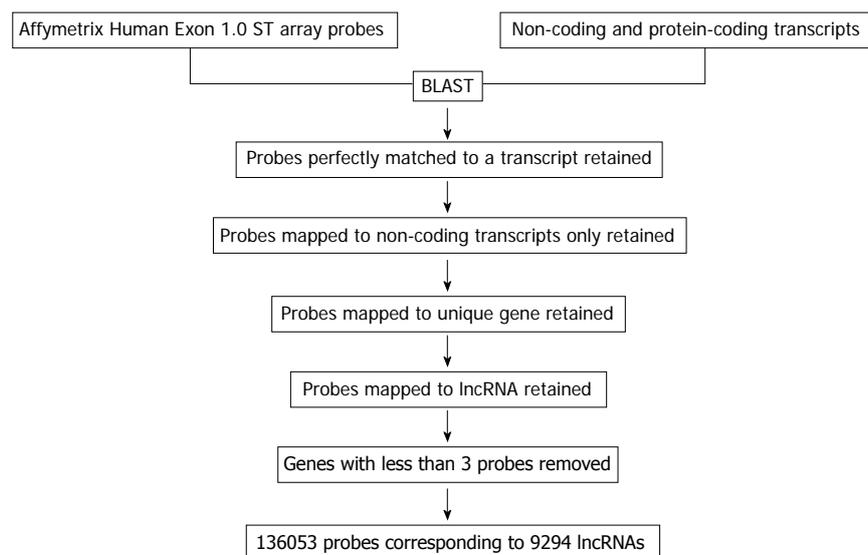


Figure 1 Computational pipeline for re-annotating the probes of the Affymetrix Human Exon 1.0 ST array. lncRNA: Long non-coding RNA.

short ncRNAs and pseudogenes were then discarded, leaving 136,533 probes mapping to lncRNAs, which were annotated with Ensembl (processed\_transcript, lincRNA, antisense, non\_coding, sense\_intronic, ncna\_host, sense\_overlapping and 3prime\_overlapping\_ncrna). To further increase accuracy, genes matched by less than three probes were discarded. Finally, we obtained 136053 probes uniquely mapping to lncRNAs at the gene level, corresponding to 9294 lncRNAs. The number of probes mapping to lncRNAs ranged from 3 to 257 and the average was 18.

### Identification of differentially expressed lncRNAs in gastric cancer

The CEL files were processed by Affymetrix Power Tools for background correction, normalization, and summarizations with RMA algorithm. Using LIMMA with an adjusted *P*-value of less than 0.01 as a threshold, we identified 88 lncRNAs that were differentially expressed in gastric cancer as compared to normal gastric tissue (Figure 2). The top 30 lncRNAs differentially expressed in gastric cancer are listed in Table 1. Of 88 differentially expressed lncRNAs, 71 lncRNAs were found to be upregulated and 17 to be downregulated. Most of these lncRNAs do not have an official Human Genome Nomenclature Committee symbol and their function is unknown. But some have been reported to play a role in cancer, including LINC00152<sup>[21]</sup>, taurine upregulated 1 (TUG1)<sup>[22]</sup>, urothelial cancer associated 1 (UCA1)<sup>[23,24]</sup>, Pvt1 oncogene (PVT1)<sup>[25]</sup>, small nucleolar RNA host gene 1 (SNHG1)<sup>[26]</sup>, and LINC00261<sup>[27]</sup>.

### Validation in an independent data set

To independently validate our results, we conducted the same analysis on GSE33335 and found that 59% of the differentially expressed lncRNAs identified by above analysis showed significant expression changes (adjusted *P* < 0.01) with the same direction. As shown in Figure 3,

the distribution of expression differentials between the experimental data set and the validation data set is significantly concordant, reflecting a high consistence in expression patterns of these genes among different sample sets.

## DISCUSSION

lncRNAs have comprehensive functions in biological processes through various mechanisms<sup>[6,7]</sup>. The expression patterns of lncRNAs are of great importance in the cancer field and are often investigated with tiling arrays<sup>[28,29]</sup>, RNA sequencing<sup>[30]</sup> or lncRNA-specific microarrays<sup>[31,32]</sup>, which are relatively expensive and inflexible. Recently, studies have suggested that lncRNA expression profiling may be achieved by mining existing microarray data because some probes uniquely mapping to lncRNAs are fortuitously represented on these arrays<sup>[33,34]</sup>. The Affymetrix Human Exon 1.0 ST array consists of over 6.5 million individual probes designed along the entire length of the gene as opposed to just the 3' end, providing a unique platform for mining lncRNA profiles<sup>[35,36]</sup>.

The lncRNA list used in this study was retrieved from Ensembl and is equivalent to the GENCODE lncRNA data set. This data set utilizes a combination of manual curation, computational analysis and targeted experimental approaches, and is the largest catalog of human lncRNAs to date<sup>[22]</sup>. To filter out potentially unrecognized probes mapping to protein-coding genes, we generated a merged known protein-coding gene list from RefSeq, UCSC and Ensembl. Still, some probes could potentially hybridize to other undiscovered transcripts or genes.

We identified 136053 probes from the Affymetrix Human Exon 1.0 ST array uniquely mapping to lncRNAs at the gene level. These probes correspond to 9294 lncRNAs, covering nearly 76% of the GENCODE lncRNA data set. This analysis revealed a set of lncRNAs that were differentially expressed between gastric cancer and normal gastric tissue, some of which have been pre-

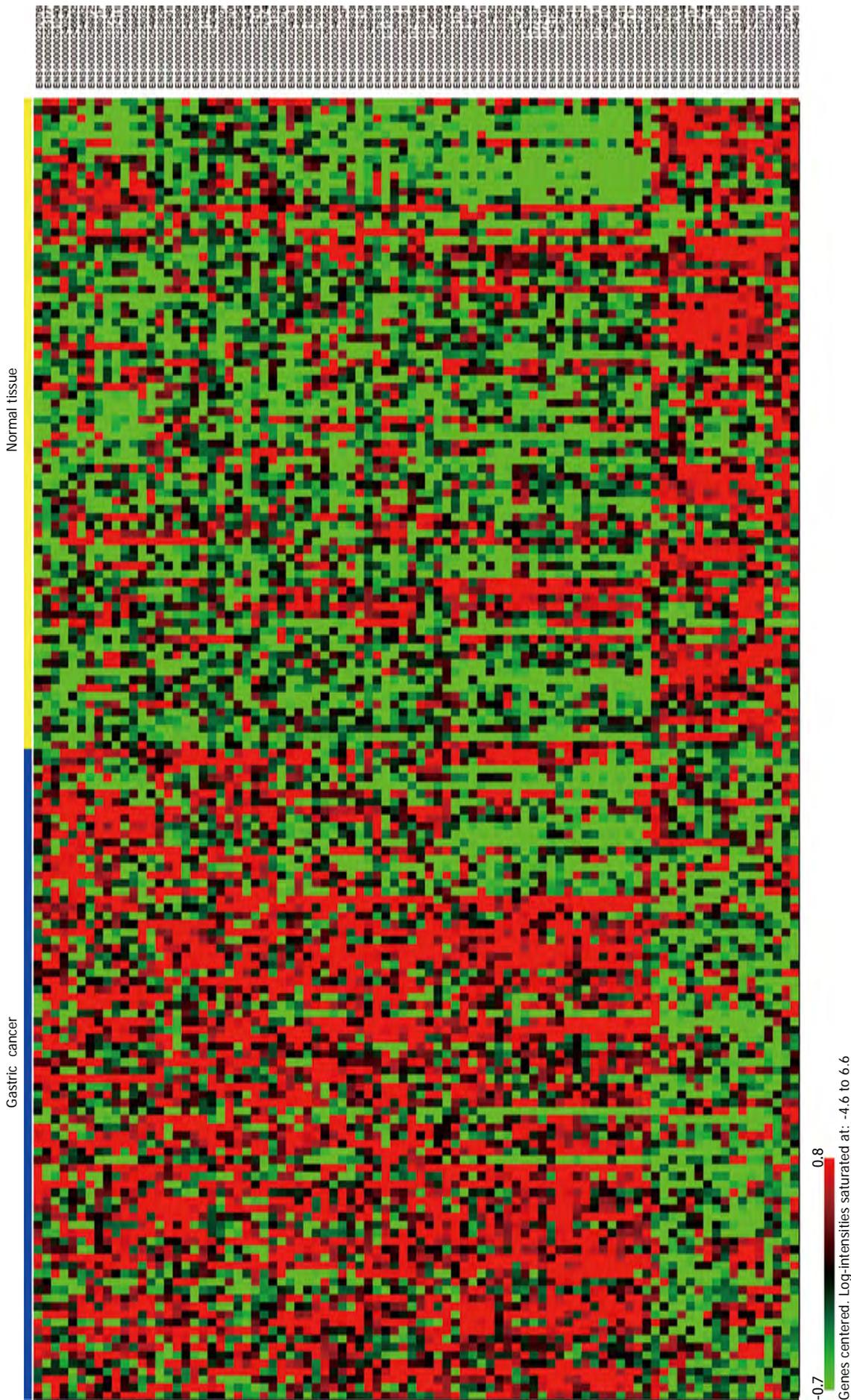
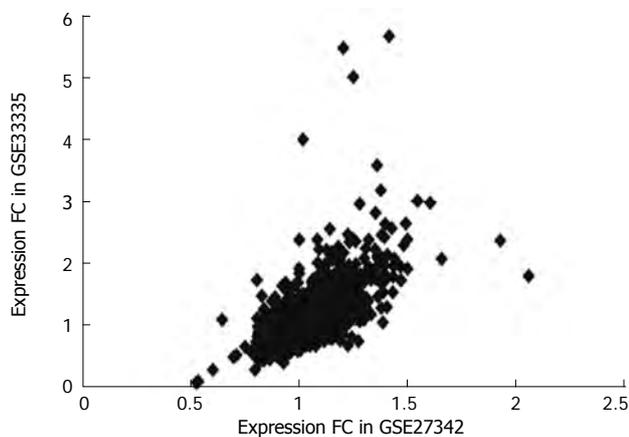


Figure 2 Clustering heatmap of 80 paired samples based on the 88 differentially expressed long non-coding RNAs. Each column represents one sample and each row represents one long non-coding RNA. Gene expression levels are indicated as follows: red, high expression; green, low expression.

**Table 1** Top 30 long non-coding RNAs differentially expressed in gastric cancer

| Ensembl gene ID | Fold change | Adjusted <i>P</i> -value | Gene biotype         | HGNC symbol  |
|-----------------|-------------|--------------------------|----------------------|--------------|
| ENSG00000222041 | 1.92678     | 6.89E-09                 | lincRNA              | LINC00152    |
| ENSG00000177410 | 1.54450     | 2.32E-06                 | lincRNA              | ZNFY1-AS1    |
| ENSG00000242125 | 1.65582     | 9.09E-06                 | processed_transcript | SNHG3        |
| ENSG00000196756 | 1.31942     | 1.26E-05                 | lincRNA              |              |
| ENSG00000255717 | 1.49039     | 2.30E-05                 | ncrna_host           | SNHG1        |
| ENSG00000226637 | 1.43434     | 2.30E-05                 | lincRNA              |              |
| ENSG00000177337 | 1.40114     | 2.30E-05                 | lincRNA              |              |
| ENSG00000249859 | 1.35920     | 2.98E-05                 | lincRNA              | PVT1         |
| ENSG00000197989 | 1.20284     | 6.10E-05                 | lincRNA              | SNHG12       |
| ENSG00000172965 | 1.23947     | 7.60E-05                 | processed_transcript |              |
| ENSG00000234741 | 1.37852     | 9.11E-05                 | non_coding           | GAS5         |
| ENSG00000177133 | 0.79465     | 9.11E-05                 | processed_transcript |              |
| ENSG00000255850 | 1.38946     | 1.45E-04                 | antisense            |              |
| ENSG00000232131 | 0.69562     | 1.77E-04                 | antisense            |              |
| ENSG00000244306 | 1.20990     | 2.64E-04                 | lincRNA              |              |
| ENSG00000234608 | 1.20458     | 2.72E-04                 | lincRNA              | C12orf47     |
| ENSG00000259758 | 1.37499     | 2.72E-04                 | antisense            |              |
| ENSG00000262001 | 1.24714     | 2.72E-04                 | lincRNA              |              |
| ENSG00000259974 | 0.53383     | 3.56E-04                 | lincRNA              | LINC00261    |
| ENSG00000237248 | 1.32118     | 3.89E-04                 | lincRNA              |              |
| ENSG00000249395 | 1.49799     | 4.04E-04                 | lincRNA              |              |
| ENSG00000175061 | 1.21582     | 4.68E-04                 | non_coding           | C17orf76-AS1 |
| ENSG00000253352 | 1.39461     | 4.68E-04                 | lincRNA              | TUG1         |
| ENSG00000260920 | 1.46802     | 5.75E-04                 | sense_overlapping    |              |
| ENSG00000248309 | 0.88939     | 5.94E-04                 | lincRNA              |              |
| ENSG00000231806 | 1.36155     | 6.48E-04                 | lincRNA              |              |
| ENSG00000223482 | 1.23001     | 6.48E-04                 | antisense            |              |
| ENSG00000227076 | 1.22709     | 6.48E-04                 | sense_intronic       |              |
| ENSG00000225241 | 1.20664     | 6.48E-04                 | lincRNA              | NBPF8        |
| ENSG00000236744 | 0.74935     | 6.48E-04                 | processed_transcript |              |

HGNC: Human Genome Nomenclature Committee; TUG1: Taurine upregulated 1; SNHG: Small nucleolar RNA host gene.



**Figure 3** Distribution of expression differentials between experimental data set GSE27342 and validation data set GSE33335.

viously reported in human cancers. For example, TUG1 is upregulated in bladder urothelial carcinoma and high TUG1 expression levels were associated with high grade and stage carcinomas<sup>[22]</sup>. Knockdown of TUG1 induced cell proliferation inhibition and apoptosis. Another candidate, UCA1, is dramatically upregulated in bladder cancer, suggesting it may be a very sensitive marker for bladder cancer<sup>[23]</sup>. Exogenous expression of UCA1 enhanced tumorigenicity, invasive potential, and drug resistance in

BLS-211 cells<sup>[24]</sup>. Also, PVT1, located in 8q24 and amplified and overexpressed in ovarian and breast cancer, increases cell proliferation and inhibits apoptosis<sup>[25]</sup>. In light of published results in other cancers, we hypothesize that these lncRNAs may play an important role in the development of gastric cancer and are potential candidates for new biomarkers and therapeutic targets in gastric cancer.

Recently, H19 was shown to be upregulated in gastric cancer and its overexpression contributes to proliferation of gastric cancer cells<sup>[37]</sup>. In our study, the fold change of H19 in gastric cancer versus normal tissue is 1.378 with an adjusted *P*-value of 0.043. Though the difference was not statistically significant with our threshold (an adjusted *P*-value less than 0.001), the trend is consistent with the early report. However, our analysis may miss some lncRNAs that other groups have demonstrated to be involved in the development of gastric cancer due to the different distributions of the patient populations in terms of age, gender and cancer subtype and stage. We are also interested in exploring which lncRNAs are differentially expressed in different stages of gastric cancer. Unfortunately, the majority of gastric cancer samples in GSE27342 are stage III (54/72), making it is challenging to identify differentially expressed lncRNAs based on the stage of disease.

In conclusion, we presented global lncRNA expression profiles in gastric cancer by mining existing microar-

ray data sets. We identified a set of lncRNAs that were differentially expressed in gastric cancer, revealing potential candidates for gastric cancer biomarkers, potentially improving diagnosis and therapy.

## COMMENTS

### Background

Long non-coding RNAs (lncRNAs) are an important class of regulatory transcripts involved in a variety of biological functions. While they are aberrantly expressed in many types of cancers, their expression patterns and biological functions in gastric cancer remain unknown.

### Research frontiers

lncRNA expression profiles are often investigated with tiling arrays, RNA sequencing or lncRNA-specific microarrays. Existing microarray data represent unique probes specific to lncRNAs, suggesting lncRNA expression profiling may be achieved by mining existing microarray data.

### Innovations and breakthroughs

The authors re-annotated the probes of the Affymetrix Human Exon 1.0 ST array and identified probes uniquely mapping to lncRNAs at the gene level. By analyzing a publicly available data set, a set of lncRNAs differentially expressed in gastric cancer were identified.

### Applications

The study results suggest lncRNAs play an important role in the development of gastric cancer and have the potential to be used as molecular diagnostic markers and therapeutic targets in gastric cancer.

### Terminology

lncRNAs are non-protein coding transcripts having a size greater than 200 nucleotides. This limit distinguishes lncRNAs from small non-coding RNAs such as microRNAs, short interfering RNAs, Piwi-interacting RNAs, and small nucleolar RNAs.

### Peer review

The authors investigated the expression patterns of lncRNAs in gastric cancer by mining existing microarray data sets. A set of lncRNAs differentially expressed in gastric cancer were identified. These results are interesting and suggest that lncRNAs may play an important role in gastric cancer.

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**P-Reviewers** Chen WX, Mann O, Martin-Villa JM  
**S-Editor** Zhai HH **L-Editor** A **E-Editor** Zhang DN

## Double-balloon enteroscopy in small bowel tumors: A Chinese single-center study

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Received: February 4, 2013 Revised: March 30, 2013

Accepted: May 16, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To analyze the clinical characteristics of small bowel tumors detected by double-balloon enteroscopy (DBE) and to evaluate the diagnostic value of DBE in tumors.

**METHODS:** Four hundred and forty consecutive DBE examinations were performed in 400 patients (250 males and 150 females, mean age  $46.9 \pm 16.3$  years, range 14-86 years) between January 2007 and April 2012. Of these, 252 patients underwent the antegrade approach, and 188 patients underwent the retrograde approach. All the patients enrolled in our study were suspected of having small bowel diseases with a negative etiological diagnosis following other routine examinations, such as upper and lower gastrointestinal endoscopy and radiography tests. Data on tumors, such as clinical information, endoscopic findings and opera-

tion results, were retrospectively collected.

**RESULTS:** Small bowel tumors were diagnosed in 78 patients, of whom 67 were diagnosed using DBE, resulting in a diagnostic yield of 16.8% (67/400); the other 11 patients had negative DBE findings and were diagnosed through surgery or capsule endoscopy. Adenocarcinoma (29.5%, 23/78), gastrointestinal stromal tumor (24.4%, 19/78) and lymphoma (15.4%, 12/78) were the most common tumors. Among the 78 tumors, 60.3% (47/78) were located in the jejunum, and the overall number of malignant tumors was 74.4% (58/78). DBE examinations were frequently performed in patients with obscure gastrointestinal bleeding (47.4%) and abdominal pain (24.4%). The positive detection rate for DBE in the 78 patients with small bowel tumors was 85.9% (67/78), which was higher than that of a computed tomography scan (72.9%, 51/70). Based on the operation results, the accuracy rates of DBE for locating small bowel neoplasms, such as adenocarcinoma, gastrointestinal stromal tumor and lymphoma, were 94.4%, 100% and 100%, respectively. The positive biopsy rates for adenocarcinoma and lymphoma were 71.4% and 60%, respectively.

**CONCLUSION:** DBE is a useful diagnostic tool with high clinical practice value and should be considered the gold standard for the investigation of small bowel tumors.

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**Key words:** Double-balloon enteroscopy; Small bowel tumors; Diagnosis; Capsule endoscopy; Endoscopic findings

**Core tip:** This was a single-center study with a large sample size of patients who underwent 440 consecutive double-balloon enteroscopy (DBE) examinations. The detection rates of various tumors, location of the

lesions, histological analyses and reasons for DBE were evaluated. Differences in the rates of detecting small bowel tumors between abdominal computed tomography, capsule endoscopy and DBE were compared. Based on the operation results, we analyzed the accuracy of DBE for locating neoplasms in addition to its positive biopsy rate. DBE's high clinical practice value indicated that it should be considered as the gold standard for small bowel tumors.

Chen WG, Shan GD, Zhang H, Li L, Yue M, Xiang Z, Cheng Y, Wu CJ, Fang Y, Chen LH. Double-balloon enteroscopy in small bowel tumors: A Chinese single-center study. *World J Gastroenterol* 2013; 19(23): 3665-3671 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3665.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3665>

## INTRODUCTION

Long considered to be a "black box" in the GI tract, the small bowel has been inaccessible to the endoscopist because of its anatomy, location and tortuosity. Small bowel tumors are relatively rare disorders and account for 3%-6% of all digestive neoplasms, most of which are malignant, and represent only 1.1%-2.4% of gastrointestinal malignancies<sup>[1]</sup>. This low incidence may be ascribed to its unique physiological features, which include an alkaline environment, fluidity, low bacterial count and a high level of IgA in the small intestine<sup>[2]</sup>. The diagnosis and management of small bowel tumors are formidable tasks for physicians.

The advent of capsule endoscopy (CE) and double-balloon enteroscopy (DBE) has completely changed our approach and launched a new era for small bowel diseases. DBE is a relatively noninvasive method, with a diagnostic yield of approximately 43%-80%<sup>[3,4]</sup>. Total enteroscopy can be achieved through antegrade and retrograde procedures. Compared with the use of CE in small bowel tumors, DBE has the particular advantage of biopsy and therapeutic potential, such as endoscopic stenting, balloon dilatation and localization before operation<sup>[5]</sup>. Additionally, DBE is not contraindicated in patients with stenosis of the intestine or an obstruction caused by neoplasms.

From January 2007 to April 2012, 440 examinations were performed in 400 patients, of whom 78 were diagnosed with small bowel tumors. However, little data involving large patient samples are available regarding the diagnostic value of DBE for small bowel tumors in China. In this context, our study was conducted to determine the characteristics of small bowel tumors in patients undergoing DBE and to evaluate the clinical value of DBE.

## MATERIALS AND METHODS

### Patients

A retrospective, descriptive study involving all patients

who were admitted to our hospital for DBE from January 2007 to April 2012 was conducted. Four hundred patients were enrolled in the present series (250 males and 150 females with a mean age of  $46.9 \pm 16.3$  years, range 14-86 years). The indications included the following: obscure gastrointestinal bleeding (OGIB) in 149 cases, abdominal pain in 123 cases, chronic diarrhea in 40 cases. The other 88 cases involved weight loss, abnormalities on computed tomography (CT) scan or CE, and anemia. The main characteristics of the patients are shown in Table 1. All the patients were suspected of having small bowel diseases, and other routine examinations, such as gastroscopy, colonoscopy, abdomen CT, and radiography, did not reveal an etiological diagnosis. The data collected included age, sex, the indication for DBE, the insertion length, the diagnosis and the results of the operation.

### DBE system and procedure

All DBE examinations were performed with a Fujinon enteroscope (EN450-P5/20, Fujinon, Inc, Saitama, Japan). The operating system consisted of a mainframe, an enteroscope, an overtube and an air pump. Two soft latex balloons, which could be inflated and deflated, were attached to the tip of the enteroscope and overtube. The balloons were connected to a pump that modulated the air automatically through an air channel in the endoscope, according to the different balloon pressures required. To reduce friction between the enteroscope and the overtube, olive oil and water were added as lubricants to the lacuna between them during the operation. When the procedure was performed as described by Yamamoto<sup>[6]</sup>, the endoscope achieved deep advancement into the small bowel using the overtube in coordination with the serial inflation and deflation of the balloons.

DBE was performed *via* the oral, anal or both approaches at the discretion of the endoscopist and according to the presumed location of the suspected lesions. When the location was uncertain, the oral approach was preferred.

### Preoperative preparation

For both the antegrade and retrograde approaches, preparation included overnight fasting and the consumption of three boxes of polyethylene glycol electrolyte (69.56 g  $\times$  3) diluted in 3000 mL of water 5-6 h before the examination.

DBE was carried out under conscious or deep sedation when required. Sedation was achieved with the help of an anesthesiologist. Conscious sedation required the intravenous injection of midazolam and meperidine. General anesthesia was indicated for select patients who were administered a combination of propofol and fentanyl. Patients who underwent DBE *via* the oral approach with deep sedation requested a tracheal cannula. The cardiovascular risk status of the patients was evaluated before the examination. During DBE, oxygen was administered along with electrocardiographic monitoring when necessary.

**Table 1 Patient characteristics *n* (%)**

| Characteristic          | <i>n</i> = 400      |
|-------------------------|---------------------|
| Age, yr, median (range) | 46.9 ± 16.3 (14-86) |
| Sex (male/female)       | 250/150             |
| Reasons for DBE         |                     |
| OGIB                    | 149 (37.3)          |
| Abdominal pain          | 123 (30.7)          |
| Chronic diarrhea        | 40 (10.0)           |
| Others                  | 88 (22.0)           |
| Tumors detected by DBE  | <i>n</i> = 78       |
| OGIB                    | 37 (47.4)           |
| Abdominal pain          | 19 (24.4)           |
| Intestinal obstruction  | 8 (10.3)            |
| Others                  | 14 (17.9)           |

DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding.

### Statistical analysis

The SPSS 16.0 software package was used for statistical analysis. Count data were expressed as a percentage, and measurement data were expressed as the mean ± SD. Differences were evaluated with the  $\chi^2$  test. We used Fisher's exact probability when the theoretical frequency was less than 5.  $P < 0.05$  (two sided) was considered to be statistically significant.

## RESULTS

In this study, 440 DBE procedures were performed in 400 patients (252 antegrade, 188 retrograde); 40 patients underwent both antegrade and retrograde procedures. Two patients completed the entire small intestine examination all at once *via* the oral approach. Seventy-eight cases of small bowel tumors were detected, giving a positive rate of 19.5% (78/400). Clinically positive DBE findings were observed in 67 patients.

All procedures were successfully performed, except for three patients who had a perforation after the examination. No hemorrhage, acute pancreatitis or other serious complications occurred. Uncomfortable feelings, such as nausea, abdominal distension and abdominal pain, occurred in some cases during the examination. However, these symptoms were transient and tolerable. The complication rate was 0.68% (3/440) in our study group.

### Tumors detected in our study

More than 10 types of tumors (Table 2) were found in our study. The majority of these tumors were adenocarcinoma, followed by gastrointestinal stromal tumor (GIST) and lymphoma. In contrast, some tumors had a low detection rate, such as lipoma, metastatic carcinoma and hamartoma. Some extremely rare cases, such as duodenal gangliocytic paraganglioma, jejunal mesangial fibrosarcoma, inflammatory myofibroblastic tumor and serosa fibromatosis, were also found in our group. The typical endoscopic images of the main tumors are shown in Figure 1.

In the 11 patients with negative DBE results, tumors

**Table 2 Tumors detected in the present study**

| Tumor                | DBE (positive/negative) | Detection rate | Benign/malignant | Duodenum/jejunum/ileum |
|----------------------|-------------------------|----------------|------------------|------------------------|
| Adenocarcinoma       | 23 (22/1)               | 29.50%         | 0/23             | 6/16/1                 |
| GIST                 | 19 (16/3)               | 24.40%         | 6/13             | 3/12/4                 |
| Lymphoma             | 12 (9/3)                | 15.40%         | 0/12             | 1/6/5                  |
| Lipoma               | 8 (7/1)                 | 10.30%         | 8/0              | 0/8/0                  |
| Metastatic carcinoma | 8 (8/0)                 | 10.30%         | 0/8              | 3/2/3                  |
| Hamartoma            | 2 (2/0)                 | 2.60%          | 2/0              | 1/0/1                  |
| Others <sup>1</sup>  | 6 (3/3)                 | 7.70%          | 4/2              | 2/3/1                  |
| Total                | 78 (67/11)              | 100%           | 20/58            | 16/47/15               |

<sup>1</sup>1 duodenal Brunner's adenoma, 1 ileal hemangioma, 1 duodenal gangliocytic paraganglioma, 1 jejunal mesangial fibrosarcoma, 1 jejunal inflammatory myofibroblastic tumor, 1 jejunal serosa fibromatosis. DBE: Double-balloon enteroscopy; GIST: Gastrointestinal stromal tumor.

were detected through surgery or capsule endoscopy and included three lymphomas, three GIST, one adenocarcinoma and one lipoma (Table 2). The reasons for the missed diagnoses were as follows: the depth of insertion was inadequate (five cases), the choice of insertion approach was not optimal and the tumors were located at the opposite end of the intestine (four cases), and the tumors were exophytic growths with normal intestinal mucosa (two cases).

### Delineation results

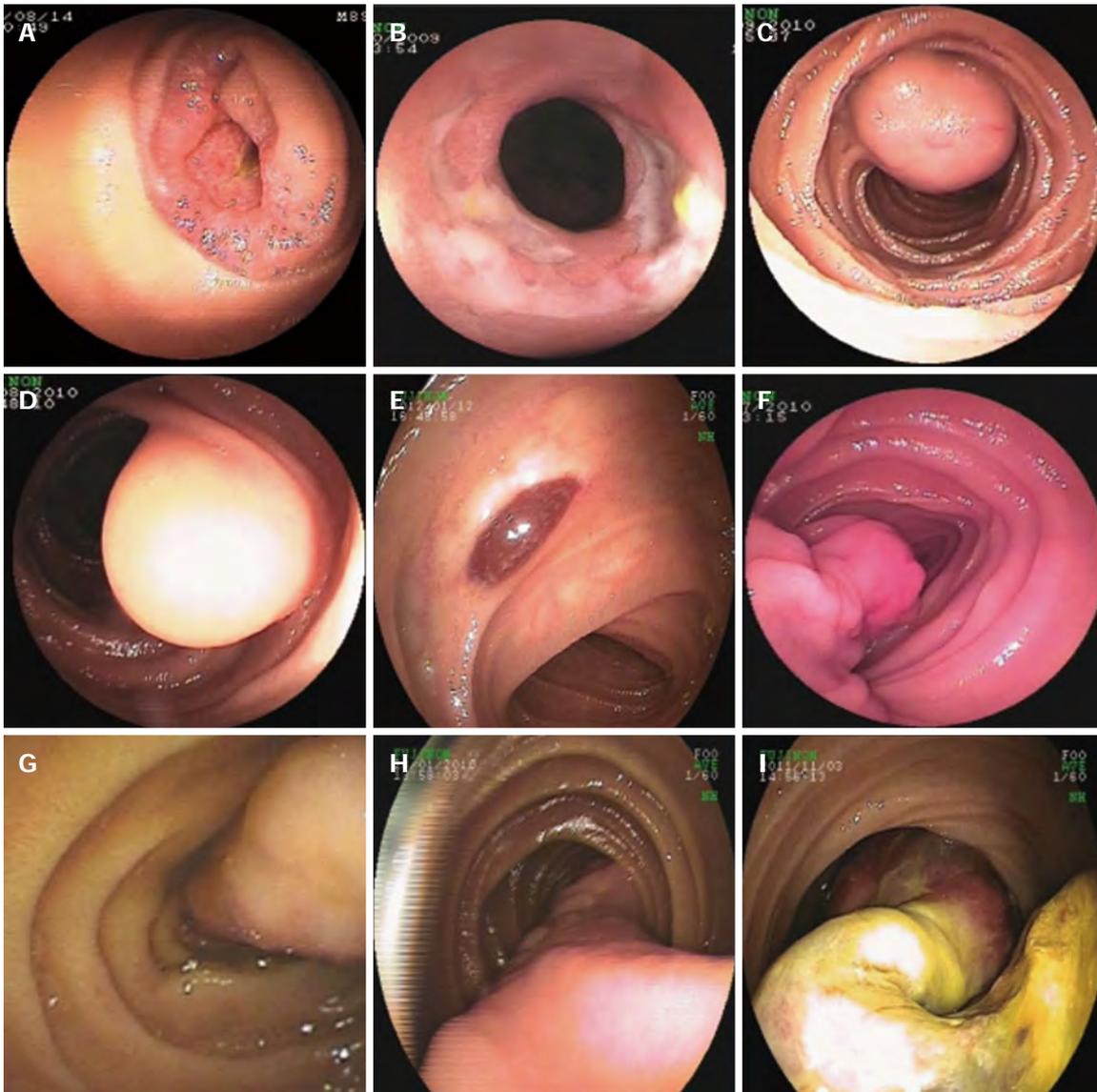
**Location of lesions:** In general, we determined the approximate location through the inserted depth of the endoscope, the size of the enteric cavity, and the shape of the mucosal fold and villi. Among the 78 tumors identified in the patients, those located in the jejunum had the highest detection rate (60.3%, 47/78). The detection rate of tumors in the duodenum was 20.5% (16/78), which was similar to that of tumors located in the ileum (19.2%). Most of the tumors, such as adenocarcinoma, lymphoma, GIST and lipoma, had a high incidence rate in the jejunum.

**Histological analysis:** Malignant tumors were found in 58 patients, with a detection rate of 74.4% (58/78). The distribution of tumors was as follows: 23 adenocarcinoma, 13 malignant GIST, 12 lymphoma, eight metastatic carcinoma and two others. In our study, compared with malignant tumors, benign tumors had lower detection rates (25.6%, 20/78); the primary benign tumors were eight lipomas and six GIST.

**Reasons for DBE:** Of the 78 patients with small bowel tumors, OGIB was the most common reason for DBE, followed by abdominal pain, intestinal obstruction and others, which included abdominal distention, vomiting, diarrhea and weight loss (Table 1).

### Comparisons between DBE and other imaging modalities

The positive detection rate for DBE in the 78 patients



**Figure 1 Typical gastrointestinal imaging.** A: Adenocarcinoma; B: Lymphoma; C: Gastrointestinal stromal tumor; D: Lipoma; E: Hemangioma; F: Gangliocytic paraganglioma; G: Brunner's adenoma; H and I: Hamartoma.

with small bowel tumors was 85.9% (67/78). Seventy of those 78 cases underwent an abdominal CT. If the CT showed a small mural mass, wall thickening with enhancement or luminal narrowing, then the results were considered positive. The positive rate for CT was 72.9% (51/70). CE was used to examine 27 cases, with a positive rate of 77.8% (21/27). Twenty-two patients underwent CE examinations before DBE; only five cases had the DBE examination first. We performed a statistical analysis of the detection rates for DBE, CE and abdominal CT. The results indicated that DBE had a higher detection rate compared with CT ( $P < 0.05$ ). There were no significant differences between DBE and CE or CE and CT ( $P > 0.05$ ).

**Operation results**

In general, we determined the types of tumors through the endoscopic features, imaging results and other aux-

iliary examinations performed before the operation. An endoscopic biopsy was carried out for some tumors. Fifty-three cases (68.0%) underwent an operation in this series of patients.

As a result of the operation, 18 cases were confirmed to have an adenocarcinoma, which was the most common tumor type in this group, followed by GIST (14 cases) and lymphoma (8 cases); the other 13 cases included metastatic carcinoma and hamartoma. Of the 53 patients who underwent an operation in our study, the positive rate of DBE in the 18 cases of adenocarcinoma was 100% (18/18), whereas the positive rate was 78.6% (11/14) for GIST and 62.5% (5/8) for lymphoma. The accuracy rates of DBE in locating small bowel neoplasms, such as adenocarcinoma, GIST and lymphoma, were 94.4%, 100% and 100%, respectively. The positive biopsy rates for adenocarcinoma and lymphoma were 71.4% and 60%, respectively.

## DISCUSSION

Although the small intestine comprises nearly 75% of the GI tract extension and nearly 90% of its mucosal surface, small bowel neoplasms are rare<sup>[7]</sup>. Recent epidemiological studies have indicated that the incidence of small bowel neoplasms has increased, particularly for malignant tumors, because of changes in diet and lifestyle<sup>[8]</sup>. Currently, the development of CE and DBE has made endoscopic examination of the entire small bowel practical. Although CE examination has revolutionized the standard small bowel evaluation<sup>[9]</sup>, some technical limitations hamper its potential usefulness and effectiveness, including its inability to sample tissue and perform therapeutic procedures. These drawbacks have been overcome with the introduction of DBE, which allows dynamic observation with the controlled movement of the endoscope, the collection of biopsies and many types of interventional procedures<sup>[10]</sup>. Under some circumstances, it can be assumed that most investigational laparotomies will be replaced by DBE.

Among the 400 patients who were submitted to examination with DBE, 67 of the 78 patients (78/400, 16.8%) who were eventually found to have small bowel tumors were detected with DBE. The results revealed that adenocarcinoma, GIST and lymphoma were the three most common tumors. In our study, adenocarcinoma had the highest incidence among the malignant neoplasms, while lipoma was the most common benign tumor. Some extremely rare tumors reported only in single cases<sup>[11,12]</sup>, including duodenal gangliocytic paraganglioma, jejunal mesangial fibrosarcoma, jejunal inflammatory myofibroblastic tumor and jejunal serosa fibromatosis, were also detected in our group. There are differences in reports regarding the incidence of small bowel tumors. In the United States, the most common small bowel tumors registered with the National Cancer Data Base are neuroendocrine (carcinoid) (44%), adenocarcinoma (33%) and lymphoma (17%)<sup>[13]</sup>. In contrast, in a Japanese multicenter study, small bowel tumors were identified in 144 of 1035 subjects (13.9%) who underwent DBE, of which lymphoma and GIST were the most frequent<sup>[14]</sup>. The hypothesis that adenocarcinoma is the most common tumor was corroborated by Safatle-Ribeiro *et al.*<sup>[15]</sup>. These differences may be ascribed to racial differences and geographical distribution.

To date, approximately 40 different histological types of small bowel tumors, of which approximately two-thirds are malignant, have been identified<sup>[16]</sup>. Of the 78 small bowel tumors of our study, the detection rate for malignancy was 74.4%. The majority of the lesions were located in the jejunum, followed by the duodenum and ileum, which is similar to the distribution reported in the literature<sup>[17]</sup>. Most of the small bowel tumors that have been reported occurred primarily in the proximal small bowel (duodenum and jejunum), except for lymphomas, sarcomas and carcinoids<sup>[18,19]</sup>. In our study, 95.7% of the adenocarcinoma (22/23) cases were found in the proximal small bowel, and only one case was located in the ileum. Among the GIST cases, 78.9% (15/19) were

detected in the proximal small bowel. In the 12 patients with lymphomas, the incidence in the jejunum was similar to that of the ileum. All of the lipomas were in the jejunum. Therefore, for patients with no clinical evidence indicating the tumor location, DBE *via* the oral approach is recommended in patients suspected of having tumors, especially adenocarcinoma, GIST and lipoma.

In large-sample studies, OGIB is the leading indication for DBE, and the diagnostic yield for OGIB is 43%-75%<sup>[20]</sup>. In our 400 patients, OGIB (37.3%, 149/400) was the main reason for DBE, which agreed with previously reported results, followed by abdominal pain (30.8%, 123/400) and chronic diarrhea (10%, 40/400). For small bowel tumors, early symptoms are often absent or nonspecific. The study by Talamonti MS indicated that obstruction, anemia and obscure bleeding were the most common symptoms of primary lesions<sup>[21]</sup>. In our group of 78 patients with small bowel tumors, the symptoms were not obviously different from those of the other patients; the top three causes for DBE were OGIB, abdominal pain and intestinal obstruction. Therefore, our research indicates that OGIB and abdominal pain were the most common reasons for DBE in both patients with small bowel tumors and patients with other diagnoses.

DBE and CE have diagnostic superiority over other routine procedures, such as push enteroscopy, abdominal CT and small bowel angiography, in detecting small bowel lesions<sup>[22]</sup>. In small bowel tumor patients, our study demonstrated that DBE had a higher detection rate than CT (85.90% *vs* 72.90%), whereas there was no difference between DBE and CE. Abdominal CT plays a pivotal role in the diagnosis, localization and staging of neoplasms and monitoring the treatment response<sup>[23]</sup>. At the same time, this examination is convenient and can determine the route of insertion for complementary DBE; therefore, it has become the initial screening method for tumors. However, it is not sufficient for the diagnosis of mucosal or small lesions of the small bowel. In the study of Cheung *et al.*<sup>[24]</sup>, tumors measuring less than 10 mm were missed with radiological techniques. CE examination has rapidly gained acceptance as the standard for small bowel evaluation. However, false-positive or false-negative results caused by the unique anatomical features of the small bowel are limitations of capsule endoscopy<sup>[25]</sup>. Imaoka *et al.*<sup>[26]</sup> reported that two-thirds of patients in whom small bowel tumors were identified had stenosis or ulceration; CE is an inappropriate modality for those who have stenosis. The capsule retention incidence ranges from 9.7%-25% in patients with small bowel tumors, which is higher than the retention incidence in all patients receiving CE and even higher than in patients with small bowel Crohn's disease<sup>[27]</sup>. DBE examination has no risk of obstruction and allows for the biopsy of tumors, which has high diagnostic value, especially for adenocarcinoma and lymphoma. In our group of 78 tumor patients, 53 cases who underwent an operation were compared regarding the DBE results, and the results indicated that the accuracy rate of DBE in locating small bowel tumors, such as adenocarcinoma, GIST and lymphoma, was very

high. The positive biopsy rates for adenocarcinomas and lymphomas were 71.4% and 60%, respectively. All of the above results indicate that DBE possesses a high value in the qualitative and localization diagnosis of small bowel tumors and provides marked reference values for surgery.

In summary, our study results indicate that DBE examination has high clinical practice value in the diagnosis of tumors and confirms it as a useful diagnostic and therapeutic tool for the investigation of small bowel diseases. DBE can obtain direct visualization and histological characterization of small bowel tumors. DBE should be considered the gold standard for the diagnosis of small bowel tumors because of its unique advantages compared with other procedures<sup>[28]</sup>.

## COMMENTS

### Background

Small bowel tumors are relatively rare, and the diagnosis of such tumors before surgery was difficult until the advent of double-balloon enteroscopy (DBE) and capsule endoscopy (CE). Compared with CE and other routine examinations used to identify small bowel tumors, DBE has particular advantages because of its diagnostic and therapeutic capabilities.

### Research frontiers

Studies are being performed to evaluate the diagnostic value of DBE in small bowel tumors.

### Innovations and breakthroughs

This study was a single-center experience in China with a large sample size involving 440 consecutive DBE examinations. The difference between abdominal computed tomography, CE and DBE in the positive rates of detecting small bowel tumors was evaluated. At the same time, the detection rates of various tumors, the location of the lesions, the histological analysis, the reasons for DBE, the accuracy rates for localization and the positive biopsy rates for DBE were also analyzed in detail.

### Applications

This study may encourage the use of DBE in the investigation of small bowel tumors. DBE has high diagnostic and therapeutic capabilities in clinical practice; therefore, should be considered as the gold standard for small bowel tumors. In the future, more therapies for small bowel tumors will be finished through DBE.

### Terminology

Double-balloon enteroscopy (DBE): a method of enteroscopy that can lead to the observation of the small intestine *via* the mouth or anus with the help of two balloons. One balloon is attached to the tip of the endoscope and the other balloon is attached to the distal end of a soft overtube.

### Peer review

This is an interesting paper with important results, which demonstrates the importance of DBE in the diagnosis of small bowel tumors and analyzes the clinical characteristics of 78 tumor patients who underwent DBE.

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**P- Reviewers** Figueiredo P, Hondo FY, Yamamoto S

**S- Editor** Gou SX **L- Editor** Stewart GJ **E- Editor** Zhang DN



## Case-matched comparison of laparoscopy-assisted and open distal gastrectomy for gastric cancer

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Supported by The key project Grant from the Science and Technology Department of Zhejiang Province, China, No. 2011C13036-2

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Received: January 16, 2013 Revised: May 13, 2013

Accepted: May 18, 2013

Published online: June 21, 2013

### Abstract

**AIM:** To compare short- and long-term outcomes of laparoscopy-assisted and open distal gastrectomy for gastric cancer.

**METHODS:** A retrospective study was performed by comparing the outcomes of 54 patients who underwent laparoscopy-assisted distal gastrectomy (LADG) with those of 54 patients who underwent open distal gastrectomy (ODG) between October 2004 and October 2007. The patients' demographic data (age and gender), date of surgery, extent of lymphadenectomy, and differentiation and tumor-node-metastasis stage of the tumor were examined. The operative time, intraoperative blood loss, postoperative recovery, complications, pathological findings, and follow-up data were compared between the two groups.

**RESULTS:** The mean operative time was significantly longer in the LADG group than in the ODG group ( $259.3 \pm 46.2$  min vs  $199.8 \pm 40.85$  min;  $P < 0.05$ ), whereas intraoperative blood loss and postoperative complications were significantly lower ( $160.2 \pm 85.9$  mL vs  $257.8 \pm 151.0$  mL; 13.0% vs 24.1%, respectively,  $P < 0.05$ ). In addition, the time to first flatus, time to initiate oral intake, and postoperative hospital stay were significantly shorter in the LADG group than in the ODG group ( $3.9 \pm 1.4$  d vs  $4.4 \pm 1.5$  d;  $4.6 \pm 1.2$  d vs  $5.6 \pm 2.1$  d; and  $9.5 \pm 2.7$  d vs  $11.1 \pm 4.1$  d, respectively;  $P < 0.05$ ). There was no significant difference between the LADG group and ODG group with regard to the number of harvested lymph nodes. The median follow-up was 60 mo (range, 5-97 mo). The 1-, 3-, and 5-year disease-free survival rates were 94.3%, 90.2%, and 76.7%, respectively, in the LADG group and 89.5%, 84.7%, and 82.3%, respectively, in the ODG group. The 1-, 3-, and 5-year overall survival rates were 98.0%, 91.9%, and 81.1%, respectively, in the LADG group and 91.5%, 86.9%, and 82.1%, respectively, in the ODG group. There was no significant difference between the two groups with regard to the survival rate.

**CONCLUSION:** LADG is suitable and minimally invasive for treating distal gastric cancer and can achieve similar long-term results to ODG.

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**Key words:** Stomach neoplasms; Gastrectomy; Laparoscopy; Survival; Case matched study

**Core tip:** We retrospectively analyzed patients treated with laparoscopy-assisted distal gastrectomy (LADG) and compared the surgical and long-term outcomes of LADG and open distal gastrectomy for gastric cancer. Our analysis showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effect of lymph node dissection and distance of excision margin were as good as those of

open gastrectomy. Long-term follow-up showed no obvious differences compared to open surgery. LADG can achieve a radical effect similar to that of open surgery.

Wang W, Chen K, Xu XW, Pan Y, Mou YP. Case-matched comparison of laparoscopy-assisted and open distal gastrectomy for gastric cancer. *World J Gastroenterol* 2013; 19(23): 3672-3677 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3672.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3672>

## INTRODUCTION

Since Kitano *et al*<sup>[1]</sup> described the first early gastric cancer treated by laparoscopy-assisted distal gastrectomy (LADG), this procedure has rapidly gained popularity in both international and domestic hospitals<sup>[2]</sup>. Although the minimally invasive effect of LADG is excellent, the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies. In this study, we performed a 1:1 case matched study to retrospectively analyze patients treated with LADG in our hospital and compared the surgical and long-term outcomes of LADG and open distal gastrectomy (ODG) for gastric cancer.

## MATERIALS AND METHODS

### Patients

A total of 108 (1:1 matched) consecutive patients who underwent LADG (54) or ODG (54) between October 2004 and October 2007 at the General Surgery Department of Sir Run Shaw Hospital were included in this study. The patients were matched for the following parameters: gender, age ( $\pm 5$  years), American Society of Anesthesiologists Physical Status score (ASA), time of operation ( $\pm 6$  mo), extent of lymph node resection (standard D<sub>2</sub>), and differentiation and tumor-node-metastasis (TNM) stage of tumor. Clinical and pathological staging was determined according to the American Joint Committee on Cancer (sixth edition) TNM classification scheme.

### Surgical technique for gastrectomy

The laparoscope was introduced through a 10-mm infra-umbilical trocar while the patient lay in the supine position, followed by placement of two 5-mm assistant ports in the bilateral anterior axillary line in the lower costal margin. The working port was placed to the right external rectus, 2 cm above the umbilicus through a 12-mm trocar, and another 5-mm assistant port was placed in the left corresponding position. These 5 operating trocars were placed in a V-shaped distribution. The surgeon and the second assistant (camera operator) stood on the patient's right and the first assistant stood on the patient's left. The procedure began with the division of the greater omentum and continued with the exposure of the right gastro-omental artery and vein along the gastroduodenal

artery and intermediate vein, dissecting at their roots and the infrapyloric lymph nodes. The root of the right gastric artery was exposed along the plane of the common and proper hepatic artery and the duodenohepatic ligament and suprapyloric lymph nodes were dissected. The duodenum was dissected and transected using the endoscopic gastrointestinal anastomosis (endo-GIA) stapler after thorough dissociation of the duodenal ampulla. After retracting the stomach specimen on the left, the lymph nodes along the proximal common hepatic artery, celiac axis, and the root of the splenic artery were dissected in the order described. The root of the left gastric artery was exposed and clipped. We then opened the right diaphragmatic crura and dissected the right cardiac nodes. The stomach specimen was extracted through a 6-cm vertical median incision at the epigastrium and a Billroth II gastrojejunostomy was performed after resection of the stomach specimen.

### Variables

The patients' surgical characteristics (operative time, extent of intraoperative hemorrhage, and amount of blood transfused), postoperative recovery (time to first flatus, time to initiate oral intake, complications, and length of postoperative hospital stay), and histopathologic indices (number of resected lymph nodes, surgical margins distance) were observed and compared between the two groups. Follow-up was conducted through an outpatient service, telephone call, or mail in order to determine whether recurrences, metastasis, or death occurred.

### Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS<sup>®</sup>) version 16.0 (SPSS, Inc. Chicago, IL, United States). The differences in the measurement data were compared using the Student's *t* test, and comparisons between groups were tested using the  $\chi^2$  test or the Fisher exact probability test. The survival data were estimated using the Kaplan-Meier method. *P* < 0.05 was considered statistically significant.

## RESULTS

### Preoperative characteristics and postoperative pathologic features

The preoperative characteristics and postoperative pathologic features of the LADG group and the ODG group are summarized in Table 1. There were no differences with respect to preoperative characteristics and postoperative pathologic features between the two groups.

### Comparison of surgical indices

Both surgical approaches were completed successfully with no conversion from LADG to open operation. Surgical indices are shown in Table 2. The operative time in the LADG group was longer than that in the ODG group (*P* < 0.01), whereas blood loss and blood infusion frequency were significantly lower (*P* < 0.01). There were no significant differences in the mean number of

**Table 1** Perioperative characteristics of patients undergoing gastrectomy

| Characteristics                               | LADG<br>(n = 54) | ODG<br>(n = 54) | P value |
|---|------------------|-----------------|---------|
| Age (yr)                                      | 58.2 ± 11.9      | 58.4 ± 11.6     | 0.93    |
| Gender (M/F)                                  | 40/14            | 40/14           | 1.00    |
| BMI index (kg/m <sup>2</sup> )                | 22.6 ± 3.2       | 21.6 ± 3.9      | 0.15    |
| Complication (Yes/No)                         | 16/38            | 18/36           | 0.42    |
| ASA (I / II)                                  | 24/30            | 24/30           | 1.00    |
| Tumor size (cm)                               | 3.7 ± 1.9        | 3.5 ± 1.5       | 0.17    |
| Histology (well/moderate/<br>low/signet ring) | 19/10/14/11      | 19/10/14/11     | 1.00    |
| Lymph node metastasis (+/-)                   | 27/27            | 25/29           | 0.85    |
| TNM stage (I A / I B / II / III A / III B)    | 22/8/9/10/15     | 22/8/9/10/15    | 1.00    |

ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy; TNM: Tumor-node-metastasis; ASA: American Society of Anesthesiologists Physical Status score; M: Male; F: Female.

retrieved lymph nodes between the two groups.

### Comparison of postoperative recovery

Postoperative recovery in the two groups is shown in Table 2. The time to first flatus, time to initiate oral intake, and postoperative hospital stay were significantly shorter in the LADG group than in the ODG group ( $P < 0.05$ ).

### Comparison of postoperative complications and mortality rate

Seven (13%) patients experienced postoperative complications after LADG. One patient developed short-term gastric emptying disorder and was discharged on the 16<sup>th</sup> postoperative day after treatment with gastrointestinal decompression, gastrointestinal prokinetic agents (GIPA), and total parenteral nutrition (TPN) support therapy. One patient developed chylous fistula and was discharged 14 d after surgery following treatment with short-term fasting and TPN support. Two patients developed pulmonary infections and both patients recovered after antibiotic treatment. Three patients developed abdominal cavity effusions with complicating inflammation, one was reversed after conservative therapy, the other two were treated with abscess needle puncture and drainage under computed tomographic (CT) guidance and were discharged 16, 22, and 24 d after surgery, respectively.

Sixteen (24.1%) patients in the ODG group developed postoperative complications. One patient developed an anastomotic leak and was discharged on the 36<sup>th</sup> postoperative day after undergoing re-operation. In two patients with intra-abdominal bleeding, one was reversed after conservative therapy, the other underwent re-operation, and they were discharged 12 and 13 d after surgery, respectively. Of four patients with abdominal cavity effusions with complicating infection, one underwent laparotomy, abdominal cavity flushing drainage and was discharged 40 d after surgery, and the others were treated with abscess needle puncture and drainage under CT guidance and were discharged 18, 20, and 21 d after surgery, respectively. Three patients had pulmonary infec-

**Table 2** Comparison

| Variables                                 | LADG<br>(n = 54) | ODG<br>(n = 54) | P value |
|---|------------------|-----------------|---------|
| Surgical indices                          |                  |                 |         |
| Operative time (min)                      | 259.3 ± 46.2     | 199.8 ± 40.8    | < 0.01  |
| Blood loss (mL)                           | 160.2 ± 85.9     | 257.8 ± 151.0   | < 0.01  |
| Intraoperative blood infusion<br>(yes/no) | 1/53             | 13/41           | < 0.01  |
| Number of retrieved lymph nodes           | 27.9 ± 7.8       | 27.7 ± 10.1     | 0.94    |
| Distance of the proximal margin<br>(cm)   | 3.6 ± 1.9        | 4.4 ± 2.1       | 0.27    |
| Distance of the distal margin (cm)        | 4.3 ± 2.1        | 4.8 ± 2.3       | 0.30    |
| Postoperative recovery                    |                  |                 |         |
| First flatus (d)                          | 3.9 ± 1.4        | 4.4 ± 1.5       | 0.03    |
| Initiate fluid intake (d)                 | 4.6 ± 1.2        | 5.6 ± 2.1       | < 0.01  |
| Initiate semifluid intake (d)             | 6.0 ± 1.7        | 7.4 ± 2.4       | < 0.01  |
| Hospital stay (d)                         | 9.5 ± 2.7        | 11.1 ± 4.1      | 0.02    |

ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

tions and all recovered after antibiotic treatment. Two patients had short-term gastric emptying disorder and were discharged on the 20<sup>th</sup> and 26<sup>th</sup> postoperative day, respectively, after treatment with gastrointestinal decompression, GIPA and TPN support. Two patients developed chylous fistula and were discharged 15 and 16 d after surgery, respectively, following treatment with short-term fasting and TPN support. Two patients had wound infections which were reversed after the incision was opened and dressed. No preoperative deaths occurred in either group. Comparisons of postoperative complications and mortality rates are summarized in Table 3.

### Comparison of long-term results

Of the 108 identified patients, 96 (88.9%) were followed up and 12 were lost to follow-up. Follow-up data were available for 49 (90.1%) and 47 (87.0%) of patients treated with LADG and ODG, respectively. The mean follow-up was 60 mo (range, 5-97 mo).

In the LADG group, 11 (20.4%) patients experienced recurrence and 8 died due to the disease: one stage IA patient developed recurrence and he is still alive 64 mo after operation. Another stage I B patient died 51 mo after operation. Two stage II patients had recurrence, one is alive 57 mo after operation, and the other died. Five stage III A patients experienced recurrence, four of whom died during the follow-up period, and the remaining patient is alive 56 mo after operation. Two stage III B patients died of the disease 14 and 59 mo after operation, respectively.

In the ODG group, nine (16.7%) patients had recurrence and 8 patients died of metastatic disease: three stage IB patients died due to the disease 9, 11, 49 mo after operation, respectively; two stage II patients experienced recurrence, one of whom died, and the other is alive 79 mo after operation; another four stage III A patients also had recurrence, and they died 6, 11, 15, and 31 mo after operation, respectively.

The 1-, 3-, and 5-year disease-free survival rates were

**Table 3** Comparison of postoperative complications and mortality rate *n* (%)

| Variables  | LADG     | ODG       | <i>P</i> value |
|--|----------|-----------|----------------|
| Overall  | 7 (13.0) | 16 (24.1) | 0.03           |
| Anastomotic leakage                              | 0        | 1         |                |
| Anastomotic stenosis                             | 0        | 0         |                |
| Intra-abdominal bleeding                         | 0        | 2         |                |
| Abdominal cavity effusion complicating infection | 3        | 4         |                |
| Pulmonary infection                              | 2        | 3         |                |
| Gastric emptying disorder                        | 1        | 2         |                |
| Chylous fistula                                  | 1        | 2         |                |
| Wound infection                                  | 0        | 2         |                |
| Reoperation                                      | 0 (0.0)  | 3 (5.6)   | 0.24           |
| Mortality  | 0 (0.0)  | 0 (0.0)   | 1.00           |

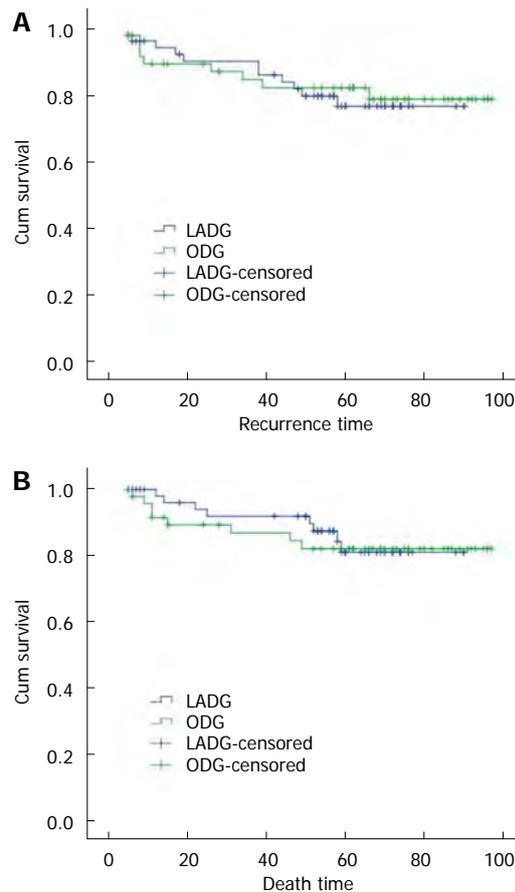
ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

94.3%, 90.2%, and 76.7%, respectively, in the LADG group and 89.5%, 84.7%, and 82.3%, respectively, in the ODG group. The 1-, 3-, and 5-year overall survival rates were 98.0%, 91.9%, and 81.1%, respectively, in the LADG group and 91.5%, 86.9%, and 82.1%, respectively, in the ODG group. There were no significant differences in these values between the two groups ( $P > 0.05$ ) (Figure 1).

## DISCUSSION

Since the first successful LADG by Kitano *et al*<sup>[1]</sup> for early gastric cancer in 1994, the use of LADG has significantly increased in both international and domestic hospitals. More and more studies, including some randomized controlled trials, have demonstrated the advantages of LADG in the treatment of early gastric cancer such as earlier recovery of ambulation and bowel movement, less pain, and a lower rate of complications. These studies confirmed that LADG is a safe and feasible surgical method<sup>[3-5]</sup>.

Kitano *et al*<sup>[6]</sup> reported the clinical findings of a multicenter trial which included 1491 cases of laparoscopy-assisted distal gastrectomy at 18 surgery centers, and found a complication rate of 12%, death rate of 0%, and recurrence rate of 0.4%. Prospective studies by Dulucq *et al*<sup>[7]</sup> revealed that postoperative complications and length of hospital stay in the LADG group were reduced compared with those in the ODG group. In the present study, complications, time to first flatus, time to initiate oral intake, and postoperative hospital stay were all significantly shorter in the LADG group than in the ODG group ( $P < 0.05$ ), showing the advantages of LADG during postoperative recovery. Several publications have reported that the rate of surgical complications following LAG are in the range of 3.8%-26.7%<sup>[8-11]</sup>, this rate was 13% in our study and was significantly lower than that in the ODG group. There was no preoperative death in our study; however, a recent survey showed that the mortality rate for open gastric cancer radical gastrectomy was 4%-6%<sup>[12]</sup>.



**Figure 1** Curves showing disease-free survival (A) and overall survival (B) after gastrectomy. ODG: Open distal gastrectomy; LADG: Laparoscopy-assisted distal gastrectomy.

Some studies have described the disadvantages of LADG compared to ODG, which include increased operative time similar to that in our study<sup>[13-16]</sup>. Operator experience, familiarity with instruments, and degree of assistant compliance are major factors influencing the operative time. During initial LADG procedures in our center, the operative time was often more than 300 min due to the above-mentioned reasons; these factors may be responsible for the significantly longer average operative time in the LADG group than in the ODG group. Other reports have also indicated that operative time with experienced surgeons was similar for LADG and ODG<sup>[17,18]</sup>.

Similar to most reports, the intraoperative blood loss in the LADG group was less than that in the ODG group<sup>[5,19]</sup>, as was blood transfusion, which reiterated the importance of careful laparoscopic manipulation during LADG. In addition, magnified vision also creates conditions for careful laparoscopic manipulation. Lack of blood is a common problem faced by many hospitals, especially in developing countries such as China; therefore, less invasive laparoscopic surgery can reduce the clinical requirement for blood and lower the rate of complications associated with blood transfusion such as virus infection and allergic reaction.

Radical resection of the tumor lymphatic drainage area should include complete resection and well incised

margins. Laparoscopic gastric cancer D<sub>2</sub> lymph node dissection is difficult and requires high technical expertise. Thus, whether laparoscopic techniques can be applied in the treatment of advanced gastric cancer is still controversial. However, the number of laparoscopically dissected lymph nodes is closely related to the surgical technique, and some studies have shown that there was no difference in the number of retrieved lymph nodes between LAG and OG<sup>[20,21]</sup>. Our analysis showed similar results in that D<sub>2</sub> lymph node dissection can be completed during laparoscopy. Park *et al.*<sup>[22]</sup> evaluated the long-term results of 239 patients who underwent LADG for the treatment of advanced gastric cancer. They found that the major recurrence was distant metastasis, whereas lymph node relapses were most frequent in para-aortic or distant lymph node metastasis; therefore, we believe that the dissection of lymph nodes around the stomach can be performed efficiently by LADG. In our study, the mean distance of the proximal and the distal margin in the LADG group was  $3.6 \pm 1.9$  cm and  $4.3 \pm 2.1$  cm, respectively, without residual cancer cells and no statistical difference compared with the OG group. We conclude that laparoscopy-assisted radical gastrectomy may help achieve results similar to those of abdominal opening on margin distance. Port-site metastasis caused by intraoperative pneumoperitoneum of LADG is another controversial issue. Port-site metastases were not reported in our study, similar to other studies, thus we conclude that pneumoperitoneum does not contribute to a higher risk of port-site metastasis.

Whether LADG can achieve effective radical cancer excision similar to that by ODG needs to be confirmed through long-term outcomes, however, large-volume long-term studies are still lacking. In a prospective trial conducted by Huscher *et al.*<sup>[11]</sup>, the long-term results of LAG and OG were similar, but the scope of lymph node dissection was identical. A retrospective analysis conducted by Hamabe *et al.*<sup>[23]</sup> revealed that the long-term results did not differ significantly between LAG and OG; however, because the application of LAG increased gradually, whereas the application of OG decreased gradually over the entire study period, the former had a short follow-up time. However, during the 10-year study, improvements in the chemotherapy program will certainly have an impact on the study results. Sato *et al.*<sup>[24]</sup> analyzed the difference between OG and LAG in relation to D<sub>1</sub>, D<sub>1+</sub>, or D<sub>2</sub> lymph node dissection using a hierarchical approach and found that the long-term results of LAG were comparable to those of OG; however, the pathological stage in the LAG group was significantly earlier than that in the OG group ( $P < 0.001$ ), which seriously affected the credibility of the analysis. Pak *et al.*<sup>[25]</sup> analyzed the follow-up of 714 patients who underwent LAG and calculated the 5-year survival rate of different TNM stages, however, their findings were not compared with those of open surgery over the same period. In addition, their follow-up duration was not long enough, as the median follow-up was less than 50 mo. Although postoperative recurrence after radical gastrectomy for cancer usually occurs within

36 mo, about 12% of cases with recurrence occur after 36 mo<sup>[26,27]</sup>. Therefore, prolonged follow-up can result in more reliable survival analysis data.

In our study, the follow-up duration was long enough as the median follow-up duration was 60 mo. In addition, we found no difference in the disease-free survival or overall survival rate between the two groups, after conducting a matched-pair design to eliminate the effects of confounding factors, including gender, age, operative period, ASA status, the extent of lymph node resection, and differentiation and TNM stage of the tumor. These results suggest that the LADG is as good as ODG with regard to long-term outcomes. The matched-pair design makes the results more credible. However, this study has several limitations, including its relatively small sample size, no survival analysis after stratification according to the TNM stage, and the use of matching research, which is a type of retrospective analysis that cannot replace prospective trials.

In conclusion, our analysis showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effect of lymph node dissection and distance of excision margin were as good as those of open gastrectomy. Long-term follow-up of LADG patients showed no obvious differences compared to open surgery. We believe that LADG can achieve a radical effect similar to that of open surgery in patients with gastric cancer.

## COMMENTS

### Background

More and more studies, including some randomized controlled trials, have demonstrated the advantages of laparoscopy-assisted distal gastrectomy (LADG) in the treatment of early gastric cancer such as earlier recovery of ambulation and bowel movement, less pain, and a lower rate of complications. These studies have confirmed that LADG is a safe and feasible surgical method, but the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies.

### Research frontiers

Although the minimally invasive effect of LADG is excellent, the therapeutic effects in adenocarcinoma still lack support from long-term follow-up studies. Whether LADG can achieve effective radical cancer excision similar to that by open distal gastrectomy (ODG) requires to be confirmed through long-term outcomes, however, large-size long-term studies are still lacking.

### Innovations and breakthroughs

In this study, the authors performed a 1:1 case matched study to retrospectively analyze the patients treated by LADG and compared the surgical and long-term outcomes of LADG and ODG for gastric cancer. This study showed that LADG is suitable and minimally invasive for treating distal gastric cancer and can achieve similar long-term results to ODG.

### Applications

This study showed that LADG has the advantages of minimally invasive surgery, rapid recovery, and fewer complications. The effects of lymph node dissection and distance of the excision margin are as good as those of open gastrectomy. Long-term follow-up showed no obvious differences compared to open surgery. LADG can achieve a radical effect similar to that of open surgery in patients with gastric cancer. These findings are helpful in decision-making for the treatment of resectable gastric cancer.

### Peer review

This is a nice paper, which is worth reading and publication. The subject and findings are significant in clinical practice, especially in the field of laparoscopy-assisted surgery. Overall, the manuscript is well prepared.

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**P- Reviewers** Catena F, Du JJ, Wang Z **S- Editor** Wen LL  
**L- Editor** A **E- Editor** Zhang DN



## Diagnostic accuracy of endoscopic ultrasound in pancreatic neuroendocrine tumors: A systematic review and meta analysis

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Received: March 2, 2013 Revised: April 6, 2013  
Accepted: April 13, 2013

Published online: June 21, 2013

**METHODS:** Only EUS studies confirmed by surgery or appropriate follow-up were selected. Articles were searched in Medline, Ovid journals, Medline nonindexed citations, and Cochrane Central Register of Controlled Trials and Database of Systematic Reviews. Pooling was conducted by both fixed and random effects model).

**RESULTS:** Initial search identified 2610 reference articles, of these 140 relevant articles were selected and reviewed. Data was extracted from 13 studies ( $n = 456$ ) which met the inclusion criteria. Pooled sensitivity of EUS in detecting a PNETs was 87.2% (95%CI: 82.2-91.2). EUS had a pooled specificity of 98.0% (95%CI: 94.3-99.6). The positive likelihood ratio of EUS was 11.1 (95%CI: 5.34-22.8) and negative likelihood ratio was 0.17 (95%CI: 0.13-0.24). The diagnostic odds ratio, the odds of having anatomic PNETs in positive as compared to negative EUS studies was 94.7 (95%CI: 37.9-236.1). Begg-Mazumdar bias indicator for publication bias gave a Kendall's tau value of 0.31 ( $P = 0.16$ ), indication no publication bias. The  $P$  for  $\chi^2$  heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

**CONCLUSION:** EUS has excellent sensitivity and specificity to detect PNETs. EUS should be strongly considered for evaluation of PNETs.

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**Key words:** Endoscopic ultrasound; Ultrasound; Endosonography; Pancreatic mass; Neuroendocrine tumors; Sensitivity; Specificity; Positive predictive value; Negative predictive value

**Core tip:** The published data on the diagnostic accuracy of endoscopic ultrasound (EUS) for detection of pancreatic neuroendocrine tumors is varied. We conducted a comprehensive review of the published literature to as-

### Abstract

**AIM:** To detect pancreatic neuroendocrine tumors (PNETs) has been varied. This study is undertaken to evaluate the accuracy of endoscopic ultrasound (EUS) in detecting PNETs.

sess the diagnostic accuracy of EUS in this setting. Our systematic review and meta-analysis has demonstrated an excellent sensitivity and specificity of EUS in this setting compared to previously published literature of other imaging modalities such as transabdominal ultrasound, computed tomography, and magnetic resonance imaging.

Puli SR, Kalva N, Bechtold ML, Pamulaparthi SR, Cashman MD, Estes NC, Pearl RH, Volmar FH, Dillon S, Shekleton MF, Forcione D. Diagnostic accuracy of endoscopic ultrasound in pancreatic neuroendocrine tumors: A systematic review and meta analysis. *World J Gastroenterol* 2013; 19(23): 3678-3684 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3678.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3678>

## INTRODUCTION

Neuroendocrine tumors of the gastrointestinal tract are rare, accounting for less than 1% of all malignancies with an estimated annual incidence of 1-4 per 100000; however, they may lead to significant morbidity and mortality<sup>[1,2]</sup>. These tumors are difficult to diagnosis, treat, and have a propensity for metastasis prior to their diagnosis given that many do not become clinically apparent until late in their course. These tumors may be found throughout the gastrointestinal tract; however, the pancreas is an area where neuroendocrine tumors are commonly discovered<sup>[3,4]</sup>.

Neuroendocrine tumors of the pancreas (PNETs) may be functional or non-functional and are mostly sporadic, although some are associated with other genetic diseases<sup>[1]</sup>. Functional PNETs often secrete active substances, such as insulin, somatostatin, gastrin, glucagon, or vasoactive intestinal peptide, which may allow them to be discovered earlier<sup>[1,5]</sup>. However, some of these PNETs are non-functional, secreting non-active substances, such as chromogranin A<sup>[1]</sup>. Serological tests have been used to determine levels of these compounds, leading to an enhanced ability to diagnosis PNET. However, these tumors tend to have metastasized by the time they are diagnosed, especially in non-functioning PNETs. Many imaging modalities have been utilized for PNETs, including trans-abdominal ultrasound (US), computed tomography (CT), and magnetic resonance imaging (MRI) with significant limitations. These imaging techniques are able to detect PNETs in 9%-48% with an estimated sensitivity of 29%-60%<sup>[6-8]</sup>. Given the need for improved imaging techniques, endoscopic ultrasound (EUS) has been evaluated as a possible diagnostic tool for PNETs.

Since its early introduction in the early 1990's, EUS has emerged as a safe and accurate technique for the diagnosis, stage, and treat a variety of lesions. A particularly useful aspect of EUS is the enhanced imaging of the pancreas. There are currently several reports of EUS in correctly detecting PNETs. However, the accuracy

of these results varies across centers. To the best of our knowledge, a meta-analysis summarizing these results has not been performed. The purpose of this investigation is to review the world literature regarding the accuracy of EUS in detecting PNET.

## MATERIALS AND METHODS

### Study selection criteria

Studies evaluating the use of EUS to characterize pancreatic neuroendocrine tumors with a gold standard (either confirmed by surgery or appropriate follow-up) were selected. From this pool, only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included.

### Data collection and extraction

Articles were searched in MEDLINE (through PubMed, an electronic search engine for published articles and Ovid), PubMed, Ovid Journals, EMBASE, Cumulative Index for Nursing and Allied Health Literature, ACP Journal Club, DARE, International Pharmaceutical Abstracts, old Medline, Medline non-indexed citations, OVID Healthstar, and Cochrane Central Register of Controlled Trials and Database of Systematic Reviews (Central). The search was performed from January 1966 to January 2012. The terms used for search were endoscopic ultrasound, EUS, ultrasound, endosonography, pancreatic mass, neuroendocrine tumors, sensitivity, specificity, positive predictive value, and negative predictive value. Study authors were contacted when the required data could not be determined from the publications. Two by two tables were constructed with the data extracted from each study. Two authors (Puli SR, Bechtold ML) independently searched and extracted the data using an abstraction form. Any differences were resolved by mutual agreement. The agreement between reviewers for the collected data was quantified using the Cohen's  $\kappa$ <sup>[9]</sup>.

### Quality of studies

Clinical trials designed with control and treatment arms can be assessed for quality of the study. A number of criteria have been used to assess the quality of a study (*e.g.*, randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)<sup>[10,11]</sup>. There is no consensus on how to assess studies designed without a control arm. Hence, these criteria do not apply to studies without a control arm<sup>[11]</sup>. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of the data and inclusion criteria. Completeness was defined as data available for true positive, false negative, false positive and true negative values of the diagnostic test (EUS). Quality Assessment of Studies of Diagnostic Accuracy Included in Systematic Reviews (QUADAS) criteria has been proposed to evaluate quality of diagnostic studies<sup>[12,13]</sup>. This was used to evaluate the studies on 14 items described in the QUADAS criteria.

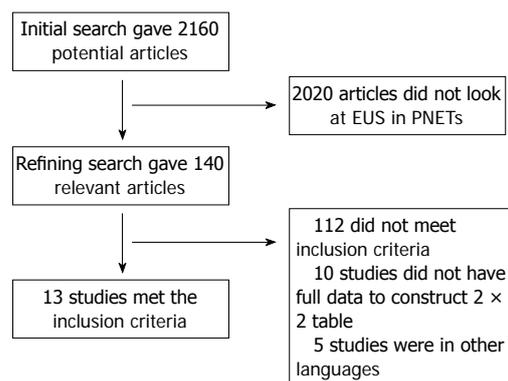


Figure 1 Search results. EUS: Endoscopic ultrasound; PNETs: Pancreatic neuroendocrine tumors.

### Statistical analysis

Meta-analysis for the accuracy of EUS in diagnosing PNETs was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. Pooling was conducted using both Mantel-Haenszel Method (fixed effects model) and DerSimonian Laird Method (random effects model). The confidence intervals were calculated using the F distribution method<sup>[14]</sup>. Forrest plots were drawn to examine how the point estimates in each study related to the summary pooled estimate. For 0 value cells, a 0.5 was added as described by Cox<sup>[15]</sup>. The heterogeneity of the sensitivities and specificities were tested by applying the likelihood ratio test<sup>[16]</sup>. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's  $Q$  test based upon inverse variance weights<sup>[17]</sup>. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate the area under the curve (AUC). The affect of publication and selection bias on the summary estimates was tested by Egger bias indicator<sup>[18]</sup>. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio<sup>[19,20]</sup>.

## RESULTS

Initial search identified 2610 reference articles, of these 140 relevant articles were selected and reviewed. Data was extracted from 13 studies<sup>[21-33]</sup> ( $n = 456$ ) which met the inclusion criteria. Search results are shown in Figure 1. All the pooled estimates given are estimates calculated by the fixed effects model. The change adjusted agreement analysis between the reviewers for data collected separately gave a kappa value of 1.0. QUADAS criteria to evaluate the quality of studies demonstrated that all the studies fulfilled 4 to 5 out of 14 described criterion.

### Accuracy of EUS to detect PNETs

Pooled sensitivity of EUS in detecting PNETs was 87.2% (95%CI: 82.2-91.2). Forrest plot in Figure 2A shows the sensitivity of EUS in individual included studies. EUS had a pooled specificity of 98.0% (95%CI: 94.3-99.6). Figure 2B shows the specificity from various studies. The

positive likelihood ratio (+LR) of EUS was 11.1 (95%CI: 5.34-22.8) and negative likelihood ratio (-LR) was 0.17 (95%CI: 0.13-0.24). The diagnostic odds ratio (DOR), the odds of having anatomic PNETs in positive as compared to negative EUS studies was 94.7 (95%CI: 37.9-236.1). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Figure 3 shows SROC curve and the area under the curve. Egger bias indicator for publication bias gave a value of 1.39 (95%CI: -1.52-4.32,  $P = 0.31$ ), indication no publication bias. Funnel plot in Figure 4 also shows that there is no publication bias in the included studies. The  $P$  for chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

Subgroup analysis was performed to see how EUS performs in detecting an Insulinoma or a Gastrinoma in the pancreas.

### Accuracy of EUS to detect an insulinoma in the pancreas

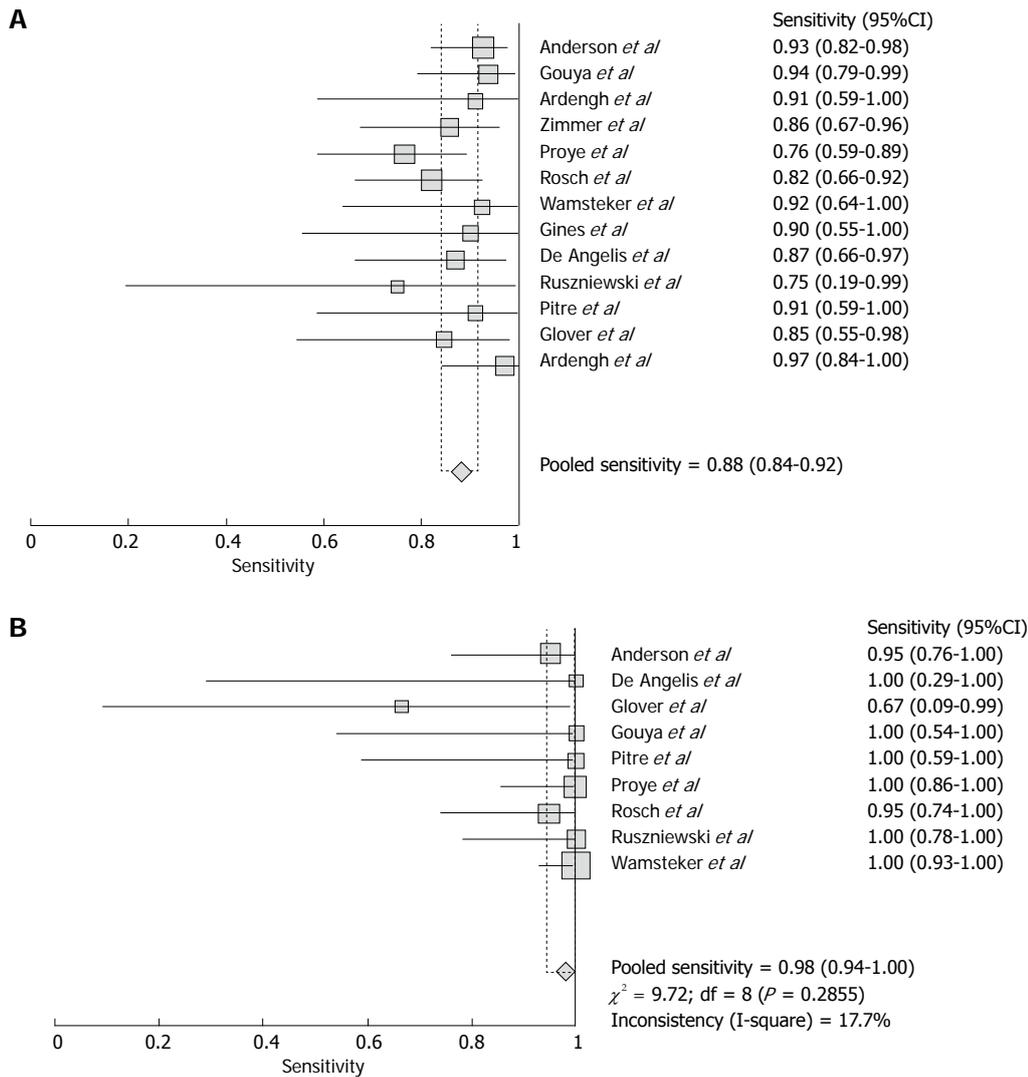
Data was extracted from 9 studies ( $n = 242$ ) which met the inclusion criteria. Pooled sensitivity of EUS in detecting a Pancreatic Insulinoma was 87.5% (95%CI: 81.2-92.3). EUS had a pooled specificity of 97.4% (95%CI: 90.8-99.7). The +LR of EUS was 8.2 (95%CI: 3.7-18.3) and -LR was 0.17 (95%CI: 0.12-0.26). The DOR, the odds of having anatomic Pancreatic Insulinoma in positive as compared to negative EUS studies was 67.6 (95%CI: 22.7-200.9). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Egger bias indicator for publication bias gave a value of -0.05 (95%CI: -4.13-4.04,  $P = 0.98$ ), indication no publication bias. The  $P$  for chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

### Accuracy of EUS to detect gastrinoma in the pancreas

Five EUS studies ( $n = 122$ ) looked at detecting Gastrinomas in the pancreas. Pooled sensitivity of EUS in detecting a gastrinoma in the pancreas was 84.5% (95%CI: 72.6-92.7). EUS had a pooled specificity of 95.3% (95%CI: 86.9-99.0). The positive likelihood ratio of EUS was 10.5 (95%CI: 4.3-25.5) and negative likelihood ratio was 0.25 (95%CI: 0.13-0.47). The diagnostic odds ratio, the odds of having anatomic gastrinoma in positive as compared to negative EUS studies was 57.3 (95%CI: 15.1-217.2). All the pooled estimates calculated by fixed and random effect models were similar. SROC curves showed an area under the curve of 0.94. Egger bias indicator for publication bias gave a value of -0.74 (95%CI: -15.19-13.72,  $P = 0.88$ ), indication no publication bias. The  $P$  for  $\chi^2$  heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

## DISCUSSION

Localizing or detecting a neuroendocrine neoplasm in the pancreas helps not only with the planning of treatment but also when detected early might improve overall prog-



**Figure 2** Forrest plot. A: Forrest plot showing sensitivity of endoscopic ultrasound (EUS) to detect pancreatic neuroendocrine tumor; B: Forrest plot showing specificity of EUS to detect pancreatic neuroendocrine tumors.

nosis. Over the past sixteen years since the introduction of EUS, this minimally invasive technique has emerged as the premiere modality to confirm pancreatic neoplasms. In this meta-analysis we sought to pool and compare the findings of 13 high quality studies concerned with the performance of EUS in the evaluation of PNETs.

The strengths of this analysis are that the literature was reviewed and data was extracted independently by two independent reviewers. Comparison of their analyses indicates excellent agreement. This meta-analysis and systematic review was written in accordance with the proposal for reporting by the Quality of Reporting of Meta-analyses statement<sup>[34]</sup>. A standardized extraction algorithm was applied and only studies which fulfilled at least four of the QUADAS criterion were included. Additionally, extensive efforts were made to ensure that the true positive, false positive, true negative and false negative results for all studies were either gleaned from the literature or acquired via direct communication with the investigators. Egger bias estimates as well as funnel plots were performed and both methods suggest that there

was no significant publication bias. Since this manuscript looks at diagnostic accuracy of a test, the study design for this meta-analysis and systematic review followed the guidelines of Standards for Reporting of Diagnostic Accuracy initiative<sup>[35]</sup>.

A core finding of this meta-analysis is that in patients with symptomatic PNETs, EUS had high sensitivity (88%) and specificity (98%) in localizing the lesion to the pancreas. EUS as a diagnostic test has a very high DOR to detect PNETs (about 95 times). If EUS localizes the lesion to the pancreas, the odds of having the correct histological neuroendocrine tumor in the pancreas is 95 times.

Additional performance characteristics for EUS were assessed in this meta-analysis which demonstrate a high +LR and low -LR. The higher the positive likelihood ratio, the better the diagnostic test performs in correctly identifying the true disease state. On the flip side, negative likelihood ratio of a diagnostic test is a measure of how well the test correctly excludes a disease stage. The diagnostic tests ability to exclude a disease state is better

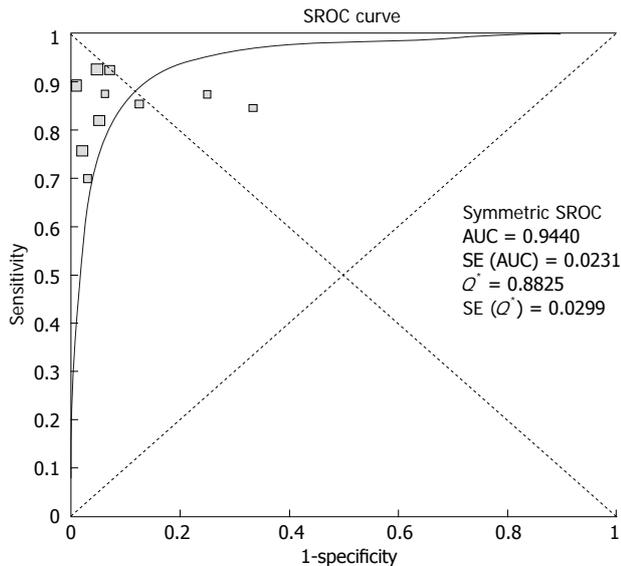


Figure 3 Summary receiver operating characteristic curves of endoscopic ultrasound to detect pancreatic neuroendocrine tumors. AUC: Area under the curve; SROC: Summary receiver operating characteristic.

if the negative likelihood ratio is lower. For PNETs, EUS has a high positive likelihood ratio and a low negative likelihood ratio. This indicates that EUS performs well in excluding as well as correctly localizing neuroendocrine tumor within the pancreas.

In a subgroup analysis to look at performance of EUS to correctly diagnose Insulinomas, EUS had high sensitivity (88%) and specificity (97%). Also, EUS had high sensitivity (85%) and specificity (95%) to detect Gastrinomas in pancreas.

A strength of this meta-analysis is that there was no heterogeneity among the studies included in this analysis. Heterogeneity among different studies was evaluated not only with test of heterogeneity but also by drawing SROC curves and finding the AUC. An AUC of 1 for any diagnostic test indicates that the test is excellent. SROC curves for EUS showed that the value of AUC was very close to 1, indicating that EUS is an excellent diagnostic test to detect PNETs.

One limitation of this meta-analysis is that none of the included studies were multicentre or randomized trials. The included studies were small in size indicating the low incidence of neuroendocrine tumors among general population. Studies on EUS with statistical significance tend to be published and cited leading to publication bias. Additionally smaller studies may show larger treatment effects due to fewer case-mix differences (*e.g.*, patients with only early localized *vs* late metastatic disease) than larger studies. This publication and selection bias may affect the summary estimates in any meta-analysis. This bias can be estimated by Egger bias indicators and construction of Funnel plots. In our meta-analysis and systematic review, bias calculations using Harbord-Egger bias indicator<sup>[18]</sup> showed no statistically significant bias. Furthermore, analysis using Funnel plots showed no significant publication bias among the studies included in

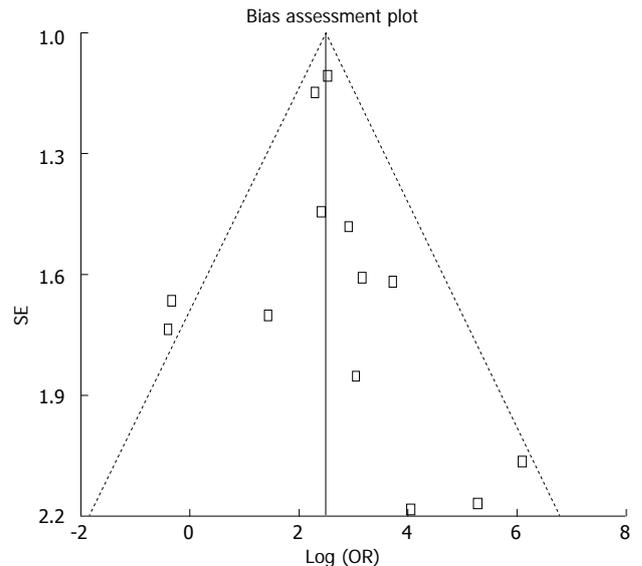


Figure 4 Funnel plot showing no publication bias in the studies included in this meta-analysis.

this analysis.

At this time the role of EUS to detect PNETs is as an adjunct to the imaging modalities such as CT scans. This is especially true when they are functional and when present in an early localized stage. More recently a single center study undertaken by Khashab *et al.*<sup>[36]</sup> showed the despite improvement in CT technology which has increased detection rates, they still missed PNETs that were smaller in size.

A subgroup analysis to see if FNA could improve the diagnostic accuracy could not be performed as there were only two studies<sup>[28,33]</sup> that included FNA data for PNETs. In a meta-analysis of 41 studies by Puli *et al.*<sup>[37]</sup>, the accuracy of EUS-FNA in the setting of solid pancreatic mass was analysed which showed a pooled sensitivity and specificity of 86.9% (95%CI: 85.5-87.9) and 95.8% (95%CI: 94.6-96.7) respectively. Given these findings it would make sense to probably conclude that EUS-FNA could replicate similar diagnostic characteristics in PNETs. In additional, Khashab *et al.*<sup>[36]</sup> also reported increased diagnostic accuracy of EUS in detected lesions to the pancreas when the were smaller in size. This was especially true for functional PNETs which tend to present early due to active peptides.

In conclusion, EUS has excellent sensitivity and specificity to localize PNETs approaching 100%. In a subgroup analysis, EUS had high sensitivity and specificity to detect Insulinoma or Gastrinoma in the pancreas. Though the studies in literature are small studies, EUS should be strongly considered for evaluation of PNETs.

## COMMENTS

### Background

Biochemically active neuroendocrine tumors often arise from the pancreas and are preceded by hormone related symptoms before metastasis. They are often small early on and could be missed on traditional imaging such as abdominal ultrasound, computed tomography (CT), and magnetic resonance imaging.

However, the performance characteristics of endoscopic ultrasound (EUS) from previously published studies have demonstrated varying results.

### Research frontiers

To our knowledge there is no published meta-analysis that has reported the diagnostic accuracy of EUS in neuroendocrine tumor of pancreas (PNETs). Several small studies have demonstrated varying results. This study is undertaken to assess pooled estimates on the diagnostic accuracy of EUS in early PNETs.

### Innovations and breakthroughs

EUS has excellent sensitivity and specificity in detecting PNETs both approaching close to 100%. A subgroup analysis is also performed for pancreatic functional PNETs *i.e.*, gastrinoma and insulinoma which showed high sensitivity and specificity. This gives additional diagnostic option in patients undergoing conventional imaging such as CT scan and scintigraphy with higher diagnostic accuracy compared to previously published data of the former tests.

### Applications

EUS can be used to identify pancreatic PNETs with high degree of diagnostic accuracy.

### Terminology

EUS has a very high sensitivity and specificity in PNETs especially in early stages aiding in early diagnosis and potential treatment.

### Peer review

The current paper of systematic review and meta-analysis investigates the diagnostic accuracy of EUS in diagnosis of PNETs. The statistical analysis performed in this study produced reliable results.

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**P- Reviewers** Bago J, Coriot R, Teoh AYB, Wong KKY, Zavoral M  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Zhang DN



## Endoscopic transluminal pancreatic necrosectomy using a self-expanding metal stent and high-flow water-jet system

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Received: November 13, 2012 Revised: February 14, 2013

Accepted: March 15, 2013

Published online: June 21, 2013

### Abstract

Walled-off pancreatic necrosis and a pancreatic abscess are the most severe complications of acute pancreatitis. Surgery in such critically ill patients is often associated with significant morbidity and mortality within the first few weeks after the onset of symptoms. Minimal invasive approaches with high success and low mortality rates are therefore of considerable interest. Endoscopic therapy has the potential to offer safe and effective alternative treatment. We report here on 3 consecutive patients with infected walled-off pancreatic necrosis and 1 patient with a pancreatic abscess who underwent direct endoscopic necrosectomy 19-21 d after the onset of acute pancreatitis. The infected pancreatic necrosis or abscess was punctured transluminally with a cystostome and, after balloon dilatation, a non-covered

self-expanding biliary metal stent was placed into the necrotic cavity. Following stent deployment, a nasobiliary pigtail catheter was placed into the cavity to ensure continuous irrigation. After 5-7 d, the metal stent was removed endoscopically and the necrotic cavity was entered with a therapeutic gastroscope. Endoscopic debridement was performed *via* the simultaneous application of a high-flow water-jet system; using a flush knife, a Dormia basket, and hot biopsy forceps. The transluminal endotherapy was repeated 2-5 times daily during the next 10 d. Supportive care included parenteral antibiotics and jejunal feeding. All patients improved dramatically and with resolution of their septic conditions; 3 patients were completely cured without any further complications or the need for surgery. One patient died from a complication of prolonged ventilation severe bilateral pneumonia, not related to the endoscopic procedure. No procedure related complications were observed. Transluminal endoscopic necrosectomy with temporary application of a self-expanding metal stent and a high-flow water-jet system shows promise for enhancing the potential of this endoscopic approach in patients with walled-off pancreatic necrosis and/or a pancreatic abscess.

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**Key words:** Acute necrotizing pancreatitis; Walled off pancreatic necrosis; Endoscopic necrosectomy; Self-expanding metal stent; Water-jet system

**Core tip:** The endoscopic transluminal management of pancreatic necrosis and/or pancreatic abscess is associated with good initial and long-term clinical success, with acceptable morbidity and mortality rates. The advantages of endoscopic management are related to its minimal invasiveness. The combination of multiple endoscopic approaches is designed to achieve the goals of any treatment strategy.

Hritz I, Fejes R, Székely A, Székely I, Horváth L, Sárkány Á, Altorjay Á, Madácsy L. Endoscopic transluminal pancreatic necrosectomy using a self-expanding metal stent and high-flow water-jet system. *World J Gastroenterol* 2013; 19(23): 3685-3692 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3685.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3685>

## INTRODUCTION

Acute pancreatitis (AP) is a disease characterized by significant morbidity and mortality. Pancreatic necrosis (PN), occurring as diffuse or focal areas of non-viable pancreatic parenchyma as defined by the 1992 Atlanta classification, is a serious complication that can develop within a few days following the onset of AP<sup>[1]</sup>.

Acute necrotizing pancreatitis (ANP) complicates 15%-20% of all cases with AP and, in patients in whom the most severe infectious complication infected necrosis develops, the mortality can be as high as 25%-70%<sup>[2]</sup>.

“Organized” or “walled-off pancreatic necrosis” (WOPN), described as relatively well-circumscribed areas of PN, evolves during the several weeks after an episode of severe ANP. This process reflects the evolution from early diffuse PN to an encapsulated, loculated form<sup>[3]</sup>.

Similarly, a pancreatic abscess (PA), a serious complication of AP, can be defined as a circumscribed intra-abdominal collection of purulent material adjacent to the pancreas, containing little or no PN, but circumscribed within a well-defined wall<sup>[1]</sup>.

The conventional treatment of infected WOPN and PA necessitates open surgical debridement, together with sump drainage and continuous lavage. The key objectives of the surgical approach are the removal of all pancreatic- and peripancreatic necrotic tissue, the evacuation of purulent infected material, and the provision of continuous and adequate drainage to promote resolution of the inflammatory processes. The best current therapeutic approach comprises complete removal of the necrotic material, but preservation of all the viable pancreatic tissue, together with the best possible supportive care, preferably at an intensive care unit (ICU).

The recommended surgical procedure of open necrosectomy is associated with a high postoperative complication rate (95%)<sup>[4]</sup>, and the median mortality rate has been reported to be 25% (range 6%-56%)<sup>[5]</sup>.

Minimally invasive approaches with high success and lower mortality rates are therefore of considerable interest, and various percutaneous and endoscopic techniques for the management of PN and PAs have been described during the last decade.

Transluminal retroperitoneal endoscopy for the debridement of infected WOPN can be considered one of the first clinical applications of natural orifice transluminal endoscopic surgery. However, the history of endoscopic necrosectomy began with the endoscopic transmural drainage of pancreatic pseudocysts and ab-

scesses<sup>[3]</sup>.

Endoscopic interventions are typically performed under conscious sedation without the need for general anesthesia. Endoscopic therapy for PN may involve endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy, stenting, and nasojejunal feeding tube placement, while a more aggressive and potent therapeutic approach is transmural (transgastric or transduodenal) endoscopic drainage<sup>[3]</sup>, with or without endoscopic ultrasonographic guidance<sup>[6,7]</sup>, followed by balloon dilation of the cystogastrostoma or cystoduodenostoma, and repeated direct endoscopic necrosectomies through the dilated fistulous tract using different accessories (*e.g.*, a polypectomy snare, Dormia basket, hot biopsy forceps, or tripod grasper)<sup>[8,9]</sup>, and stent and/or catheter placement into the necrotic cavity, followed by continuous lavage and irrigation<sup>[10]</sup> and, if required, repeated balloon dilations of the fistulous tract.

Two large, multicenter retrospective studies have demonstrated that the direct transluminal endoscopic management of PN is associated with good long-term maintenance of the high initial success rate<sup>[11,12]</sup>. Nevertheless, all of the current endoscopic techniques have obvious inherent limitations, such as the risk of air embolism, endoscopically uncontrollable bleeding, and inadequate drainage through multiple plastic stents, together with an early occlusion of the fistulous tract.

To overcome these difficulties, we demonstrate here a new and successful method of endoscopic transluminal necrosectomy (ETN); a combination of the temporary placement of a self-expanding metal stent (SEMS) into the fistulous tract and daily irrigations of the necrotic cavity with a high-flow water-jet system, using a flush knife.

## CASE REPORT

Direct endoscopic therapy was performed on 4 patients. The indication for the endoscopic transmural approach was infected PN in 3 cases and a PA in 1 case.

### Case 1

A 59-year-old female was admitted to our emergency sub-ICU with AP. At admission, she had severe upper abdominal pain that had started 24 h before. Abdominal ultrasonography revealed multiple small stones in the gallbladder, with concomitant dilation of the extrahepatic bile ducts. Laboratory tests demonstrated leukocytosis (26 g/L), and elevated blood glucose (13.4 mmol/L), liver function tests [aspartate aminotransferase (AST): 76 U/L, alanine aminotransferase (ALT): 106 U/L, gamma-glutamyl transferase (GGT): 389 U/L], and serum amylase (2262 U/L). The calculated Glasgow score was 7, which predicted a severe attack of pancreatitis. Emergency ERCP was performed, and after endoscopic sphincterotomy a small (5 mm in diameter) impacted gallstone was successfully removed from the common bile duct. Despite maximum conservative therapy, an abdominal

computed tomography (CT) scan 72 h later demonstrated severe APN with extensive necrosis and peripancreatic fluid accumulation was observed (Balthazar score: 6). After transmission to the ICU she received maximum supportive care, with mechanical ventilation, parenteral antibiotics (Imipenem and Metronidazole), parenteral volume replacement and nasojejunal feeding, epidural anesthesia and plasmapheresis. Despite the optimum therapy, her general condition was still deteriorating at the end of the third week, due to a high fever, sepsis, and multi-organ failure. Laboratory tests showed elevated C-reactive protein (CRP) and procalcitonin (PCT) levels (226 mg/L and 2.72 µg/L, respectively). A repeated CT scan demonstrated an approximately 14 cm × 11 cm × 12 cm volume of not clearly demarcated peripancreatic necrosis with accumulated fluid located between the stomach and the body of the pancreas; clinically, infection of the necrosis was assumed. After repeated surgical consultations, we decided to attempt ETN rather than open surgical necrosectomy in order to minimize the level of invasiveness in this critically-ill patient on day 21 after the onset of ANP.

### Case 2

A 62-year-old female patient was transferred from a secondary care hospital to our gastroenterological sub-ICU with upper abdominal pain and an elevated serum amylase level (4000 U/L). She had experienced the first attack of abdominal pain 2 wk earlier, though she then became asymptomatic, but the day before admission she again developed severe abdominal pain after a fatty food intake. At admission, the calculated Glasgow score was 8, indicating a severe attack of AP. On abdominal ultrasonography a large (2 cm in diameter) solitary gallbladder stone was detected. Laboratory tests demonstrated leukocytosis (21 G/L), elevated blood glucose (21 mmol/L), liver function tests (AST: 439 U/L, ALT: 339 U/L, GGT: 308 U/L), and serum amylase (1152 U/L). Emergency ERCP was performed, but, apart from the known gallbladder stone, no cholangiographic signs of biliary obstruction or gallstone pancreatitis were seen. Two days later, due to high fever and sepsis, she was transferred to the ICU. An abdominal CT scan on day 4 of hospitalization revealed ANP with extensive necrosis, duodenal compression, and an accumulation of peripancreatic fluid 45 mm in width at the bursa omentalis (Balthazar score: 7). Maximum supportive care at the ICU included parenteral antibiotics (Imipenem + Amikacin, followed by Tigecycline and Metronidazole), parenteral volume replacement, nasojejunal feeding, and epidural anesthesia; there was no need for long-term mechanical ventilation. A repeated CT scan demonstrated an 11 cm × 7 cm × 7.5 cm volume of peripancreatic necrosis with the extensive accumulation of fluid mainly between the posterior wall of the stomach and the pancreas. At the end of the third week, she still had high fever, and presented extremely high CRP (326 mg/L) and elevated PCT (2.67 µg/L) levels. From the clinical signs and laboratory results, infection of the PN was evident and therefore, after repeated surgical consul-

tations, we decided to perform ETN on day 20.

### Case 3

A 72-year-old female patient was admitted to our department with upper abdominal pain and vomiting that had persisted for 6 d before hospitalization. Her case history included hypertension and a vertebrobasilar stroke 7 years previously. At admission, laboratory tests revealed only mild leukocytosis (10.2 g/L), but high CRP (249.6 mg/L), elevated lactate dehydrogenase (741 U/L) and blood urea nitrogen (14.8 mmol/L), and mildly increased serum amylase (136 U/L). The liver function tests were normal, with the exception of mild GGT elevation (110 U/L). The clinical signs and abdominal ultrasonography (no gallstones or bile duct dilatation) led to a diagnosis of idiopathic AP. Despite maximum supportive care, with parenteral antibiotics (Ciprofloxacin and Metronidazole), parenteral volume replacement, nasojejunal feeding, and epidural anesthesia, an abdominal CT scan 3 wk later demonstrated severe ANP, with complete destruction of the pancreatic tissue, secondary antral and duodenal compression, and a 10 cm wide accumulation of peripancreatic fluid in the retrogastric region, from the liver hilum up to the spleen, but not clearly demarcated from the retroperitoneal tissue (Balthazar score: 9). After repeated surgical consultations, we decided on ETN rather than open surgical necrosectomy in order to minimize the invasiveness in this elderly patient with significant comorbidity, on day 31 after the onset of ANP.

### Case 4

A 52-year-old male patient was admitted to our department with upper abdominal pain and nausea. The symptoms had developed after a spicy and fatty food intake. The case history revealed two attacks of AP one year previously, with the consequent development of a pancreatic pseudocyst. At admission, laboratory tests indicated mild leukocytosis (12.2 g/L), CRP (15.2 mg/L), and mildly elevated blood glucose (6.5 mmol/L) levels. Liver function tests were normal, but the serum level of amylase was increased (653 U/L). On abdominal ultrasonography a large (11 cm × 4 cm) pancreatic pseudocyst was seen, without signs of bile duct dilation. A temporary improvement was achieved on supportive care, but on day 7 of hospitalization a sudden deterioration was observed and the patient was transferred to the ICU. An abdominal CT scan revealed a PA (8.5 cm × 5 cm). Despite maximum supportive care with mechanical ventilation, parenteral antibiotics (Imipenem and Metronidazole, followed by Amikacin), parenteral volume replacement, and nasojejunal feeding, no improvement was achieved. After repeated surgical consultations, we decided to perform ETN on day 13 after the onset of AP.

### Endoscopic treatment

All patients provided informed consent before each endoscopic intervention. The endoscopic procedures were performed with the patients under conscious sedation

with intravenous benzodiazepine and/or opioid administration. ERCP was carried out when indicated.

The feasibility of endoscopic drainage was assessed by endoscopic ultrasonography with the application of a linear echoendoscope (Fujinon EG-530UT). The optimum drainage site was selected, and vessel interposition was excluded by color-flow Doppler. After infiltration of the gastric wall with 20 mL of diluted adrenaline (1:10000 in saline), the cavity was punctured with a 22-gauge needle for sample collection (for microbiological analysis). Next, the gastric wall was perforated with a cystostome (Wilson-Cook, 60 W, forced coagulation) to access the necrotic cavity, followed by dilatation of the fistulous tract up to 12 F with the outer dilatation catheter of the same device. Contrast medium was then injected to assess the extent and borders of the cavity. After this, a 480 cm long, 0.035 inch diameter guidewire (Tracer Wire Guide; Wilson-Cook) was carefully advanced through the cystostome to form at least two loops inside the cavity, so as to facilitate safe catheter exchange. The puncture site was dilated with a wire-guided biliary balloon up to 10 mm and 1.5 psi (Olympus Endoscopy). Finally, a 6 cm long, 10 mm wide throughout, non-covered, biliary SEMS (Micro-Tech Europe) was positioned and opened inside the fistulous tract in order to make a permanent and wide connection between the necrotic cavity and the stomach. After opening of the SEMS, a large volume of purulent fluid and necrotic material emptied spontaneously into the stomach, and this was further facilitated with continuous flushing and suction *via* the endoscope. We finalized the first stage of the necrosectomy process with the placement of a 12 F nasobiliary pigtail catheter into the necrotic cavity through the SEMS. During the next 5 d, physiological saline was irrigated through this catheter at a flow rate of 100 mL/h to facilitate continuous lavage and optimum drainage of the necrotic material.

We entered the stomach with a double-channel therapeutic gastroscope (Fujinon EG-530D), and removed the SEMS from the necrotic cavity without any difficulty with standard foreign body forceps 5-7 d later. ETN was performed in the following steps: (1) the therapeutic gastroscope (Fujinon EG-530D) was inserted directly into the cavity; (2) with the application of a flush knife (Fujinon Flush Knife), the purulent debris was flushed from the inner cavity wall, using the high-flow water-jet system (Fujinon JW-2 Water Pump). For high-flow irrigation, we used Betadine solution (1:10 dilution in saline); continuous suction was achieved through the wide-channel therapeutic endoscope; (3) the necrotic remnant was removed by simultaneous application of the flush knife, a Dormia basket, and hot biopsy forceps; and (4) finally, placement of a 12 F nasobiliary catheter into the necrotic cavity and a suction drain into the stomach provided continuous lavage between the endoscopic sessions.

ETN and lavage were performed daily until complete evacuation of the necrotic and purulent material (Figures 1-3).

The puncture of the necrotic cavity and placement

of the SEMS into the fistulous tract, together with naso-pancreatic drainage, was successful in all patients. Access to the cavity was created by an endoscopic puncture into a clearly bulging lesion under endosonographic guidance in 3 cases, and by using a spontaneous perforation of the cyst to the gastrointestinal lumen in Case 4. Transgastric (Cases 1-3) or transduodenal access (Case 4) was chosen in 3 and 1 cases, respectively. After 5-7 days of lavage, the SEMSs were all safely removed. No SEMS-related complications were observed. After SEMS removal, the fistulous tract was sufficiently widely open (without any need for further balloon dilation) to be entered repeatedly with a double working channel therapeutic gastroscope; this allowed permanent access for transgastric necrosectomy during the next 2-3 wk. The average number of daily necrosectomies was 2.6 (range, 2-5). No endotherapy-related complications were observed. ERCP was performed in two patients. After endoscopic management, the Sequential Organ Failure Assessment score and the sepsis improved, the decreased level of consciousness ceased, CRP levels significantly decreased (Figure 4), and a CT scan showed no further evolution of peripancreatic fluid, necrosis, or abscess. All patients, except one (Case 2), were uneventfully and completely cured after endoscopic therapy and medical treatment. Following 6 wk of further basic supportive treatment and jejunal nutrition, they could leave the hospital. The average duration of hospitalization was 63 d (range 48-74 d). The artificial transluminal fistula spontaneously closed within 3 mo.

After a temporary improvement, one patient (Case 2) died 20 d after the first endoscopic intervention, on day 38 of ICU treatment. The autopsy revealed multifocal pulmonary abscesses and severe bilateral pneumonia (an obvious complication of prolonged ventilation), but no retroperitoneal abscess and only moderate inflammatory signs at the site of ETN.

## DISCUSSION

Despite receiving maximum ICU therapy, patients with ANP can succumb rapidly. In terms of the clinical disease course, severe AP is biphasic. During the first week after the onset, there is an early mortality peak, which is known to be mainly due to the systemic inflammatory response syndrome and early multiorgan failure evoked by the exaggerated pro-inflammatory response<sup>[13,14]</sup>. In contrast, 2-3 wk after the onset of the symptoms, at the time of the maximum anti-inflammatory response and consequent immunosuppression, there is a second peak of mortality, which is known to be mainly due to the infectious complications caused by bacterial translocation, followed by sepsis and late multiorgan failure<sup>[15,16]</sup>. At this point, even maximum ICU therapy may not be able to halt or reverse the disease progression in some patients.

The standard treatment for infected and complicated WOPN is surgical intervention with open necrosectomy and drainage<sup>[17,18]</sup>. If it is considered that ANP is responsible for the multiple organ dysfunction syndrome, con-

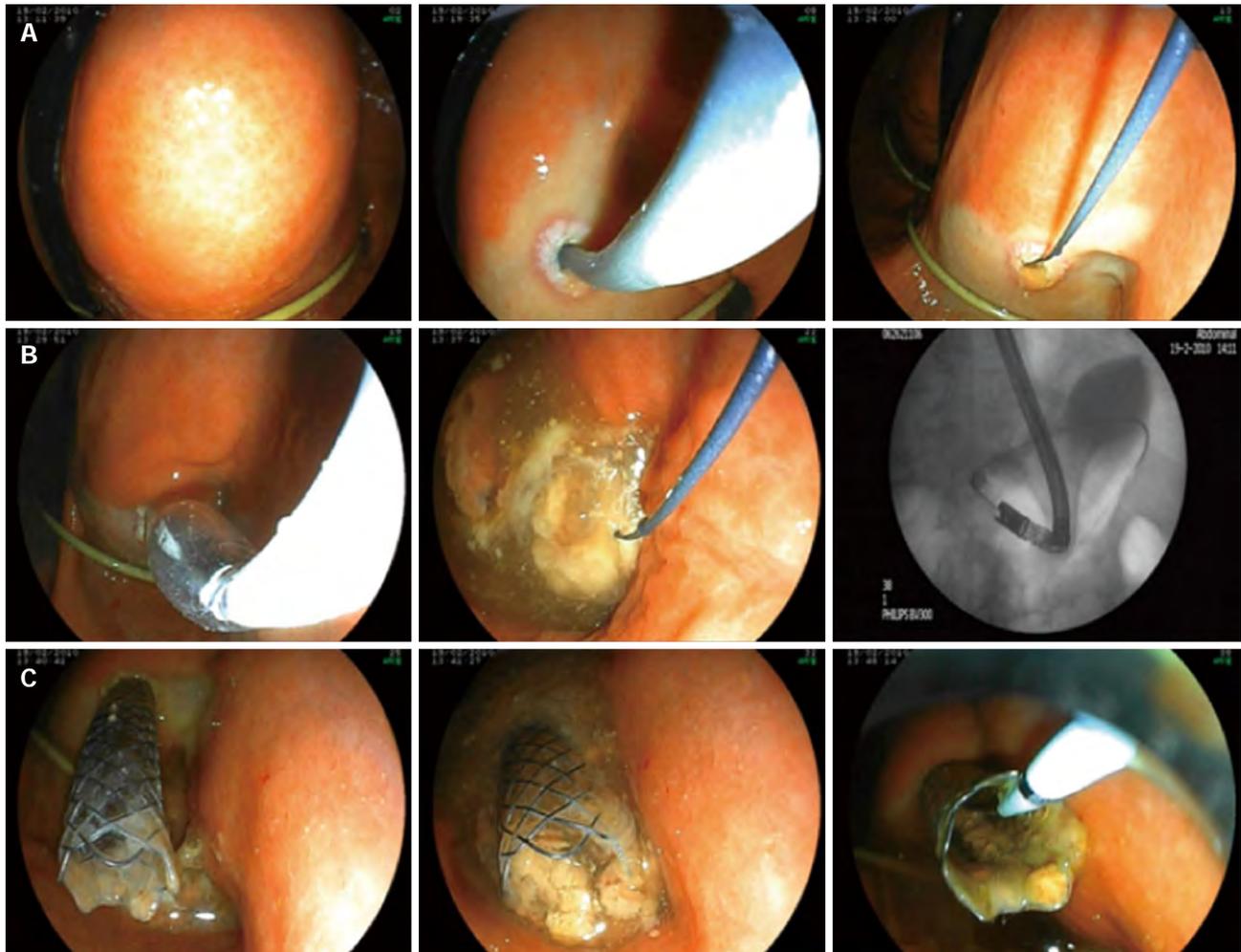


Figure 1 Endoscopic treatment of a large walled off pancreatic necrosis. A: Puncture; B: Dilatation; C: Self-expanding metal stent implantation (Case 1).

ventional early surgery (with its high complication rates) is the possible second hit, which may be the explanation, at least in part, for the high mortality. This concept is compounded by the fact that minimally invasive approaches, including minimally invasive retroperitoneal surgical necrosectomy<sup>[19]</sup>, percutaneous catheter drainage<sup>[20]</sup>, and endoscopic transluminal drainage (ETD)<sup>[3]</sup> ETN<sup>[6-12,21]</sup>, are thought to induce less physiological stress and result in less activation of the inflammatory processes than equivalent open surgery, and may therefore improve the overall outcome of patients with infected PN.

Transgastric endoscopic stent placement, mainly under endoscopic ultrasonographic guidance, is helpful for the drainage of abscesses, but its success in cases of infected WOPN is rather limited. In 2000, Seifert *et al*<sup>[6]</sup> first described transgastric access with a standard endoscope into the retroperitoneal space for necrosectomy and debridement. The comparison of ETN with ETD by Gardner *et al*<sup>[7]</sup> indicated that ETN achieves higher rates of resolution relative to ETD, without concomitant changes in the number of endoscopic procedures, the complication rate, or the time to resolution. To date, numerous workgroups have reported success and convincing re-

sults, including the low morbidity and mortality of ETN in the treatment of infected WOPN in case series<sup>[8,9,22]</sup>. Moreover, two large, multicenter retrospective studies from Germany and the United States have demonstrated that transmural minimally-invasive endoscopic treatment of WOPN is an efficacious and reproducible technique with an acceptable safety profile<sup>[11,12]</sup>. However, all studies involving transluminal pancreatic necrosectomies are challenged by the limitations in the efficacy of the endoscopic devices and accessories used for debridement (*e.g.*, Dormia baskets and polypectomy snares). Additional novel methods have recently been described that may facilitate debridement during ETN and improve the overall outcome. Belle *et al*<sup>[23]</sup> showed that, through the temporary use of a special partially-covered SEMS designed to keep the pancreaticogastrostomy open for the drainage of WOPN, clinical success (defined as the complete removal of necrotic masses without major complications) was achieved in all 4 of the study's patients. Furthermore, Antillon *et al*<sup>[24]</sup> reported one case where placement of a large-diameter removable metallic esophageal stent into the necrotic cavity, in conjunction with intensive lavage facilitated drainage, was effective; when previous multiple sessions of ETN and drainage with plastic stents had

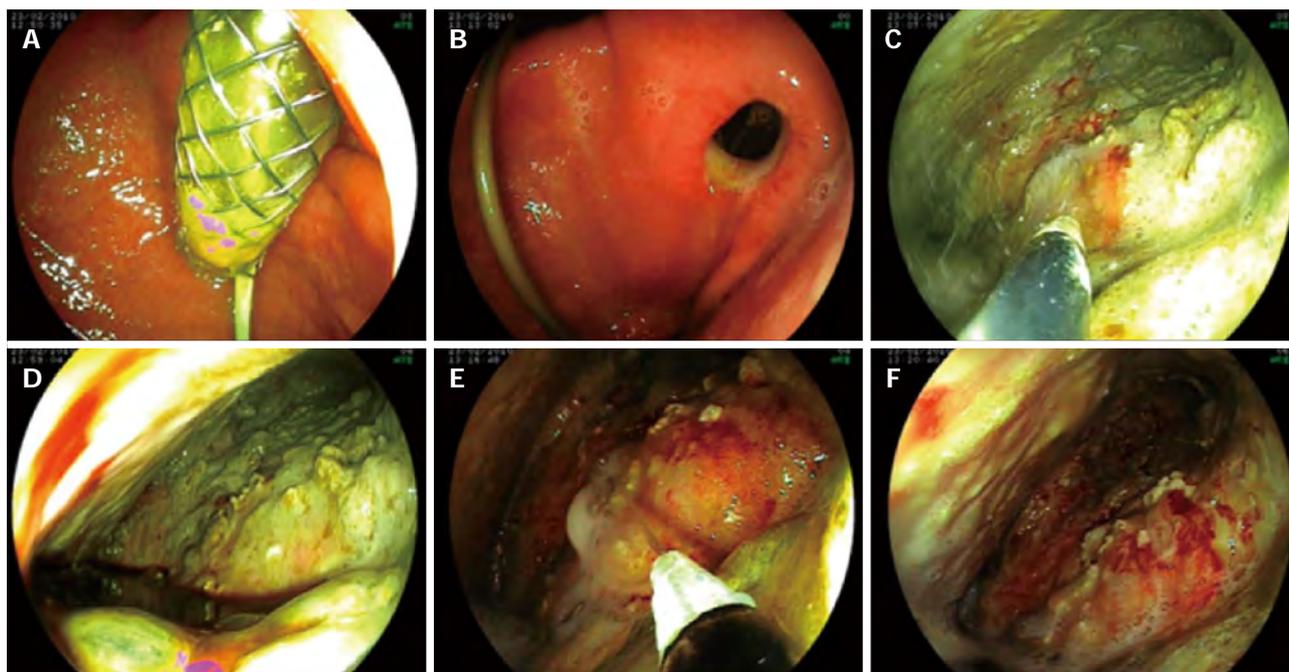


Figure 2 Endoscopic treatment. Removal of self-expanding metal stent (A, B), endoscopic transluminal necrosectomy (C, D), application of high-flow water-jet system using a flush knife (E, F) (Case 2).

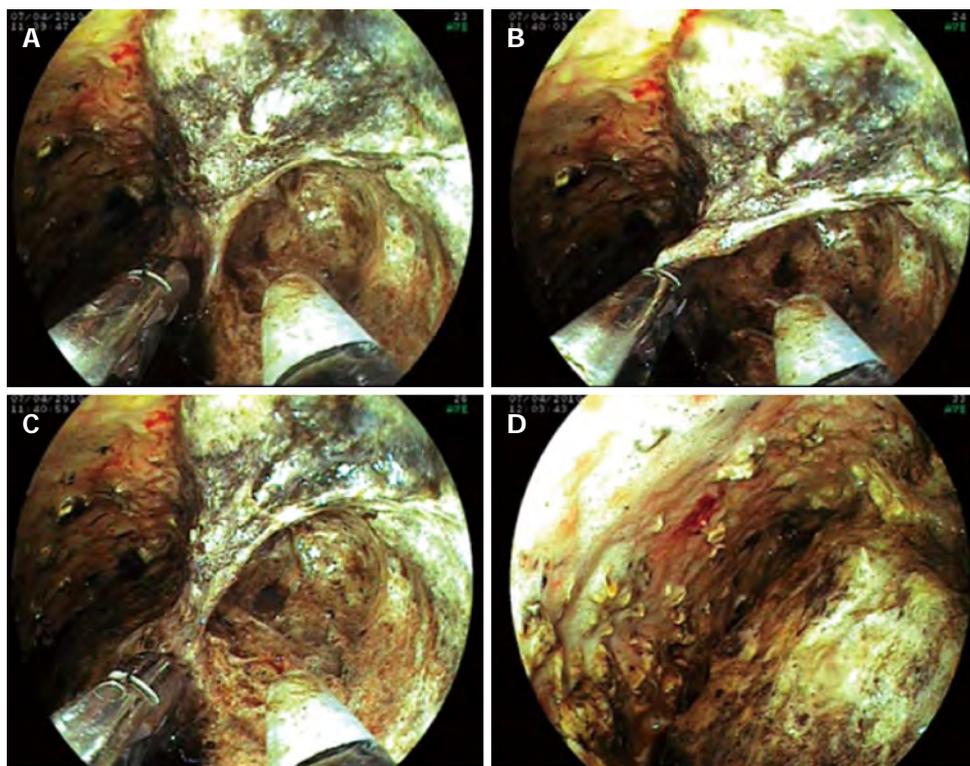


Figure 3 Endoscopic treatment. Endoscopic transluminal necrosectomy (A), application of high-flow water-jet system using a flush knife and hot biopsy forceps (B-D) (Case 3).

failed.

We have presented here a new and highly efficient method of ETN as a combination of the temporary placement of a non-covered biliary SEMS into the fistulous tract and daily irrigations of the pancreatic necrotic

cavity during direct necrosectomies with a high-flow water-jet system, using a flush knife.

The temporary placement of the SEMS resulted in a sustained, open fistulous tract with a wide orifice between the stomach and pancreatic necrotic cavity, through

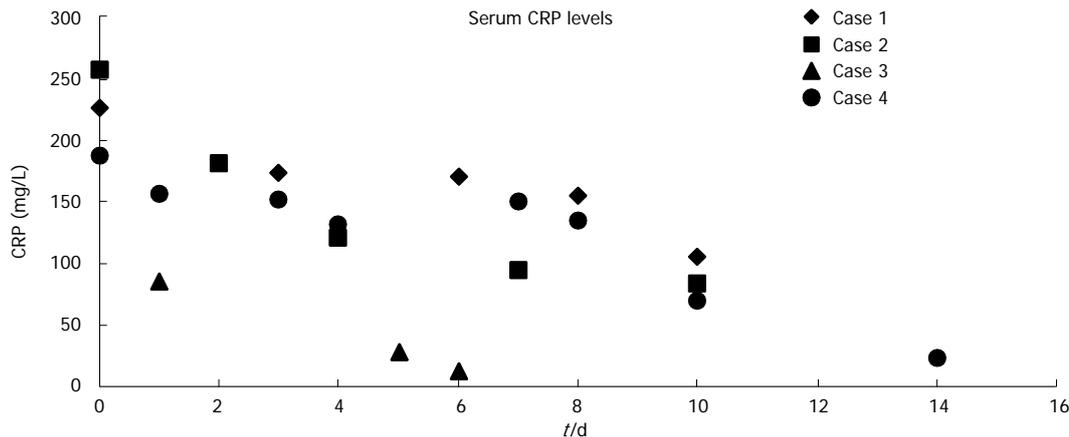


Figure 4 Effect of endoscopic transluminal therapy on C-reactive protein levels (first endoscopic intervention at day 0). CRP: C-reactive protein.

which the ETN could easily be traced and re-performed (even 2 wk after removal) without any additional plastic stent insertion. The relatively short duration in place of the SEMS may prevent stent-related potential complications such as migration, stent-end rubbing-associated cavities, or stomach wall erosions, bleeding or perforation. The necrotic tissue, including large particles, and the purulent material could be removed and/or emptied to the stomach without any complication by washing the cavity with the high-flow water-jet system, using the flush knife, and providing continuous suction *via* the double-channel therapeutic gastroscope. Only for the removal of adhesive necrotic remnants did we use other accessories (a Dormia basket and hot biopsy forceps) to facilitate the debridement. During this effective, but from a surgical aspect relatively non-aggressive approach, we experienced no procedure-related complications.

Earlier, when we have performed ETN, several hours after each direct endoscopic debridement session we observed a temporary temperature increase in the patients (data not shown). It is speculated that this may be associated with the extreme endotoxin challenge resultant from the debris removed from the necrotic cavity to the gastrointestinal tract, and the impaired gastrointestinal barrier function. We hypothesize that continuous lavage performed constantly together with suction may prevent this event.

We also included a patient with a PA (Case 4) which consisted mainly of solid particles and we had therefore decided to perform direct ETN instead of drainage alone.

We believe that this new combination of methods may lead to clinical success as a consequence of its efficacy with fewer complications and an improvement in patient comfort. However, in order to confirm and optimize this endotherapy, further studies are necessary, preferably on a larger patient population.

The endoscopic transluminal management of PN and/or PA is associated with good initial and long-term clinical success, with acceptable morbidity and mortality rates. The advantages of endoscopic management are

related to its minimal invasiveness. The combination of multiple endoscopic approaches is designed to achieve the goals of any treatment strategy.

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**P- Reviewer** Han TQ **S- Editor** Gou SX  
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## Polyarteritis nodosa clinically mimicking nonocclusive mesenteric ischemia

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Received: February 9, 2013 Revised: April 8, 2013

Accepted: April 13, 2013

Published online: June 21, 2013

### Abstract

Here, we present the case of a 74-year-old Japanese man with segmental intestinal necrosis, which developed after treatment with pulsed methylprednisolone for mononeuritis multiplex. The patient was weakly positive for myeloperoxidase (MPO)-anti-neutrophil cytoplasmic antibody (ANCA). Computed tomography and surgical findings were compatible with nonocclusive mesenteric ischemia (NOMI). He underwent small intestinal resection by emergency surgery and an intestinal fistula was made. Pathologically, necrotizing

vasculitis with fibrinoid necrosis was present in medium to small-sized arteries, which was equivalent to Arkin's classification II-IV. Most of the arteries had fibrous intimal thickening, which was considered to obstruct the arteries and thus cause segmental intestinal necrosis. A diagnosis of polyarteritis nodosa (PAN) was made, and intravenous cyclophosphamide pulse therapy was added to the therapeutic regimen. This patient was successfully treated with these multidisciplinary therapies and his stoma was finally closed. This is a very rare and indicative case of PAN weakly positive for MPO-ANCA and clinically mimicking NOMI, which occurred even after treatment with pulsed methylprednisolone.

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**Key words:** Anti-neutrophil cytoplasmic antibody; Intestinal necrosis; Myeloperoxidase; Nonocclusive mesenteric ischemia; Polyarteritis nodosa

**Core tip:** We present a patient with polyarteritis nodosa (PAN) weakly positive for myeloperoxidase-anti-neutrophil cytoplasmic antibody and clinically mimicking nonocclusive mesenteric ischemia (NOMI), which occurred after treatment with pulsed methylprednisolone for mononeuritis multiplex. The present case is not only rare but also informative, because vasculitis in medium to small-sized arteries was shown to take a few months to develop tangible signs of visceral ischemia, which can occur even after treatment with pulsed methylprednisolone, and the imaging and surgical findings of intestinal necrosis caused by PAN may resemble those of NOMI.

Shirai T, Fujii H, Saito S, Ishii T, Yamaya H, Miyagi S, Sekiguchi S, Kawagishi N, Nose M, Harigae H. Polyarteritis nodosa clinically mimicking nonocclusive mesenteric ischemia. *World J Gastroenterol* 2013; 19(23): 3693-3698 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3693.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3693>

## INTRODUCTION

Polyarteritis nodosa (PAN) is necrotizing arteritis of medium to small-sized arteries without glomerulonephritis or vasculitis in arterioles, capillaries, or venules<sup>[1]</sup>. PAN can show a wide variety of symptoms, including general symptoms, neurological manifestations, skin involvement, renal involvement, and gastrointestinal (GI) manifestations<sup>[2]</sup>. Clinically, the spectrum of GI manifestations is wide, ranging from mild transient abdominal pain to life-threatening complications requiring emergency surgery, *e.g.*, peritonitis, bowel infarction, or hemorrhage<sup>[3]</sup>. Severe GI involvements, including bowel perforation, bleeding, and pancreatitis, are independent predictive factors for poor prognosis of PAN together with age<sup>[4]</sup>. Although GI ischemia has been reported to occur at a rate of 13%-31% in PAN patients<sup>[3,5]</sup>, the prevalence of PAN itself is very low, and clinical suspicion of vasculitis is sometimes difficult in cases showing intestinal necrosis. Here, we describe a case in which a patient with PAN presented with segmental intestinal necrosis clinically mimicking nonocclusive mesenteric ischemia (NOMI) even after treatment with pulsed methylprednisolone for vasculitis.

## CASE REPORT

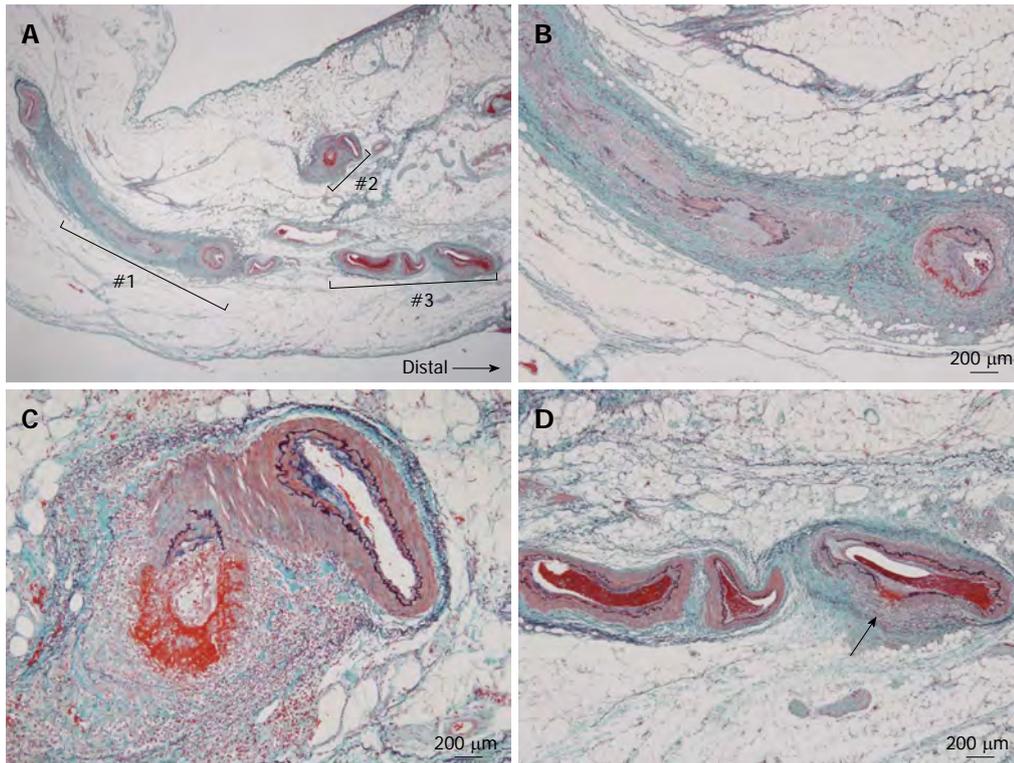
A 74-year-old Japanese man was admitted to our hospital because of mononeuritis multiplex in the left ulnar and peroneal nerves on May 26, 2012. Two months previously, he had experienced systemic muscular pain. A tentative diagnosis of polymyalgia rheumatica was made and he was treated with prednisolone (PSL) at 10 mg/d. Although steroids were partially effective in improving the patient's condition, the levels of C-reactive protein (CRP) continued to be high (18 mg/dL). He was referred to our hospital for further evaluation one month later, at which time he had no complaints other than muscular pain. His height was 172 cm and body weight was 62 kg; the patient's body weight had decreased by 3 kg from the onset of the disease. Laboratory tests indicated leukocytosis, anemia, thrombocytosis, and elevated CRP levels. The results of urine tests were negative. The patient was negative for anti-nuclear antibody and proteinase 3 (PR3)-anti-neutrophil cytoplasmic antibody (ANCA), but weakly positive for myeloperoxidase (MPO)-ANCA (23.7 U/mL; normal range 0.0-8.9 U/mL). Tests for infections, including hepatitis B surface antigen and blood culture, were negative. Torso computed tomography (CT) revealed emphysema alone, and whole-body positron emission tomography yielded negative results. Although we recommended hospitalization for diagnosis and treatment, the patient refused for personal reasons. One week later, he noticed numbness in his left hand and leg. He then developed left foot drop, so underwent a medical examination and was admitted to our hospital. On admission, his consciousness was clear, performance status was 3, body temperature was 36.3 °C, and blood pressure was 178/124 mmHg. He had general muscle weakness and sensory loss in regions supplied by the left ulnar and

peroneal nerves, and dorsiflexion of the left foot was compromised to manual muscle testing 2/5. Laboratory tests indicated elevated levels of CRP and muscular enzymes, such as creatine kinase (CK) (Table 1). Urinary analysis showed proteinuria and hematuria with a few casts. He received pulsed methylprednisolone at a dose of 1 g for 3 d followed with PSL at 60 mg/d because the underlying disease was considered to be vasculitis, and intravenous nicardipine for hypertension, which was considered to be caused by renal vascular involvement. Despite this therapy, the CRP level did not fall below 10 mg/dL and the CK level increased significantly to 9358 IU/L after 3 d of this treatment (Figure 1). At the same time, the patient complained of abdominal pain. His abdomen was flat and soft, and showed left lower quadrant pain without muscular defense or rebound. Abdominal X-ray showed a distended intestine, indicating paralytic ileus. CT revealed distended small bowel loops, gas in the small bowel, and blurred enhancement of the intestinal wall without any significant obstruction in the mesenteric arteries, suggesting NOMI (Figure 2A-C). Emergency surgery was performed on the same day. His jejunum to ileum showed segmental ischemia and necrosis over 2 m, and surgical findings were compatible with NOMI (Figure 2D). Small intestinal resection was performed and an intestinal fistula was made. Pathologically, necrotizing vasculitis with fibrinoid necrosis was present in medium to small-sized arteries, and most of the arteries had fibrous intimal thickening (Figure 3). There was no necrotizing vasculitis in arterioles, capillaries, or venules. Pathology was equivalent to Arkin's classification II-IV<sup>[6]</sup>, and a diagnosis of PAN was made. Intravenous cyclophosphamide (IVCY) pulse therapy at a dose of 500 mg/mo was added to the treatment regimen. Although various complications occurred, including deep vein thrombosis, stoma trouble, and disseminated varicella zoster virus infection, the patient recovered well and was transferred to a different hospital for rehabilitation. As his nutritional status had improved because he became able to eat a regular diet without supplemental nutrition, his stoma was closed in April 2013. He is now in complete remission with the dose of PSL tapered to 10 mg/d.

## DISCUSSION

NOMI is an acute mesenteric circulatory disorder that is not caused by organic occlusion of blood vessels<sup>[7]</sup>. The typical patient is critically ill, with severe cardiac disease or sepsis<sup>[8]</sup>. With regard to pathogenesis, intestinal vasospasm due to persistent low perfusion is thought to cause ischemic disorder due to decreased cardiac output and blood pressure<sup>[9]</sup>. Mitsuyoshi *et al*<sup>[7]</sup> reported findings that may be useful as supplemental information in diagnosis of NOMI: (1) enhancement of principal arteries could be traced to the periphery close to the marginal arteries in CT slices; and (2) the staining intensity varied in the intestinal wall in the same slice (showing a difference between regions of the intestinal wall with good and poor





**Figure 3** Histological findings of the jejunum. A: Necrotizing vasculitis of the mesenteric artery showing nodular lesions in various stages of the Arkin classification, Elastic-Masson staining; B: Higher magnification of No. 1 in A showing necrotizing vasculitis of mesenteric artery in stage II-III; C: Higher magnification of No. 2 in A showing necrotizing vasculitis of the mesenteric artery in stage II and pan-arterial necrosis with fibrinoid degeneration; D: Higher magnification of No. 3 in A showing necrotizing vasculitis of mesenteric artery in stage II, characterized by destruction of internal elastic lamina associated with fibrinoid necrosis (arrow).

biopsy to observe the vasculitis histologically<sup>[10]</sup>. In this case, necrotizing vasculitis with fibrinoid necrosis was pathologically present in medium to small-sized arteries, and fibrous intimal thickening was considered to obstruct these arteries and cause segmental intestinal necrosis. Although the pathology was equivalent to Arkin's classification II-IV, most belonged to Arkin's classification III-IV, indicating longstanding vasculitis. Therefore, it is reasonable to consider that the patient suffered from vasculitis from the beginning. Although he was treated with pulsed methylprednisolone before the onset of abdominal symptoms, fibrous intimal thickening of medium to small-sized intestinal arteries must have already occurred.

According to the Chapel Hill Consensus Conference 2012<sup>[10]</sup>, PAN is defined as necrotizing arteritis of medium to small-sized arteries without glomerulonephritis or vasculitis in arterioles, capillaries, or venules, and is not associated with ANCA, which was based on the report that ANCA was typically absent in patients with PAN<sup>[11]</sup>. In this previous report by Guillevin *et al*<sup>[11]</sup>, one patient (Table 1, No. 40) diagnosed with PAN had MPO-ANCA. Furthermore, there have been some reports presenting pathologically diagnosed PAN with MPO-ANCA positivity, all of which were reported from Japan<sup>[12-16]</sup>. The prevalence rates of ANCA and ANCA-associated vasculitis are different between Japan and Europe; microscopic polyangiitis (MPA) and MPO-ANCA are more common in Japan, while granulomatosis with polyangiitis and PR3-ANCA are more common in Europe<sup>[17,18]</sup>. These trends may be related to the reports of PAN with MPO-ANCA positivity in Japan.

Although MPO-ANCA was also weakly positive in this case, this patient clearly fulfilled the American College of

Rheumatology 1990 criteria for classification of PAN<sup>[19]</sup>, the pathological findings were typical of PAN as shown in Figure 3, and vasculitis was absent in arterioles, capillaries, and venules. Therefore, a diagnosis of PAN was made in this case. Tanaka *et al*<sup>[14]</sup> described a case of PAN with MPO-ANCA and vasculitis in mesenteric medium-sized arteries, which was confirmed by autopsy. In their case, the titer of MPO-ANCA was low and was not correlated with the severity of PAN. These points were similar to our case, and we speculated that the epitope and pathogenicity may be different from those of MPO-ANCA found in MPA. Peripheral nervous system vasculitis, hypertension, cutaneous lesions, and myalgias were reported to be more common in PAN with than without GI involvement<sup>[5]</sup>, and our patient also manifested most of these symptoms with the exception of cutaneous lesions.

The standard regimen for PAN, not related to hepatitis B virus infection, is based on a combination of corticosteroids (CS) and CY<sup>[20]</sup>. The addition of CY to CS particularly benefits patients presenting with factors associated with poor prognosis, such as GI involvement. Intermittent pulse-therapy may be as efficacious as oral CY for inducing remission, while generating fewer side effects. Treating PAN patients positive for factors associated with poor prognosis with 12 rather than 6 CY pulses significantly decreased the relapse rate and significantly increased the probability of event-free survival<sup>[20]</sup>. Plasma exchange can be prescribed for severe life-threatening PAN as combined rescue therapy, although trials have not proven its benefits when prescribed systematically for all patients with PAN<sup>[21]</sup>.

In addition, the surgical management of patients with acute abdominal syndromes has also improved, and now

**Table 1 Laboratory findings**

|                           | May 2012 | Pre-surgery | Post-surgery | Mar 2013 |
|---------------------------|----------|-------------|--------------|----------|
| Urinalysis                |          |             |              |          |
| Protein                   | 1+       | 2+          | 1+           | -        |
| Occult blood              | 3+       | 3+          | 3+           | -        |
| Red blood cell            | 5-9/HPF  | 5-9/HPF     | 5-9/HPF      | -        |
| Casts                     | +        | +           | +            | -        |
| Granular                  | < 4/HPF  | < 4/HPF     | < 4/HPF      |          |
| WBC (/μL)                 | 23700    | 40700       | 20800        | 9500     |
| Segmented cells           | 89%      | 87%         | 93%          | 56%      |
| Band cells                | 0%       | 6%          | 0%           | 0%       |
| Eosinophils               | 3%       | 0%          | 0%           | 1%       |
| Basophils                 | 0%       | 0%          | 0%           | 0%       |
| Lymphocytes               | 6%       | 2%          | 5%           | 33%      |
| Monocytes                 | 2%       | 5%          | 2%           | 10%      |
| Hb (g/dL)                 | 11.5     | 11.4        | 8.4          | 10.1     |
| PLT (10 <sup>3</sup> /μL) | 49.1     | 32.8        | 22.7         | 24.1     |
| T-Bil (mg/dL)             | 0.6      | 1.1         | 0.8          | 1.3      |
| AST (IU/L)                | 39       | 352         | 89           | 36       |
| ALT (IU/L)                | 32       | 181         | 107          | 45       |
| LDH (IU/L)                | 304      | 641         | 427          | 217      |
| ALP (IU/L)                | 368      | 403         | 301          | 326      |
| CK (IU/L)                 | 374      | 9063        | 1796         | 23       |
| TP (g/dL)                 | 6.0      | 5.6         | 5.3          | 5.1      |
| Alb (g/dL)                | 2.1      | 2.1         | 2.6          | 3.0      |
| BUN (mg/dL)               | 40       | 60          | 52           | 18       |
| Cr (mg/dL)                | 0.98     | 1.11        | 1.02         | 1.00     |
| CRP (mg/dL)               | 19.7     | 27.6        | 11.0         | 0.1      |
| MPO-ANCA (U/mL)           | 19.2     | 5.1         |              | Negative |

HPF: High-power field; WBC: White blood cells; Hb: Hemoglobin; PLT: Platelets; T-Bil: Total bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: Lactate dehydrogenase; ALP: Alkaline phosphatase; CK: Creatine kinase; TP: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; Cr: Creatinine; CRP: C-reactive protein; MPO: Myeloperoxidase; ANCA: Anti-neutrophil cytoplasmic antibody.

includes more aggressive surgical management, bowel rest, parenteral nutrition, intensive care unit support, and better wound care<sup>[5]</sup>. This patient was successfully treated with these multidisciplinary therapies, including emergency surgery followed by PSL and IVCY against PAN. Although small intestinal stoma, central venous catheter, sub nutrition, and immunosuppressive therapy caused many life-threatening complications, close monitoring and appropriate treatment resulted in complete remission of PAN and eventual closure of his stoma.

In conclusion, vasculitis in medium to small-sized arteries takes a few months to show tangible signs of visceral ischemia, and the CT and surgical findings of intestinal necrosis caused by PAN may resemble those of NOMI. Clinical awareness of vasculitis and ANCA measurement (never exclusive) are important in managing patients showing segmental intestinal necrosis.

## ACKNOWLEDGMENTS

We thank the staff of the Department of Hematology and Rheumatology, Tohoku University, for help and discussion.

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**P- Reviewers** Camara CR, Grundmann RT

**S- Editor** Gou SX **L- Editor** O'Neill M **E- Editor** Lu YJ



## Esophageal mucosal metastasis from adenocarcinoma of the distal stomach

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Received: February 4, 2013 Revised: March 22, 2013

Accepted: April 27, 2013

Published online: June 21, 2013

### Abstract

Dissemination of gastric cancer may usually occur by direct spread through the perigastric tissues to adjacent organ, lymphatic spread, and hematogenous spread. We report a rare case of gastric cancer with mucosal metastatic lesion on the upper esophagus that was diagnosed by endoscopy and endosonography. A biopsy of the esophageal mass was performed and the pathologic findings with immunohistochemical stain for Mucin-5AC are proved to be identical to that of gastric adenocarcinoma, suggesting metastasis from main lesion of the gastric cancer. The lesion could not be explained by lymphatic or hematogenous spread,

and its metastasis mechanism is considered to be different from previous studies. We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach.

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**Key words:** Stomach cancer; Neoplasm metastasis; Esophagus

**Core tip:** I believe the paper may be of particular interest to your readers because the reason is as follows. First, there has been rarity of case reports about esophageal metastasis from gastric cancer without any evidence of lymphatic involvement or direct spread from the primary lesion. Second, gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach, and this case proves the possibility of direct implantation of gastric adenocarcinoma cells refluxed on esophagus.

Ki SH, Jeong S, Park IS, Lee DH, Lee JI, Kwon KS, Kim HG, Shin YW. Esophageal mucosal metastasis from adenocarcinoma of the distal stomach. *World J Gastroenterol* 2013; 19(23): 3699-3702 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3699.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3699>

### INTRODUCTION

Dissemination of gastric cancer may usually occur through one of following three pathways: (1) direct spread through the perigastric tissues to adjacent organ; (2) lymphatic spread; and (3) hematogenous spread<sup>[1,2]</sup>.

Herein we report a rare case of gastric cancer with

mucosal metastatic lesion on the upper esophagus that was diagnosed by endoscopy and endosonography. The lesion could not be explained by lymphatic or hematogenous spread, and its metastasis mechanism is considered to be different from previous ones.

## CASE REPORT

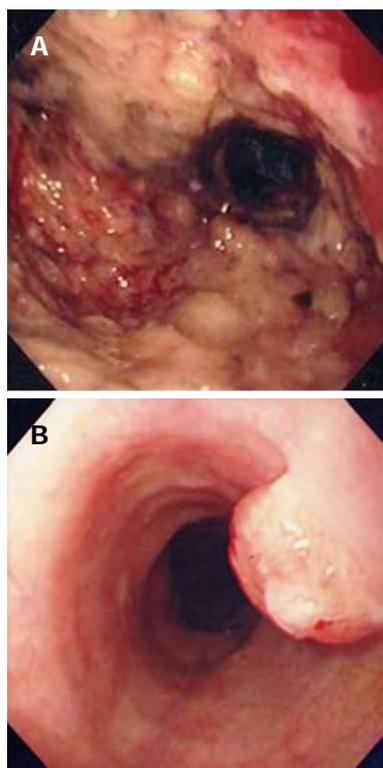
A 60-year-old man was admitted to our institution for systemic chemotherapy. Fourteen months prior to the admission, he was diagnosed with advanced gastric carcinoma on the antrum (Figure 1A). Palliative subtotal gastrectomy was performed with gastrojejunostomy to relieve pyloric obstruction, and the pathologic finding of surgically resected stomach disclosed adenocarcinoma.

Follow-up abdominal computer tomography was done a week prior to the current admission which revealed multiple hepatic metastases. The patient complained of dysphagia, and therefore endoscopy was performed. The endoscopic examination of the upper digestive tract showed a single, 1 cm-sized, polypoid mass which located 26 cm below the upper incisor (Figure 1B). Evidence of tumor recurrence in remnant stomach was not found. A biopsy of the polypoid mass of esophagus was performed and the pathologic findings with immunohistochemical stain for Mucin-5AC are proved to be identical to that of gastric adenocarcinoma, suggesting metastasis from main lesion of the gastric cancer (Figure 2). Endoscopic ultrasonography (EUS) with a miniature probe of 20 MHz frequency revealed hypoechoic wall thickening of upper esophagus, confined only to mucosal layer without any lymph node enlargement around esophagus (Figure 3).

He was treated with second line of systemic chemotherapy, consisted of docetaxel and cisplatin. However the disease progressed even after 3 cycles of the chemotherapy.

## DISCUSSION

Intramural spread of upper gastrointestinal tract tumors usually occurs *via* abundant lymphatic channels within the submucosal and subserosal layers of the gastric channel and prominent submucosal lymphatic plexus in esophagus. Regardless of the histologic type of the tumor, the tumor is able to infiltrate into submucosal or subserosal layer and spread to adjacent organ *via* lymphatic communication between stomach and esophagus<sup>[3-5]</sup>. It is considered that esophageal metastasis from the gastric cancers would also be seen as submucosal tumor in gross appearance since it shares same lymphatic channel. In our patient, follow-up endoscopy revealed polypoid mass in upper esophagus instead of appearance of submucosal tumor. EUS of the esophagus performed for assessment of the infiltration depth of the metastatic tumor evidently showed that the tumor was confined to mucosal layer and there was no disruption of muscularis mucosa or enlarged lymph nodes. If there was a pathologic confirma-

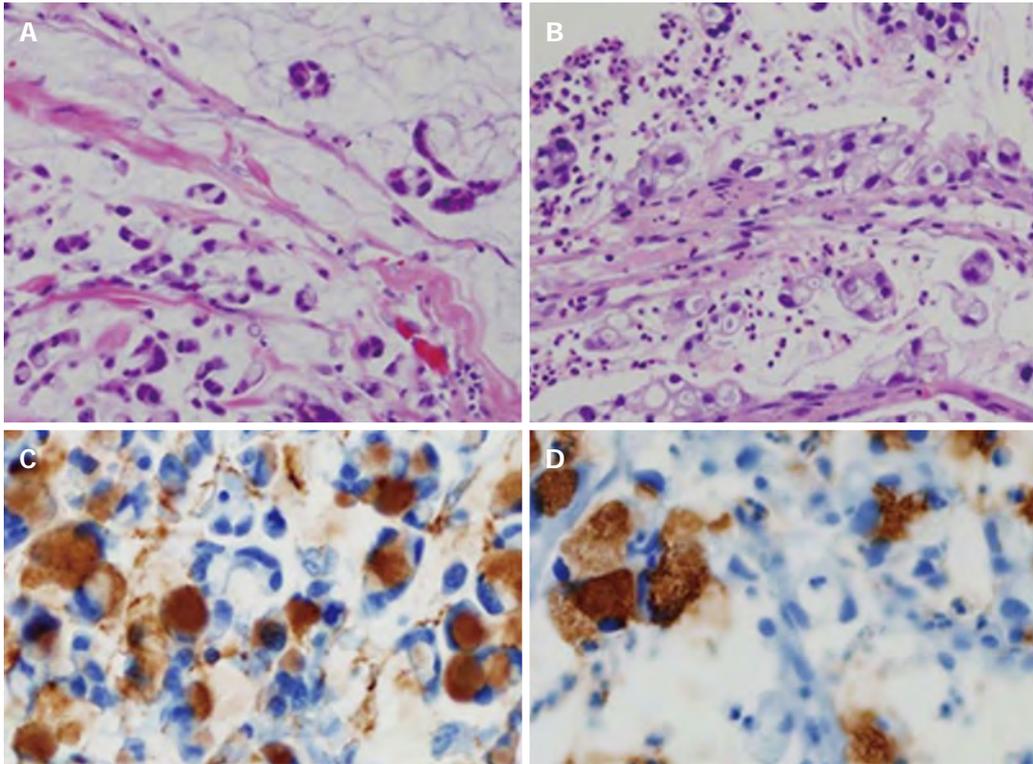


**Figure 1 Endoscopic findings.** A: Initial endoscopic finding: On the antrum, huge ulceroinfiltrative lesion with irregular margin and uneven dirty base was noted; B: Endoscopic findings 6 mo later: a 1 cm-sized polypoid mass was appeared at 26 cm below the upper incisor.

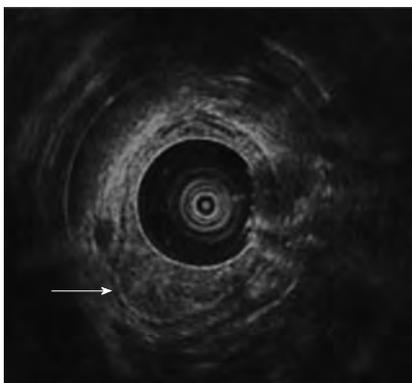
tion such as endoscopic submucosal dissection or esophagectomy it must have been definite that the esophageal tumor was confined to mucosal layer. But the resection could not be performed, considering his performance is poor and the disease is markedly progressed. EUS is currently the most accurate means available for tumor staging and locoregional nodal staging<sup>[6,7]</sup>. Therefore we could conclude that the esophageal tumor was confined to mucosal layer using by EUS.

There has been a case report of gastric cancer with esophageal metastasis which showed very similar finding of esophagus in EUS<sup>[8]</sup>. The wall of the esophagus at the level of the polypoid lesion was hypo-echoic and thick due to thickened mucosa. In this case total gastrectomy and esophagectomy was performed and the esophageal polypoid lesion was proved to be adenocarcinoma, identical to the primary gastric cancer. In this case report the author speculated that esophageal implantation metastasis from the gastric adenocarcinoma might have taken place by the gastro-esophageal reflux since gastro-esophageal reflux has been documented in various numbers of patients after distal gastrectomy.

Symptoms of gastroesophageal reflux disease have been previously reported to occur in about 30% of patients undergoing distal gastrectomy with Billoth I reconstruction<sup>[9]</sup>. Distal gastrectomy with Billoth I reconstruction causes two anatomical changes which promote gastroesophageal reflux; the presence of abnormal find-



**Figure 2 Light microscopic findings.** Specimens of stomach (A) and esophagus (B) revealed chains and irregular clusters of tumor cells floating freely in mucous lakes with scattered signet-ring cells [hematoxylin and eosin (HE), × 200]. Mucin-5AC (HE, × 400) is positive in the intracytoplasmic mucin of signet-ring cells of both stomach (C) and esophagus (D) in immunohistochemical staining.



**Figure 3 Endoscopic ultrasonographic finding.** Hypoechoic wall thickening of esophagus (arrow) was confined to mucosal layer.

ings in the cardia affected by the enlarged angle of Hiss and the high positioning of the remnant stomach in the supine position<sup>[9]</sup>. This patient underwent palliative subtotal gastrectomy and it is most likely that he had at least gastroesophageal reflux due to the anatomical alterations after surgery and it could have affected the direct implantation of gastric cancer cells on the esophagus.

According to previous study in adenocarcinoma of gastric cancer six patients among a total of 143 patients were verified to have intramural esophageal metastasis<sup>[10]</sup>. Most of these metastases would have been mediated by lymphatic channels between stomach and esophagus but few could have been done by direct implantation of

tumor cells. All patients had gastric cancer from cardia with lymphatic invasion. The distance from the primary tumor of the metastases was 20-50 mm. The metastases were appeared as multiple small submucosal tumors with intact mucosa in some patients. Most of these metastases would have been mediated by lymphatic channels between stomach and esophagus but few could have been done by direct implantation of tumor cells.

In our case, previous multiple hepatic metastases can arouse another possible mechanism of esophageal metastasis, but the esophageal lesion could not be explained by lymphatic or hematogenous spread, and its metastasis mechanism is considered to be different from previous studies.

In hematogenous or lymphatic spread, the esophageal metastasis involves submucosa and usually present as multiple masses, but in our case, esophageal metastasis was single solitary mass and was confined only to mucosal layer without any lymph node enlargement around esophagus. Most intramural esophageal metastases from gastric cancer originate from gastric cardia *via* lymphatic channels. But in our case, gastric cancer had occurred from antrum that was not close to esophagus and esophageal tumor was located at mid esophagus, far from stomach. It was difficult to metastasize from gastric antrum to mid esophagus without adjacent invasion if it metastasized *via* lymphatic channels.

We confirmed that the esophageal mass was metastasized from gastric cancer by pathology using immuno-

histochemical stain. We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach.

There has been rarity of case reports about esophageal metastasis from gastric cancer without any evidence of lymphatic involvement or direct spread from the primary lesion.

We suggest that the gastroesophageal reflux of cancer cells could be one of the possible metastatic pathways for metastasis of esophagus from an adenocarcinoma of the stomach, and this case proves the possibility of direct implantation of gastric adenocarcinoma cells refluxed on esophagus.

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**P- Reviewers** Rajeshwari K, Shehata MM **S- Editor** Zhai HH  
**L- Editor** A **E- Editor** Zhang DN



## Gastric body diaphragm-like stricture as a rare complication of nonsteroidal anti-inflammatory drugs

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Telephone: +86-10-55499007 Fax: +86-10-55499007  
Received: December 18, 2012 Revised: February 27, 2013  
Accepted: April 3, 2013  
Published online: June 21, 2013

### Abstract

Increased risk due to nonsteroidal anti-inflammatory drugs (NSAIDs) therapy has been observed in patients. Although diaphragm-like stricture in the small bowel and colon induced by NSAIDs therapy has been rarely reported, gastric body diaphragm-like stricture has not been reported. We describe the first case of gastric body diaphragm-like stricture due to NSAIDs in a 44-year-old male patient who was successfully treated by an endoscopic approach to avoid complicated surgery. This case highlights new insight into the disadvantages of NSAIDs and provides new data for future clinical studies.

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**Key words:** Gastric; Gastroscopy; Diaphragm; Stricture; Nonsteroidal anti-inflammatory drug

**Core tip:** The major disadvantage of nonsteroidal anti-inflammatory drugs (NSAIDs) therapy is the potential to induce adverse gastrointestinal effects. However, diaphragm disease is a rare complication of long-term

NSAIDs use. In this study, the first case of NSAIDs-induced diaphragm-like stricture in the gastric body is reported which was successfully treated by an endoscopic approach to avoid a complicated surgical intervention.

Wu LL, Yang YS, Cai FC, Wang SF. Gastric body diaphragm-like stricture as a rare complication of nonsteroidal anti-inflammatory drugs. *World J Gastroenterol* 2013; 19(23): 3703-3706 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3703.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3703>

### INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are known to cause erosion, ulceration, occult bleeding and subsequent stricture formation in the gastrointestinal tract. A rare NSAIDs-induced complication is the formation of diaphragm-like strictures in the intestine<sup>[1-4]</sup>. Herein, we report, to our knowledge, the first case of NSAIDs-induced diaphragm-like stricture in the gastric body successfully treated by an endoscopic approach to avoid a complicated surgical intervention.

### CASE REPORT

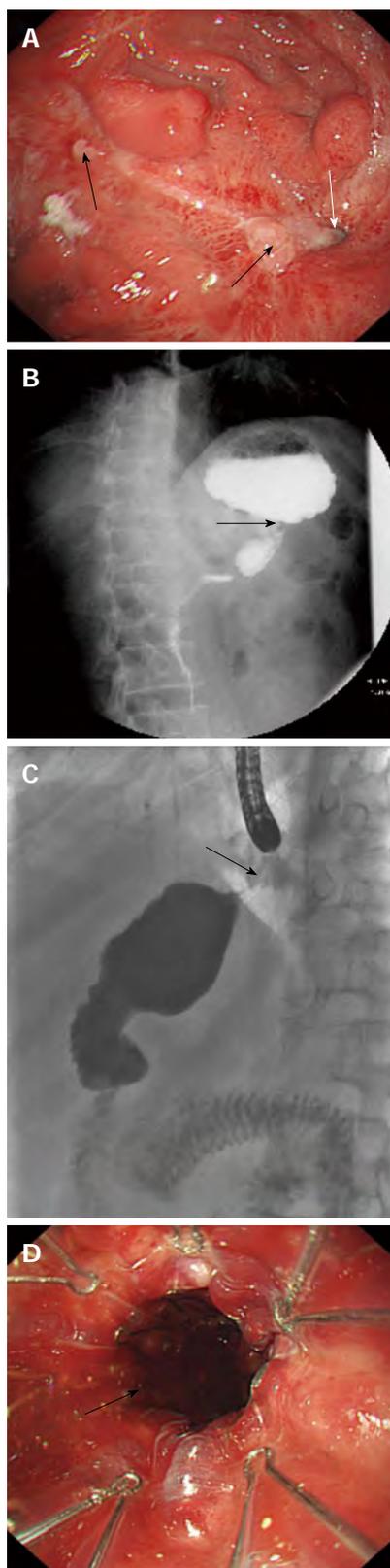
A 44-year-old male patient presented with a 2-mo history of abdominal distention and vomiting. There was temporary relief after vomiting and the vomitus was composed of gastric contents. The patient had taken more than 15 g of compound aminopyrine phenacetin tablets by mistake whilst drunk three months previously. On physical examination, his abdomen was tender without any other relevant physical findings. Routine blood and biochemical tests were normal, and tumor markers were within the normal ranges. Gastroscopy showed multiple erosions, ulcers and nodular changes in the proximal gastric body, in which the largest ulcer was

about 1.0 cm × 1.5 cm (Figure 1A, black arrow). Deformation and stricture of the gastric body was noted, and the gastroscope was unable to pass through the stricture (Figure 1A, white arrow). Biopsy pathology at the ulcer edges revealed inflammation and other benign changes. Organic iodine solution radiography of the upper gastrointestinal tract showed a very thin stricture in the gastric body (Figure 1B). Subsequently, a pediatric gastroscope was used, but also failed to pass through the stricture. Repeat biopsy pathology confirmed the previous histology of benign inflammation, and immunohistochemistry showed weakly positive results for CD3, CD43, CD20, Ki-67 and CK, which were consistent with the drug-induced gastric body benign ulcers and stricture. Endoscopic transcatheter radiography was then performed which demonstrated that the gastric body stricture was a diaphragm-like stricture (Figure 1C). The patient underwent balloon dilation and placement of a metal stent (Figure 1D); his symptoms resolved after these endoscopic procedures. On follow-up 2 mo later, the patient had no symptoms.

## DISCUSSION

NSAIDs, such as aspirin, indomethacin, diclofenac, and compound aminopyrine phenacetin, are the most commonly prescribed drugs for inflammation, arthritis and cardiovascular protection. However, a major disadvantage of NSAIDs therapy is the potential to induce adverse gastrointestinal effects, particularly in the stomach and duodenum. It has been reported that as many as 25% of chronic NSAIDs users may develop ulcer disease, and 2%-4% of these ulcers may bleed or perforate<sup>[5,6]</sup>. NSAIDs are absorbed into enterocytes and then uncouple mitochondrial oxidative phosphorylation, resulting in the dysfunction of tight intracellular junctions and intestinal permeability. Enterocytes are thereby exposed to luminal aggressive contents, leading to inflammation and ulceration<sup>[7]</sup>. Recent clinical research shed light on NSAID-induced small intestinal mucosal damage including erosions and ulcerations, which occur more often than previously expected<sup>[8]</sup>. Graham *et al*<sup>[9]</sup> reported that 70% of patients who took NSAIDs for > 3 mo had small intestinal ulcers and erosions shown by capsule endoscopy.

Diaphragm disease induced by NSAIDs, first described by Lang *et al*<sup>[1]</sup> in 1988, is a rare and severe complication of long-term NSAIDs use, especially in elderly patients<sup>[2]</sup>. Although diaphragm-like stricture of the small bowel was not associated with the use of NSAIDs in recent reports<sup>[10]</sup>, diaphragm disease was thought at one time to be a unique form of intestinal pathology associated with NSAIDs administration. Disease may occur in 2% of NSAIDs users in the small bowel<sup>[3]</sup>, commonly in the ileum<sup>[2,4]</sup>, or in the duodenum in some cases<sup>[11,12]</sup>. In the past decade, diaphragm-like strictures in the large intestine due to adverse effects of NSAIDs have been



**Figure 1** Imaging features of diaphragm-like stricture. A: Gastroscopy showed multiple erosions, ulcers, and nodular changes in the proximal gastric body (black arrow) with a very thin stricture (white arrow) which the gastroscope was unable to pass through; B: Organic iodine solution radiography of the upper gastrointestinal tract showed a tight stricture in the gastric body (arrow); C: Endoscopic transcatheter radiography demonstrated a diaphragm-like stricture (arrow); D: After balloon dilation placement of a metallic stent was undertaken (arrow).

increasingly reported<sup>[13,14]</sup>.

The precise pathogenesis of diaphragm disease is unclear, however, the main histological abnormalities include thickening and chaotic arrangement of muscular bundles in the muscularis mucosae, fibrosis of the lamina propriae and mucosal ulceration<sup>[15]</sup>. Therefore, affected patients frequently present with gastrointestinal obstructive symptoms and often require surgical treatment.

Although diaphragm-like strictures have been reported in the small bowel and colon, strictures in the gastric body have not been documented in the literature. This may be because the gastric body not only has a wider lumen or space, but also has a thick muscularis. We recently experienced a rare case which occurred after ingestion of a large quantity of NSAIDs. It is known that endoscopic dilation, surgical resection and suspension of NSAIDs administration are common treatment options depending on the position, length and severity of the stricture. In the present middle-age patient, we successfully used the minimally invasive treatment modalities endoscopic dilation and placement of a metal stent, which avoided complicated surgical management.

More and more cases induced by NSAIDs have been reported in the literature, including gastrocolic fistula<sup>[16]</sup>, Brar *et al*<sup>[17]</sup> reported a case of perforation, and even a case of Crohn's disease was reported which was endoscopically and histologically misinterpreted<sup>[18]</sup>. Due to the increasing world-wide use of NSAIDs, NSAID-related gastrointestinal complications still continue to be a major concern and require more therapeutic strategies<sup>[19-24]</sup>. This study, for the first time, reports a rare case of diaphragm-like gastric body stricture which was successfully treated by an endoscopic approach. The endoscope is a useful tool for the diagnosis and treatment of suspected NSAIDs-related gastrointestinal complications.

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**P- Reviewers** Higuchi K, Slomiany BL **S- Editor** Zhai HH  
**L- Editor** Webster JR **E- Editor** Zhang DN



## Ileocecal endometriosis and a diagnosis dilemma: A case report and literature review

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Received: February 4, 2013 Revised: March 27, 2013

Accepted: April 27, 2013

Published online: June 21, 2013

### Abstract

Bowel endometriosis affects between 3.8% and 37% of women with endometriosis. The evaluation of symptoms and clinical examination are inadequate for an accurate diagnosis of intestinal endometriosis. We describe the case of a 41-year-old woman who presented to our hospital because of six months of recurrent abdominal pain, vomiting and diarrhea, without previous history of bowel disease. Physical examination revealed a palpable 3 cm × 5 cm mass in the right lower quadrant abdomen. Laboratory tests showed slightly elevated levels of CA19-9 and CA125. Small bowel computer tomography scanning revealed an ileocecal mass with bowel wall thickening and luminal narrowing. Small bowel endoscopy identified a deep longitudinal ulcer and mucosal edema in the distal ileum. All these findings supported the diagnosis of Crohn's disease. The patient underwent a laparotomy, which identified a 5

cm × 5 cm ileocecal mass with severe mucosal edema and luminal stricture in the distal ileum. Histopathological examination confirmed a diagnosis of ileocecal endometriosis without other areas involved. After one-year follow-up, there was no recurrence of the symptoms.

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**Key words:** Ileus; Bowel obstruction; Longitudinal ulcer; Crohn's disease; Endometriosis

**Core tip:** We describe the case of a 41-year old woman who had recurrent abdominal pain with vomiting and diarrhea on presentation to our hospital. The results of computer tomography scanning and small bowel endoscopy were strongly suspicious for Crohn's disease. However, surgery and histopathological examination confirmed a diagnosis of ileocecal endometriosis without other areas involved.

Tong YL, Chen Y, Zhu SY. Ileocecal endometriosis and a diagnosis dilemma: A case report and literature review. *World J Gastroenterol* 2013; 19(23): 3707-3710 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3707.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3707>

### INTRODUCTION

Intestinal endometriosis affects 12%-15% of menstruating women, and is generally an asymptomatic condition<sup>[1]</sup>. Ileal involvement is very rare and the patients generally present with an asymptomatic or painful mass<sup>[2]</sup>. Symptoms of bowel endometriosis are numerous, ranging from being asymptomatic to a constellation of symptoms like painful bowel movements, cramps, constipation, diarrhea, vomiting, rectal pain, infertility, abdominal mass, increased urinary frequency and cyclical hematochezia<sup>[3]</sup>.

Classically, the symptoms become worse during menses, but this is not always the case. This myriad of symptoms makes the condition difficult to diagnose. Small bowel endometriosis tends to affect bowel serosa and only 10% of intestinal cases have mucosal involvement<sup>[1,4,5]</sup>. It can be difficult to discern between ileal Crohn's disease (CD) and endometriosis.

In this report, we describe a further case of ileocecal mass with longitudinal ulcer which was suspected as being CD. Surgery and histopathological examination confirmed a diagnosis of ileocecal endometriosis with no other areas involved. This report serves as a reminder of this rare condition as well as highlighting the diagnostic difficulties it can pose.

## CASE REPORT

A 41-year-old lady with no significant past medical history was presented to our hospital because of six months of recurrent abdominal pain, vomiting and diarrhea. The patient had no past history of tuberculosis or other infectious diseases. She also denied radiation exposure, a poisonous chemical contact history and genetic history. Her family had no history of bowel disease. Physical examination revealed a palpable 3 cm × 1.5 cm mass in the right lower quadrant abdomen.

Laboratory tests showed slightly elevated levels of C-reactive protein, CA19-9 and CA125. No other abnormalities were found in tests such as erythrocyte sedimentation rate, immune index or tuberculosis series check. Routine stool test was normal with occult blood negative.

Computer tomography (CT) scanning revealed an ileocecal mass with multiple mesenteric lymph nodes enlarged. A colonoscopy she underwent three months previously showed introverted mucosa surrounding the appendix hole without colon abnormalities. Small bowel endoscopy identified a deep longitudinal ulcer in the distal ileum, mucosal edema and luminal stricture which the endoscope couldn't go through (Figure 1). All these findings supported the diagnosis of CD. To evaluate other intestinal lesions, we undertook small bowel CT scanning, which revealed an ileocecal mass with bowel wall thickening and luminal narrowing, without other intestinal areas being involved (Figure 2).

Due to the bowel obstruction, the patients underwent a laparotomy, which revealed an ileocecal mass, 5 cm × 5 cm in size, with severe mucosal edema and luminal stricture in the distal ileum. No other organs were invaded. The frozen-section diagnosis was endometriosis. En bloc resection was taken and the histopathological examination confirmed ileocecal endometriosis. No subsequent medical treatment was undertaken. The patient recovered well after the surgery, and her quality of life has been significantly improved. After one-year follow-up, there was no recurrence of the symptoms.

## DISCUSSION

Endometriosis is defined as the presence of ectopic en-

dometrial tissue in extrauterine sites. It affects 10%-15% of women of reproductive age and usually becomes apparent in the reproductive years when the lesions are stimulated by ovarian hormones<sup>[6]</sup>. Intestinal endometriosis occurs in 12%-15% of cases, and the incidence of the involvement of different intestinal sites varies greatly in the literature, with the rectosigmoid colon, small bowel, appendix and cecum affected in 50%-90%, 2%-16%, 3%-18% and 2%-5% of cases, respectively<sup>[4]</sup>. As in our case, ileocecal involvement is rare with an incidence of 4.1% in intestinal cases<sup>[7]</sup>.

The etiology of endometriosis is still elusive. The most widely accepted theory is that "retrograde menstruation" causes the implantation and growth of endometriosis on the serosal surface of extra-uterine organs or that this occurs secondary to metaplasia in the pelvic peritoneum<sup>[2,8-10]</sup>.

Symptoms of bowel endometriosis can be associated with the patient's menstrual cycle in 18%-40% of cases but may become permanent when the lesions progress<sup>[2,11,12]</sup>. Under cyclical hormonal influences, serosal implants may proliferate and infiltrate the bowel wall, and lead to inflammation, fibrosis, and metaplasia or hyperplasia of intestinal smooth muscles that can involve the serosa, submucosa and (uncommonly) mucosa<sup>[13]</sup>. This then leads to introverted mucosa surrounding the appendix hole, luminal stricture, longitudinal ulcer, and ileocecal mass as we believe happened in our case.

Symptoms range from an asymptomatic state to a constellation of symptoms like painful bowel movements, cramps, constipation, diarrhea, vomiting, rectal pain, infertility, abdominal mass, increased urinary frequency and cyclical hematochezia<sup>[3]</sup>. Those symptoms can mimic a wide spectrum of diseases, including irritable bowel syndrome, infectious diseases, ischemic enteritis/colitis, inflammatory bowel disease and neoplasm, so it is difficult to establish a preoperative diagnosis of bowel endometriosis<sup>[2,14,15]</sup>.

Laboratory tests such as CA125 detection are not sensitive enough for diagnosis<sup>[9]</sup>. Transvaginal sonography should be used as the first-line diagnostic technique; this has shown a sensitivity and specificity of 43.7% and 50%, respectively<sup>[16,17]</sup>. Saline contrast sonovaginography was more accurate in diagnosing the condition than was transvaginal ultrasonography, with a sensitivity and specificity of 90.6% and 85.7%, respectively<sup>[16-18]</sup>. Contrast CT with enteroclysis protocols can be useful in diagnosis as this may demonstrate focal or constricting bowel lesions<sup>[4,9]</sup>. Magnetic resonance imaging (MRI) is currently the best imaging modality for enteric endometriosis with a sensitivity of 77%-93%<sup>[1,9]</sup>. Endoscopy may provide no valuable results because of the intact mucosa, but it is still recommended in all patients with suspected endometriosis to rule out mucosal involvement and malignant lesions with the help of biopsies, if needed. In our patient, symptoms relapsed irregularly and were not related with menses. The imaging results showed an ileocecal mass and longitudinal ulcer with luminal stricture under endoscopy, which strongly suggested CD. Due to the bowel



**Figure 1** Enteroscopic findings of the patient. A: Mucosal edema of the distal ileum; B: A deep longitudinal ulcer and luminal stricture in distal ileum; C: Multiple ulcers, mucosal edema in distal ileum.



**Figure 2** Small bowel computer tomography scan of the patient. Ileocecal bowel wall thickening and luminal narrowing with proximal lumen expansion. Contrast enhancement pattern showed marked enhancement. No other parts of the intestine involved.

obstruction, surgery was recommended.

Histopathological confirmation required presence of both glandular and stromal tissue. In our patient, the pathologist's findings showed that the annular lesion of endometriosis and mucosa was not involved.

The treatment of uncomplicated intestinal endometriosis depends on the patient's age and intention to conceive. Medical treatment with hormonal therapy such as the oral contraceptive pill, danazol or gonadotropin antagonists can be attempted for intestinal disease when there is no obstruction<sup>[1,2,19]</sup>. Bowel resection is indicated if there are symptoms of obstruction or bleeding, and if malignancy cannot be excluded. Post-operative hormonal therapy does not demonstrate benefits, according to a recent meta-analysis<sup>[20]</sup>.

In summary, bowel endometriosis should be borne in mind when a woman of reproductive age presents with episodic gastrointestinal symptoms. A careful history may elicit symptoms related to the patient's menses. Small bowel CT and MRI is indicated, and endoscopy is still recommended in all patients to rule out mucosal involvement and malignant lesions. In our case, the final diagnosis could only be given by the pathologist's report. Multidisciplinary care should be encouraged to ensure correct evaluation and improve the management of these patients.

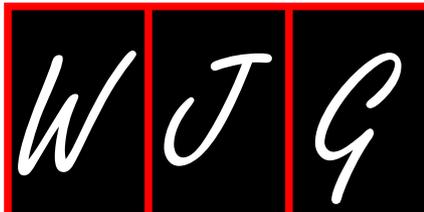
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**P- Reviewers** Cosmi E, Petraglia F **S- Editor** Zhai HH  
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## Response to Abadi and Kusters, *World J Gastroenterol* 19: 429-430

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Received: March 15, 2013 Revised: April 16, 2013

Accepted: May 16, 2013

Published online: June 21, 2013

### Abstract

In a recent study, Rafiei *et al*, reported a link between a C150T polymorphism in the human inducible nitric oxide gene and *Helicobacter pylori* infection as a risk factor for gastric cancer among an Iranian population. Subsequently, Abadi and Kusters published a letter to the editor questioning the validity of the study because of a supposed flaw in primer design. Here we respond to the claims of Abadi and Kusters and show that the results reported in the original article are valid.

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**Key words:** Inducible nitric oxide synthetase; *Helicobacter pylori*; Gastric cancer

**Core tip:** In a recent Letter to the Editor, Abadi and Kusters brought into question the validity of a study published by Rafiei *et al*. Herein we respond to the claims made by Abadi and Kusters, and show that the results reported in the article originally published by Rafiei *et al*, are valid.

Rafiei A, Gilbreath JJ, Merrell DS. Response to Abadi and Kusters, *World J Gastroenterol* 19: 429-430. *World J Gastroenterol* 2013; 19(23): 3711-3712 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i23/3711.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i23.3711>

### TO THE EDITOR

In a recent letter to the editor, entitled "Association of inducible nitric oxide synthetase genotype and *Helicobacter pylori* infection gastric cancer risk may be due to faulty primer design"<sup>[1]</sup>, Amin Talebi Bezmin Abadi and Johannes Kusters questioned the validity of the results published in our manuscript<sup>[2]</sup>, the design of which was based on a previous study<sup>[3]</sup>. In their letter<sup>[1]</sup>, the authors appraised our article<sup>[2]</sup> by two main points; variation in T allele frequency and primer design. In response to their first comment regarding the T allele frequency seen in our population, we stand by the conclusion that genetic polymorphisms depend on ethnic differences; the frequency of mutant genotypes varies in different human populations. Therefore, it is likely no surprise that the frequency of the T allele seen in our Caucasian population<sup>[2]</sup> differed from that of the East Asian population<sup>[3]</sup>.

In regards to the second point made by Abadi *et al*<sup>[1]</sup>, concerning the primer specificity, we note that there is a misprint in one primer sequence in our article<sup>[2]</sup>. The primer sequence published in our paper (5'-GTCTCTGCGGGTCTGAAG-3') differs from that of Shen *et al*<sup>[3]</sup> in that it is missing two base pairs in the 3' end of the sequence. An erratum has been submitted to the journal to note the misprinted primer sequence [On page 4919<sup>[2]</sup>, the primer sequence given for iNOS-R (5'-174 GTCTCTGCGGGTCTGAAG-3') is missing two nucleotides and should appear as iNOS-R (5'-GTCTCTGCGGGTCTGAGAAG-3')]. However, this 2 base misprint appears not to be the core issue since the correct primer sequence (5'-GTCTCTGCGGGTCTGAGAAG-3') was

GCCCATATGTAACCAACTTCGGTGGTGGGCTGTGAGCCTTCTCTGCAAGCTGTGGCCAGGTTTC  
 AG AAGAAAGGAAAAACAGTGTATCATCCTGGCTTGAGAACTGTGATTCCTCTCTTCTAGAAAC  
 TGAAGAAATCGCTCTTCATGCTGAAAGAGCTCAACAACAATTCAGGTAAGCTTCTCCGGTGTCTTAC  
 TCCTAGCCCTGCCCTGGGGCCCCAGTGTCTGGTACAGGGGATAGCTCTGGGTGTACACAGGGGGTCTTC  
**TCAGACCCGCAGAGAC**ACAGAGC

**Figure 1 Location of inducible nitric oxide synthetase-specific primer annealing sites.** The sequence of exon 16 of the inducible nitric oxide synthetase (*iNOS*) gene (<http://www.ncbi.nlm.nih.gov/nucleotide/x85772>) is shown. The sequence of forward primer used in our study and by Shen *et al.*<sup>[3]</sup> is in bold italics at the 5' end of the sequence above: 5'-TGTAACCAACTTCGGTGGT-3'. The sequence of the reverse primer used in our study and by Shen *et al.*<sup>[3]</sup> (5'-GTCTCTGCGGGTCTGAGAAG-3') is the reverse complement of the bold italicized bases in the 3' end of the sequence above. The C150T polymorphism is indicated by the bold underlined "C".

previously published by Shen *et al.*<sup>[3]</sup> and this publication was also called into question by Abadi and Kusters. We note that as shown in Figure 1, both of the primers used in the study are specific to exon 16 of the *iNOS* gene (Figure 1), which has been mapped to chromosome 17q11.2. Direct sequence analysis of exon 16 as submitted to GenBank by Shen *et al.*<sup>[3]</sup> (<http://www.ncbi.nlm.nih.gov/nucleotide/x85772>) shows that the primers used in our studies are completely conserved in this sequence. Furthermore, blast analysis of the inducible nitric oxidase synthase against the human database returns 3 hits to Homo sapiens chromosome 17. Analysis of the resulting alignments shows complete conservation of the forward primer in all 3 samples. Conservation of the reverse primer is not as high; the 5' end of the primer shows several mismatches. However, the last 9 nucleotides found in the 3' end of the primer are completely conserved across all 3 samples. Thus, there should be no overt obstacles to

amplification of the gene in question. In conclusion, we stand by the results reported in our original study<sup>[2]</sup> and posit that the supposition by Abadi and Kusters that our study design is flawed is incorrect.

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# World Journal of *Gastroenterology*

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|                         |             |  |
|-------------------------|-------------|--|
| <b>EDITORIAL</b>        | <b>3713</b> | Green tea and the risk of gastric cancer: Epidemiological evidence<br><i>Hou IC, Amarnani S, Chong MT, Bishayee A</i>  |
| <b>FIELD OF VISION</b>  | <b>3723</b> | Hepatocellular carcinoma and food contamination: Ochratoxin A as a great prompter<br><i>Felizardo RJF, Câmara NOS</i>  |
| <b>MINIREVIEWS</b>      | <b>3726</b> | Small-bowel capsule endoscopy: A ten-point contemporary review<br><i>Koulaouzidis A, Rondonotti E, Karargyris A</i>  |
| <b>ORIGINAL ARTICLE</b> | <b>3747</b> | Predominant mucosal expression of 5-HT <sub>4(+h)</sub> receptor splice variants in pig stomach and colon<br><i>Priem EKV, De Maeyer JH, Vandewoestyne M, Deforce D, Lefebvre RA</i>                           |
|                         | <b>3761</b> | Assessment of vascular invasion in gastric cancer: A comparative study<br><i>Gresta LT, Rodrigues-Júnior IA, de Castro LPF, Cassali GD, Cabral MMDA</i>  |
|                         | <b>3770</b> | Interaction of 14-3-3 $\sigma$ with KCMF1 suppresses the proliferation and colony formation of human colon cancer stem cells<br><i>Zou J, Mi L, Yu XF, Dong J</i>  |
|                         | <b>3781</b> | Glycyrrhizic acid attenuates CCl <sub>4</sub> -induced hepatocyte apoptosis in rats <i>via</i> a p53-mediated pathway<br><i>Guo XL, Liang B, Wang XW, Fan FG, Jin J, Lan R, Yang JH, Wang XC, Jin L, Cao Q</i> |
|                         | <b>3792</b> | Annexin A2 silencing inhibits invasion, migration, and tumorigenic potential of hepatoma cells<br><i>Zhang HJ, Yao DF, Yao M, Huang H, Wang L, Yan MJ, Yan XD, Gu X, Wu W, Lu SL</i>                           |
|                         | <b>3802</b> | Role of activin A in carbon tetrachloride-induced acute liver injury<br><i>Wang DH, Wang YN, Ge JY, Liu HY, Zhang HJ, Qi Y, Liu ZH, Cui XL</i>   |
| <b>BRIEF ARTICLE</b>    | <b>3810</b> | Quality of life following laparoscopic Nissen fundoplication: Assessing short-term and long-term outcomes<br><i>Kellokumpu I, Voutilainen M, Haglund C, Färkkilä M, Roberts PJ, Kautiainen H</i>               |

- 3819** Is alcoholic pancreatitis associated with enteroviral infection?  
*Khan J, Nordback I, Seppänen H, Lappalainen-Lehto R, Järvinen S, Oikarinen S, Tauriainen S, Rätty S, Hyöty H, Sand J*
- 3824** Characteristics of allergic colitis in breast-fed infants in the absence of cow's milk allergy  
*Molnár K, Pintér P, Györfly H, Cseh Á, Müller KE, Arató A, Veres G*
- 3831** Active treatments are a rational approach for hepatocellular carcinoma in elderly patients  
*Suda T, Nagashima A, Takahashi S, Kanefuji T, Kamimura K, Tamura Y, Takamura M, Igarashi M, Kawai H, Yamagiwa S, Nomoto M, Aoyagi Y*
- 3841** Anus-preserving resection *via* telescopic colorectal mucosal anastomosis for low rectal cancer: Experience from a Chinese cohort  
*Li SY, Chen G, Bai X, Zuo FY, Chen G, Du JF, Wei XJ, Cui W*
- 3847** Mitochondrial ATP 6 and 8 polymorphisms in irritable bowel syndrome with diarrhea  
*Wang WF, Li X, Guo MZ, Chen JD, Yang YS, Peng LH, Wang YH, Zhang CY, Li HH*
- 3854** Laparoscopic splenectomy for treatment of splenic marginal zone lymphoma  
*Wu Z, Zhou J, Wang X, Li YB, Niu T, Peng B*
- 3861** Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations  
*Yang JH, Zhang H, Chen XB, Chen G, Wang X*

**META-ANALYSIS**

- 3866** Clinical prognostic factors for disabling Crohn's disease: A systematic review and meta-analysis  
*Dias CC, Rodrigues PP, da Costa-Pereira A, Magro F*
- 3872** Meta-analysis of radiofrequency ablation in combination with transarterial chemoembolization for hepatocellular carcinoma  
*Ni JY, Liu SS, Xu LF, Sun HL, Chen YT*
- 3883** Meta-analysis comparison of endoscopic papillary balloon dilatation and endoscopic sphincterotomy  
*Zhao HC, He L, Zhou DC, Geng XP, Pan FM*

## CASE REPORT

- 3892** Foreign body impaction in the sigmoid colon: A twenty euro bet  
*Müller KE, Arató A, Lakatos PL, Papp M, Veres G*
- 3895** Early-stage primary signet ring cell carcinoma of the colon  
*Kim JH, Park SJ, Park MI, Moon W, Kim SE*
- 3899** Severe irinotecan-induced toxicity in a patient with *UGT1A1\*28* and *UGT1A1\*6* polymorphisms  
*Xu JM, Wang Y, Ge FJ, Lin L, Liu ZY, Sharma MR*
- 3904** Pathological diagnosis is maybe non-essential for special gastric cancer: Case reports and review  
*Song W, Chen CY, Xu JB, Ye JN, Wang L, Chen CQ, Zhang XH, Cai SR, Zhan WH, He YL*
- 3911** Traumatic rupture of a type IVa choledochal cyst in an adult male  
*Duan YF, Yang B, Zhu F*

**APPENDIX** I-VI Instructions to authors

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**NAME OF JOURNAL**  
*World Journal of Gastroenterology*

**ISSN**  
ISSN 1007-9327 (print)  
ISSN 2219-2840 (online)

**LAUNCH DATE**  
October 1, 1995

**FREQUENCY**  
Weekly

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**PUBLISHER**  
Baishideng Publishing Group Co., Limited  
Flat C, 23/F, Lucky Plaza, 315-321 Lockhart Road, Wanchai, Hong Kong, China

Fax: +852-65557188  
Telephone: +852-31779906  
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**PUBLICATION DATE**  
June 28, 2013

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## Green tea and the risk of gastric cancer: Epidemiological evidence

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Received: January 10, 2013 Revised: April 14, 2013

Accepted: May 9, 2013

Published online: June 28, 2013

### Abstract

Gastric cancer (GC) is one of the leading causes of cancer death in the world. Numerous efforts are being made to find chemoprotective agents able to reduce its risk. Amongst these, green tea has been reported to have a protective effect against stomach cancer. This article aims to critically evaluate all epidemiological studies reporting an association between green tea consumption and GC risk. MEDLINE, EBSCOHOST and Google Scholar were used to search for clinical trials of green tea and its correlation to stomach cancer. Studies include cohort and case-control studies. Outcome of interests are inverse association, no association, and positive association. Seventeen epidemiologic studies were reviewed. Eleven studies were conducted in Japan, five in China, and one with Japanese descent in Hawaii. Ten case-control studies and seven cohort

studies were included. The relative risks or odds ratio of GC for the highest level of green tea consumption was compared. Seven studies suggested no association, eight an inverse association, and one a positive association. One study had shown a significantly lowered GC risk when tea was served warm to cold. Another study also showed a significantly risk with lukewarm tea. All studies that analyzed men and women separately have suggested a reduced risk in women than in men, albeit no significant difference. This review demonstrates that there is insufficient information to support green tea consumption reduces the risk of GC. More studies on the subject matter are warranted.

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**Key words:** Gastric cancer; Green tea; Epidemiology; Case-control study; Cohort study

**Core tip:** Gastric cancer (GC) is one of the leading causes of cancer death in the world. Numerous efforts are being made to find chemoprotective agents able to reduce its risk. This review demonstrates that there is insufficient information to support green tea consumption reduces the risk of GC. More studies on the subject matter are warranted.

Hou IC, Amarnani S, Chong MT, Bishayee A. Green tea and the risk of gastric cancer: Epidemiological evidence. *World J Gastroenterol* 2013; 19(24): 3713-3722 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3713.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3713>

### INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and second leading cause of death from cancer throughout the world. A 2011 world analysis showed that 989600 new GC cases and 738000 deaths were estimated to have

occurred in 2008<sup>[1]</sup>. The incidence of GC varies up to 10-fold across the world with the greatest percentage in China, followed by South Korea, South American countries and Japan<sup>[1]</sup>. These variations may be due to differences in environmental and lifestyle factors.

An excessive intake of protein, fat, salt or meat increases the risk of stomach cancer. Contrarily, dietary fiber, vegetables, fruit, and soy have been shown to play an important role in prevention of GC<sup>[2]</sup>. Tea, one of the most commonly consumed beverages in the world, has been reported in pre-clinical and epidemiological studies to provide protective effects against GC<sup>[3]</sup>. Green tea and its constituents have been shown to exhibit multiple health benefits<sup>[4-6]</sup>. Green tea and its bioactive constituents inhibit tumorigenesis in many animal models, including those for cancer of the skin, lung, oral cavity, esophagus, stomach, small intestine, colon, liver, pancreas, bladder, breast, and prostate<sup>[7-11]</sup>.

Polyphenols, which include flavanols, flavandiol, flavonoids, and phenolic acids, constitute the most interesting group of green tea leaf components. Most green tea polyphenols (GTPs) are flavonoids. Flavonoids are phenol derivatives synthesized in substantial amounts (0.5%-1.5%) and variety (more than 4000 flavonoids identified), and widely distributed among plants<sup>[12]</sup>. The main flavonoids present in green tea are catechins. Catechins are colorless, astringent, water soluble compounds, and are readily oxidizable. Since green tea does not undergo fermentation, it contains greater amounts of various catechins than in black or oolong tea. Traces of catechins are also found in grapes, wine, and chocolate<sup>[13]</sup>. The four kinds of catechins found mainly in green tea include: (-)-epicatechin-3-gallate (ECG), (-)-epigallocatechin, (-)-epicatechin, and (-)-epigallocatechin-3-gallate (EGCG). Out of the above, EGCG accounts for more than 40% of the total catechin content. Figure 1 shows the chemical structure of the four major catechins present in green tea. EGCG is the most abundant polyphenol in green tea and has gained most attention as the active constituent responsible for the anticarcinogenic activity of this tea. One cup of brewed green tea (2.5 g of green tea leaves/200 mL of water) may contain 90 mg of EGCG<sup>[14]</sup>. In black tea, the catechins compounds such as theaflavins and thearubigins predominate. Black and green tea both contains similar amounts of flavonoids, however they differ in their chemical structure; green tea contains more catechins (simple flavonoids). Conversely, the oxidation undergone by black tea processing converts these simple flavonoids into theaflavins and thearubigins<sup>[12]</sup>.

The first green tea clinical trial with cancer patients as a phase I study using green tea capsules was performed in 1997 and later published in 2001<sup>[15]</sup>. The purpose of this study was to determine the maximum-tolerated dose, toxicity, and pharmacology of oral green tea extract. A total of 49 cancer patients with solid tumors were studied. There were two treatment arms in this two years study: 0.05-5.05 g/m<sup>2</sup> once daily dose and 1.0-2.2 g/m<sup>2</sup> three-times-daily for 6 mo. The maximum tolerated dose was 4.2 g/m<sup>2</sup> once daily or 1.0 g/m<sup>2</sup> three times a day. This study

recommended that 1.0 g/m<sup>2</sup> for three times daily should be considered for future clinical trials and that doses studied can be taken safely for at least 6 mo<sup>[15]</sup>. Thereafter, several phase I studies on healthy volunteers have also been conducted to define the basic pharmacokinetic parameters and safety profile for oral consumption of various types of green tea preparations<sup>[16,17]</sup>. In 2003, a phase I study investigated polyphenon E<sup>TM</sup> (a defined, decaffeinated GTP mixture) in healthy individuals<sup>[18]</sup>. The study concluded that greater oral bioavailability of EGCG can be achieved by taking the polyphenon E<sup>TM</sup> capsules on an empty stomach after an overnight fast. Recent phase I studies have concluded that the consumption of green tea appears to be relatively safe (8-16 cups of green tea once a day or in divided doses twice a day for 4 wk)<sup>[3]</sup>. However, up to 2 g orally twice per day was observed to be well tolerated in patients with stage 0 to II chronic lymphocytic leukemia<sup>[19]</sup>. Bettuzzi *et al*<sup>[20]</sup> had reported that 600 mg of daily catechin extract had a statistically significant protective effect in patients with high-grade prostate intraepithelial neoplasia. EGCG delivered in the capsule form (200 mg/d for 12 wk) has also been reported to be effective in patients with human papilloma virus-infected cervical lesions<sup>[21]</sup>.

Although many clinical trials have been conducted to explore the safety and efficacy of green tea extract in cancer patients, similar studies in GC patients have not yet been executed. Over the last three decades, a number of epidemiological studies were conducted to investigate the association between green tea consumption and stomach cancer risk in human subjects<sup>[22-27]</sup>. Nonetheless, the epidemiologic studies have not yielded clear conclusions concerning the protective effects of tea consumption against cancer formation in humans. The aim of this systematic and up-to-date review was to critically evaluate all epidemiological studies published so far to report an association between green tea consumption and GC risk.

## SEARCH STRATEGY AND METHODS

### Database and search strategy

Research articles presented in this review includes epidemiological studies of green tea consumption in relation to GC risks. Epidemiological studies were retrieved through three computerized literature search engines: PubMed (Medline), American University of Health Science's literature database EBSCOHOST, and Google Scholar. All 5490 journals in PubMed and 32 publications in EBSCOHOST are utilized in the search. Only articles published between 1990 and 2012 were retrieved. The major descriptor key words used for the search were stomach cancer, GC, green tea, *Camellia sinensis*, EGCG, polyphenol, catechin and the minor descriptor key words used were tumor, cancer, lesion, polyp, adenocarcinoma. The identifiers used were risk, prevention, treatment, prolong disease. In addition, relevant publications retrieved from reference lists were manually searched to be included in this review. Abstracts were reviewed and studies retrieved in full.

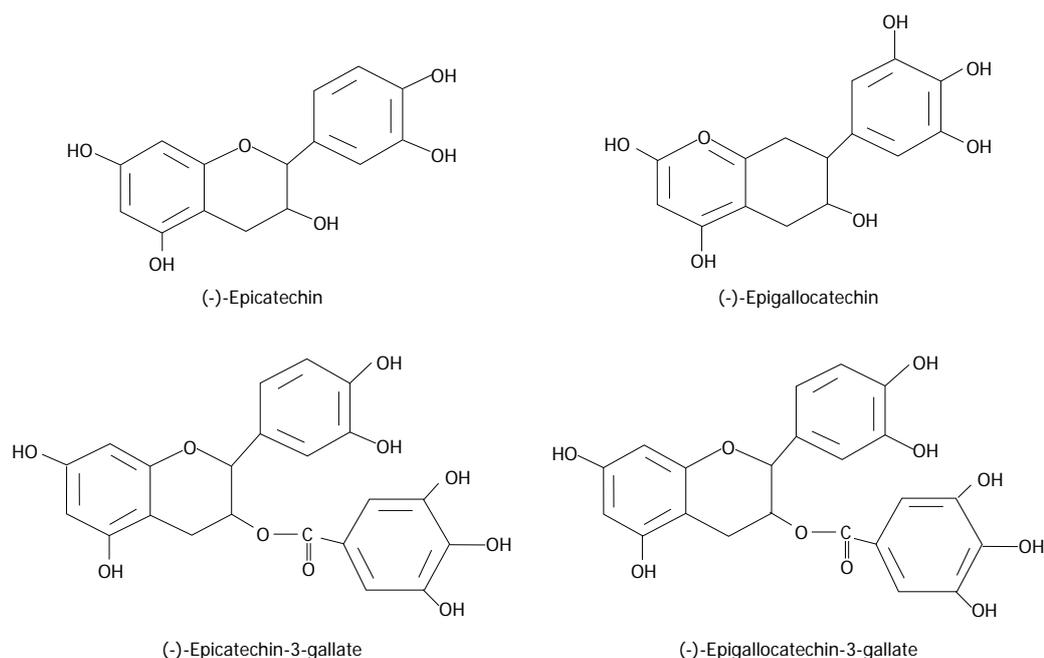


Figure 1 Structures of green tea catechins.

### Inclusion criteria

Only case-control studies, cohort studies, and prospective studies reporting association between green tea and stomach cancer risk were included. Articles specifying the number of GC cases and controls for case-control studies, odds ratio (OR) or relative risk (RR) and its corresponding 95%CI for highest versus non/lowest levels of tea intake are presented in this review.

### Exclusion criteria

*In vitro* cell culture and animal studies were excluded. Papers that failed to report the number of individuals (cases and controls) involved in the study and the level of green tea consumption (amount or frequency of green tea consumed) were also excluded. Articles written in foreign languages, as well as abstracts, were excluded since the complete publications were not available for this review work.

### Data extraction

All articles were read in full. From those studies finally selected, the following data were extracted: study design, study name (first author, publication year), region and study period (years), population size, green tea consumption level, OR or RR with 95%CI of the highest consumption level, and adjustments.

## GREEN TEA AND GC RISK

This review included a total of 17 epidemiological studies (10 case-control studies<sup>[28-37]</sup> and 7 cohort studies<sup>[38-44]</sup> published between 1988 and 2010. Figure 2 displays a flow diagram of the procedure used to identify the studies included in this review. There were 210 papers relevant to the words used for the search. Through the

steps of title screening, 125 studies were excluded (16 duplicate articles, 31 articles not in English, and 78 were not epidemiologic studies). Abstracts from 85 articles were reviewed and an additional 67 studies were excluded (33 were not epidemiologic studies, 34 were not conducted in humans), resulting in 18 articles for full publication review. Of these, 7 were excluded (5 were not green tea, 2 did not report usable data). Of the remaining 11 articles, six articles were identified from the reference lists and are included in this review. As a result, 17 articles were found to meet the inclusion criteria described above.

Eleven studies were conducted among the Japanese population in Japan<sup>[28-30,33,35,39-44]</sup>, five studies were conducted among the Chinese population in China<sup>[31,32,34,36,37]</sup>, and others were conducted among the Japanese-born population in Hawaii, United States<sup>[38]</sup>. All of the Chinese studies were case-control studies. RR, OR, 95%CI of the highest green tea consumption levels were included. Table 1 represents the main characteristics of the studies included in this review.

Three hospital-based case control studies were carried out in Japan<sup>[29,33]</sup> and China<sup>[37]</sup>. In all investigations, all cases were confirmed histologically and controls were free of gastrointestinal diseases. Information on green tea consumption, as well as lifestyle habits and family history, was obtained through a self-administered questionnaire before the final diagnosis. Adjustments were made for age, gender, place of residence<sup>[29]</sup>, age, gender, year and season at first hospital visit, smoking and alcohol drinking status, physical exercise, intake of coffee, black tea, fruit, rice and beef<sup>[33]</sup>, or age, gender, education level, and smoking status<sup>[37]</sup>. The RRs or ORs were calculated using, as reference category, subjects who drank less than 1 cup/d, or non-drinking subjects<sup>[33,37]</sup>. Although Kato *et al.*<sup>[29]</sup> did not find an association between green tea

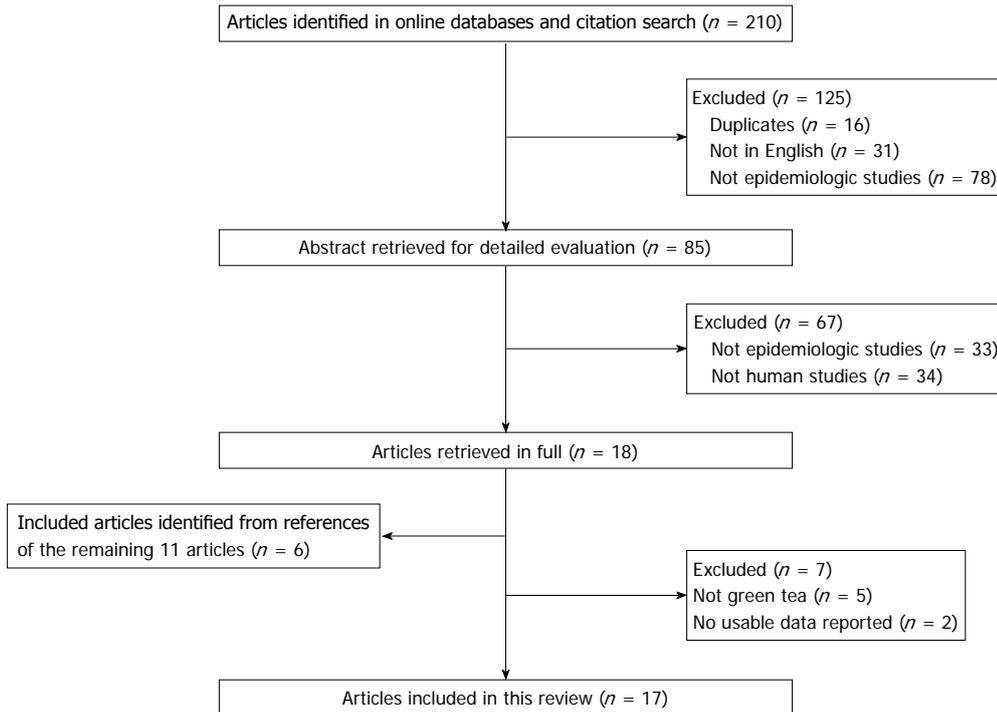


Figure 2 Flow diagram of identification of relevant studies.

consumption and GC risk, Inoue *et al.*<sup>[33]</sup> and Deandrea *et al.*<sup>[37]</sup> showed that the frequent consumption of green tea ( $\geq 7$  cups/d and  $\geq 750$  g/year, respectively) decreased the risk of GC. Furthermore, Deandrea *et al.*<sup>[37]</sup> observed a significant decrease in GC risks amongst drinkers of green tea at lukewarm temperature.

Five population-based case control studies were conducted in Japan<sup>[35]</sup> and China<sup>[31,32,34,37]</sup>. Cancer cases were confirmed pathologically<sup>[34,37]</sup>, histologically<sup>[31,32]</sup> or by other methods<sup>[35]</sup>. In some epidemiological studies, controls were randomly selected<sup>[32,34,36]</sup> and matched according to age and gender<sup>[31,32,35]</sup>, or study area<sup>[35]</sup>. Information was either obtained through interview<sup>[31,32,34,37]</sup> or administration of questionnaires<sup>[35]</sup>. Adjustments were made for age, education, birthplace, alcohol and cigarette usage, fresh fruit, vegetable and preserved fruit intake<sup>[31]</sup>; age, income, education among women and further adjusted for smoking and alcohol drinking among men<sup>[32]</sup>; age, gender, body mass index, cigarette smoking, and alcohol drinking<sup>[34]</sup>; and age, gender, education, income, body mass index, smoking and alcohol drinking, very hot food eating habit, *Helicobacter pylori* (*H. pylori*) infection, stomach disease, family history of GC<sup>[35]</sup>. The RRs or ORs were calculated using, as reference category, non-tea drinkers<sup>[31,32,34,36]</sup> or low consumption of green tea<sup>[35]</sup>. Four of these five studies reported an inverse association between green tea consumption and the risk of GC<sup>[31,32,34,36]</sup>. The investigation conducted in Japan did not report any association<sup>[35]</sup>. Yu *et al.*<sup>[31]</sup> reported that drinking warm or cold tea was associated with significant decreased GC risk compared with non-drinkers. This coincides with the findings of Deandrea *et al.*<sup>[37]</sup> that there is a significant inverse association with consuming lukewarm green tea and GC risk.

Two population-hospital-based case control studies were conducted in Japan<sup>[28,30]</sup>. In both studies, cases were histologically diagnosed and hospital controls were recruited. Information from cases and hospital controls, as well as population controls, on the frequency and amount of green tea consumption was obtained by interview. Interviews were performed by the public health nurses and hospital staff<sup>[28]</sup> or by the authors and colleagues<sup>[30]</sup>. In both studies, the interview on cases and hospital controls were conducted before diagnostic procedures. The results were adjusted for age, gender, smoking, intake of mandarin oranges and other fruits<sup>[28]</sup>, and age, gender, residence, and smoking status<sup>[30]</sup>. The RRs for both studies were compared intermediate and high consumption with low consumption of green tea. Kono *et al.*<sup>[28]</sup> observed a significantly decreased GC risk with high consumption of green tea (10 or more cups/d) compared to both hospital and population controls. However, Hoshiyama *et al.*<sup>[30]</sup> observed a minimal to positive association in hospital and population controls.

All of the seven cohort studies were carried out among the Japanese population<sup>[38-44]</sup>. However, one was conducted in Hawaii, United States<sup>[38]</sup>. Information on the frequency and amount of green tea consumed and on other lifestyle factors was obtained by a self-administered postal questionnaire. In two of the seven cohort studies, the validity of the food frequency questionnaire was evaluated<sup>[42,44]</sup>, and in two of the seven cohort studies the questionnaire was checked by interviewers<sup>[40,41]</sup>. In the study by Galanis *et al.*<sup>[38]</sup> of 11907 randomly selected Japanese residents of Hawaii, 108 participants developed GC (follow-up period of 14.8 years on average). The study by Nakachi *et al.*<sup>[39]</sup> on 8552 residents in Saitama Prefecture,

Table 1 Characteristics of epidemiological studies on green tea consumption and stomach cancer risk

| Ref.  | Region and observation period         | Study population <sup>1</sup>            | Green tea consumption levels <sup>2</sup>   | RR or OR (95%CI) <sup>3</sup>  | Direction of association                        | Adjustments   |
|---|---------------------------------------|--|---|--|---|---|
| Case-control studies                          |                                       |  |   |  |   |   |
| Kono <i>et al</i> <sup>[28]</sup> , 1988      | Kyushu, Japan, 1979-1982              | 139 GC cases<br>2574 HC<br>278 PC        | ≤ 4 cups/d<br>5-9 cups/d<br>≥ 10 cups/d   | HC: 0.5 (0.3-1.1)<br>PC: 0.3 (0.1-0.7)   | Inverse (significant) with high consumption     | Age, gender, cigarette smoking, mandarin oranges and other fruits   |
| Kato <i>et al</i> <sup>[29]</sup> , 1990      | Aichi, Japan, 1985-1989               | 427 GC cases<br>3014 HC                  | 1-4 cup/d<br>≥ 5 cups/d   | Males: 1.01 (0.70-1.47)<br>Females: 0.81 (0.51-1.27)   | None  | Age, gender, residence (metropolitan area in Aichi prefecture, other areas of Aichi prefecture, and other prefectures)  |
| Hoyoshima <i>et al</i> <sup>[30]</sup> , 1992 | Saitama, Japan, 1984-1990             | 294 GC cases<br>202 HC<br>294 PC         | ≤ 4 cups/d<br>5-7 cups/d<br>≥ 8 cups/d  | HC: 1.3 (0.8-2.1)<br>PC: 0.8 (0.5-1.3)   | Minimal to positive                             | Age, gender, residence, cigarette smoking   |
| Yu <i>et al</i> <sup>[31]</sup> , 1995        | Shanghai, China, 1991-1993            | 711 GC cases<br>711 matched PC           | Non drinkers<br>Drinkers  | Temperature: 0.71 (0.54-0.93)<br>Boiling hot: 1.18 (0.75-1.86)<br>Hot: 0.63 (0.46-0.87)<br>Warm/cold: 0.51 (0.29-0.91) | Inverse (significant) with warm/cold green tea) | Age, education, birthplace, alcohol drinking, cigarette smoking, intake of fresh fruit, vegetables and preserved fruit  |
| Ji <i>et al</i> <sup>[32]</sup> , 1996        | Shanghai, China, 1988-1989            | 1124 GC cases<br>1451 matched PC         | Non drinkers<br>Males:<br>≤ 1200 g/yr<br>≤ 2000 g/yr<br>≤ 3000 g/yr<br>Females:<br>≤ 1200 g/yr<br>> 1200 g/yr | Males: 0.76 (0.55-1.27)<br>Females: 0.81 (0.46-1.43)   | Inverse   | Age, income, and education among women; further adjusted for smoking and alcohol drinking among men   |
| Inoue <i>et al</i> <sup>[33]</sup> , 1998     | Nagoya, Japan, 1990-1995              | 893 GC cases<br>21128 HC                 | Rarely<br>≥ 7 cups/d  | 0.69 (0.48-1.00)   | Inverse   | Age, gender, year and season at first hospital visit, habitual smoking, habitual alcohol drinking, regular physical exercise, intake of coffee, black tea, fruit, rice and beef   |
| Setiawan <i>et al</i> <sup>[34]</sup> , 2001  | Yangzhong, China, 1995                | 133 GC cases<br>433 PC                   | Non drinkers<br>1-21 cups/wk<br>> 21 cups/wk  | 0.39 (0.15-1.01)   | Inverse (significant)                           | Age, gender, body mass index, cigarette smoking, alcohol drinking   |
| Hoshiyama <i>et al</i> <sup>[35]</sup> , 2004 | Japan, 1988-1990                      | 157 GC cases<br>285 PC                   | < 1 cup/d<br>1-2 cups/d<br>3-4 cups/d<br>5-9 cups/d<br>≥ 10 cups/d  | 1.20 (0.6-2.5)   | None  | Age, gender, cigarette smoking, <i>H. pylori</i> infection, history of peptic ulcer, family history of stomach cancer, educational level, consumption of rice, miso soup, green-yellow vegetables, white vegetables, fruits, preference for salty foods |
| Mu <i>et al</i> <sup>[36]</sup> , 2005        | Taixing, China, 2000                  | 206 GC cases<br>415 PC                   | Non-drinkers<br>> 250 g/mo  | 0.39 (0.17-0.91)   | Inverse   | Age, gender, education, income, body mass index, cigarette smoking, alcohol drinking, very hot food eating habit, <i>H. pylori</i> infection, stomach disease, family history of stomach cancer   |
| Deandrea <i>et al</i> <sup>[37]</sup> , 2010  | Harbin, China, 1987-1989              | 266 GC cases<br>533 HC                   | Non-drinkers<br>< 750 g/yr<br>≥ 750 g/yr  | Temperature: 0.87 (0.60-1.25)<br>Hot: 1.27 (0.85-1.90)<br>Lukewarm: 0.19 (0.07-0.49)                                   | Inverse (significant) with lukewarm green tea)  | Age, gender, education level, cigarette smoking   |
| Prospective cohort studies                    |                                       |  |   |  |   |   |
| Galanis <i>et al</i> <sup>[38]</sup> , 1998   | Hawaii, Unites States, 1975-1994      | 108 GC cases<br>11907 Japanese residents | Non-drinkers<br>1 cup/d<br>> 2 cups/d   | 1.5 (0.9-2.3)<br>Males: 1.6 (0.9-2.9)<br>Females: 1.3 (0.6-2.6)  | Positive  | Age, gender, years of education and Japanese place of birth   |
| Nakachi <i>et al</i> <sup>[39]</sup> , 2000   | Saitama, Japan, 2000                  | 488 GC cases<br>8552 adults              | ≤ 3 cups/d<br>4-9 cups/d<br>≥ 10 cups/d   | 0.59 (0.35-0.98)<br>Males: 0.54 (0.22-1.34)<br>Females: 0.57 (0.34-0.98)   | Inverse   | Cigarette smoking, alcohol drinking, intake of green and yellow vegetables, intake of rice  |
| Tsubono <i>et al</i> <sup>[40]</sup> , 2001   | Miyagi, Japan, 1984-1992              | 419 GC cases<br>26311 adults             | < 1 cups/d<br>1-2 cups/d<br>3-4 cups/d<br>≥ 5 cups/d  | 1.2 (0.9-1.6)<br>Males: 1.5 (1.0-2.1)<br>Females: 0.8 (0.5-1.3)  | None  | Age, gender, type of health insurance, history of peptic ulcer, cigarette smoking, alcohol consumption, consumption of rice, black tea, coffee, meat, green or yellow vegetables, pickled vegetables, other vegetables, fruits and bean-paste soup      |
| Nagano <i>et al</i> <sup>[41]</sup> , 2001    | Hiroshima, Nagasaki, Japan, 1979-1981 | 901 GC cases<br>37639 adults             | 0-1 cups/d<br>2-4 cups/d<br>≥ 5 cups/d  | 0.95 (0.76-1.2)  | None  | Age, gender, city of residence, radiation exposure, cigarette smoking, alcohol drinking, body mass index, education level   |

|   |   |                                |   |  |      |   |
|---|---|--------------------------------|---|--|------|---|
| Hoshiyama <i>et al</i> <sup>[42]</sup> , 2002 | Japan (nationwide), 1988-1997                                   | 359 GC deaths<br>72851 adults  | < 1 cups/d<br>1-2 cups/d<br>3-4 cups/d<br>5-9 cups/d<br>≥ 10 cups/d | Men: 1.0 (0.5-2.0)<br>Women: 0.7 (0.3-2.0)           | None | Age, smoking, history of peptic ulcer, family history, consumption of rice, miso soup, green-yellow vegetables, fruits and preference for salty foods |
| Fujino <i>et al</i> <sup>[43]</sup> , 2002    | Japan (nationwide), 1988-1990                                   | 379 GC deaths<br>328030 adults | Everyday<br>≤ 3 times/d<br>> 3 times/d                              | Males: 1.11 (0.75-1.63)<br>Females: 1.43 (0.78-2.62) | None | Age, gender, cigarette smoking, alcohol drinking, diet, sporting activities, medical history, education level   |
| Sasazuki <i>et al</i> <sup>[44]</sup> , 2004  | Japan (nationwide) Cohort I : 1990-2001<br>Cohort II: 1993-1999 | 892 GC cases<br>72943 adults   | < 1 cups/d<br>1-2 cups/d<br>3-4 cups/d<br>≥ 5 cups/d                | Males: 0.97 (0.77-1.22)<br>Females: 0.70 (0.47-1.05) | None | Age, area, cigarette smoking, consumption of fruit, green-yellow vegetables, fish gut, miso soup, rice, black tea and coffee                          |

<sup>1</sup>Number of gastric cancer (GC) cases observed over the study period; <sup>2</sup>Frequency of green tea consumption reported in each study; <sup>3</sup>Relative risk (RR) or odds ratio (OR) for the highest green tea (GT) consumption level studies. HC: Hospital controls; PC: Population controls; *H. pylori*: *Helicobacter pylori*.

**Table 2 Summary of findings: Number of epidemiological studies with its reported direction of association between green tea consumption and gastric cancer risk**

| Study design         | Direction of association |      |          | Total |
|----------------------|--------------------------|------|----------|-------|
|                      | Negative/inverse         | None | Positive |       |
| Case-control studies | 7                        | 3    | 0        | 10    |
| Cohort studies       | 1                        | 5    | 1        | 7     |
| Total                | 8                        | 8    | 1        | 17    |

Japan found 488 deaths from GC (follow-up period of 11 years). In the study by Tsubono *et al*<sup>[40]</sup> on 26311 residents of the Miyagi Prefecture, 419 participants developed GC (follow-up period of 9 years). Nagano *et al*<sup>[41]</sup> examined 37639 atomic bomb survivors, and of those 901 participants developed GC (follow-up period of 15 years). In the study by Hoshiyama *et al*<sup>[42]</sup> on 72851 Japanese residents, 359 subjects died of GC (follow-up period of 7 years). In the study by Fujino *et al*<sup>[43]</sup> on 328030 Japanese residents, 345 subjects died of cancer (follow-up period of 7 years). Finally, in the study conducted by Sasazuki *et al*<sup>[44]</sup> of 72943 Japanese residents, 892 participants developed GC (follow-up period of (follow-up period of 10 years). In all cohorts studies, some adjustments were made: age<sup>[38-44]</sup>, gender<sup>[38-41,44]</sup>, years of education<sup>[38,41,43]</sup>, cigarette smoking (for men only)<sup>[38-44]</sup>, alcohol consumption (for men only)<sup>[39-41,43]</sup>, place of birth<sup>[38]</sup>, history of peptic ulcer<sup>[40,42]</sup>, body mass index<sup>[41]</sup>, coffee consumption and several other foodstuffs<sup>[39,40,42,43]</sup>. RRs of the highest green tea consumption levels were included instead for all consumption levels. In all studies, some adjustments were made: gender, age, years of education, cigarette smoking, alcohol consumption, place of birth, family history of GC, body mass index, coffee consumption and several other foodstuffs. The RRs were calculated using, as reference category, either non-drinkers<sup>[38]</sup> or the lowest level of green tea consumption<sup>[39-44]</sup>. In the first cohort study, Galanis *et al*<sup>[38]</sup> reported a non-significant increased risk of GC associated with green tea consumption. In contrast, Nakachi *et al*<sup>[39]</sup> reported a slight inverse association between green tea consumption and GC risks. The other five cohort studies found no association between green tea consumption and GC incidence<sup>[40-44]</sup>.

## CRITICAL ANALYSIS OF EPIDEMIOLOGICAL FINDINGS

Green tea contains polyphenols, powerful antioxidants that act as chemopreventive agents. EGCG is considered the major polyphenol that constitute to green tea's preventive constitute. Significant amounts of *in vitro* studies with human cancer cells as well as *in vivo* studies with animal models and have investigated the mechanism of EGCG for its anticarcinogenic properties. EGCG blocks multistage carcinogenesis by modulating a wide spectrum of signal transduction pathways, including mitogen-activated protein kinases, Janus kinase-signal transducer and activator of transcription, phosphoinositide 3-kinase/protein kinase B, Notch, nuclear factor-κB and Wnt/β-catenin, involved in cell proliferation, transformation, apoptosis, inflammation, invasion and metastasis<sup>[45]</sup>. Accumulative studies show another green tea catechin ECG can also interfere with multiple cell signaling pathways and has multiple cellular targets which are likely to interact in concert to reduce the risk of cancer<sup>[46]</sup>. Several clinical studies with human subjects have also demonstrated that consumption of green tea as well as EGCG exert beneficial effects<sup>[45]</sup>. However, epidemiologic studies are somewhat limited with mixed results. Of the 17 epidemiological studies investigated in this review, eight have no association<sup>[29,30,35,40-44]</sup>, eight have inverse association<sup>[28,31-34,36,37,39]</sup>, and one has positive association<sup>[38]</sup>. Table 2 provides a summary of findings. A trend observed is that decreased risk was associated with an increased green tea consumption level. The studies conducted in China showed a stronger reduction in GC among green tea drinkers than those conducted in Japan. A few authors have argued that the relative lack of subjects in Japan who do not drink green tea may have resulted in an insufficient number of non-drinkers, and this might be an explanation for the weaker association among Japanese studies<sup>[36,41,47]</sup>. In terms of study design, prospective/cohort studies are considered to be more reliable than case-controlled studies because of the large population size. However, the value of a study depends not only on the type of design, but also on the overall quality. This review identified seven cohort studies which examined the association between cancer risk and green tea consumption.

Of these, the smallest study observed an inverse association<sup>[39]</sup>, whereas the five largest studies observed no association<sup>[40-44]</sup>. One study found a non-significant positive association<sup>[38]</sup>.

A negative association is stronger in case-control studies than in cohort studies. Seven of the ten case-control studies have suggested an association of green tea consumption and reduced GC risk<sup>[28,31-34,36,37]</sup>, whereas only one of the seven cohort studies showed a reduced GC risk<sup>[39]</sup>. These discrepancies may be partly associated with the limitations of case-control design: recall, information, selection, and confounding biases. Cancer patients may recall their dietary habits differently from healthy controls, and healthy controls are rarely representative of the population as a whole and tend to report a healthy dietary habit<sup>[36]</sup>. In retrospective studies like case control studies, the decreased consumption of green tea after abdominal symptoms due to GC may have biased the patient's recall of past consumption, resulting in underestimating the patient's true intake on green tea. Moreover, present dietary habits might influence the accuracy of recalling past dietary habits. In case-control studies, there may be a problem with the reliability of information, because information that exposes the past history is collected after cancer is diagnosed. In all studies included in this review, selection and confounding bias was minimized (*i.e.*, cases and controls were drawn from the same population; adjustment for certain factors). However, in hospital-based case control studies, the controls were not free of diseases. Furthermore, self-selection bias could not be excluded because information was obtained by questionnaire survey. Only one cohort study (reporting no association between green tea consumption and GC risk) showed limitations due to selection bias<sup>[30]</sup>.

The non-significant findings regarding the effects of green tea consumption on GC risk in this review contradicts the results of previous experimental studies on this topic using *in vivo* animal models and *in vitro* cancer cell lines. The experimental studies have suggested that GTPs might have a protective effect against GC due to apoptosis-inducing, antimutagenic, anti-inflammatory, and antioxidant activities. The reason for this discrepancy between the results of experimental studies and this review is unclear. However, there might be a few possible explanations. First is the difference in causative factors between the cancers in humans and animals. It is possible that green tea may be only effective against GC in certain animal species. Second, in *in vivo* studies, the doses of green tea used in animal models are much higher in comparison to human consumption. Lastly, in the context of GC, there might be a difference in the biological activities of polyphenols as an independent compound rather than green tea taken as a whole. Despite the protective role of an independent compound against the development of cancer, it is possible that the adverse effect of green tea taken as a whole is due to the interactions and complex biological mechanisms of its multiple constituents.

In this review, eight studies have analyzed men and women separately, provided RRs or ORs for each gen-

der<sup>[18,22,28-30,32-34]</sup>. In these eight studies, men were considered to show no relation at all. Women, however, seemed to show lower risk, though there was no statistically significant difference. It should also be noted that cigarette smoking and alcohol consumption are important confounding factors. For example, in a case-control study by Tsubono *et al.*<sup>[40]</sup>, a protective effect of green tea consumption was observed in women, mostly non-smokers, with an OR of 0.8 and a 90%CI of 0.5-1.3. However, no protective effect was found in men, who were mostly smokers (OR = 1.5, 95%CI: 1.0-2.1). In Asia, tea drinking is commonly associated with cigarette smoking in men. Although the study by Tsubono *et al.*<sup>[40]</sup> and other studies included in this review tried to correct for smoking, the interaction between these two factors is difficult to assess.

It is noted by Yu *et al.*<sup>[31]</sup> that among green tea drinkers, the risk of developing GC did not depend on the age when routine tea drinking started. This implied that green tea may disrupt gastric carcinogenesis at the intermediate and late stages. In addition to finding a negative association between green tea drinking and GC risk, Yu *et al.*<sup>[31]</sup> noted that a lowered risk is observed when the tea was served warm/cold (boiling hot, OR = 1.18, 95%CI: 0.75-1.86; warm/cold, OR = 0.51, 95%CI: 0.29-0.91). This observation was further supported by another case-control study by Deandrea *et al.*<sup>[37]</sup> (hot, OR = 1.27, 95%CI: 0.85-1.90; lukewarm, OR = 0.19, 95%CI: 0.07-0.49).

In one of the cohort studies, Galanis *et al.*<sup>[38]</sup> suggested that tea might have a mutagenic effect (OR = 1.5, 95%CI: 0.9-2.3). However, the number of cases in this study was very small, and that may have resulted in the exaggerated risk estimates.

Overall, these data do not seem to suggest a protective effect of green tea on GC. The inconsistent results between the epidemiologic studies may be due to variables, such as differences in tea preparation and consumption, the methods of tea production, the bioavailability of tea compounds, and genetic variation in how the human body responds to tea consumption.

All research findings from tumor cell cultures, animal models, and epidemiological studies have shown the effects of green tea and tea polyphenols in GC prevention. However, this should be confirmed in clinical trials in order to gain more knowledge of the relationship between green tea consumption and GC risks. This review shows that the overall evidence for protective effects of green tea against cancer is inconclusive. Therefore, further epidemiologic studies and clinical trials are warranted. Adequate sample sizes, better descriptions of populations and/or clear definitions of green tea consumption may be required for conclusive studies. It is also important to consider the type of tea or its preparation (*e.g.*, short time *vs* long brewing time and hot tea *vs* iced tea) due to the marked impact of these factors on polyphenol content and concentration. It is also important to draw attention on the need of further in-depth studies on the nature and mechanisms of the active green tea compounds, on the bioavailability of the different catechins in human and

appropriate dose level to act as functional food. Further epidemiological research, designed specifically to study the effect of green tea on GC, is needed. Because many green tea drinkers brew more than one cup of tea from each batch of dried leaves, in future studies, green tea consumption should be assessed in terms of the amount of active ingredient consumed in a given period. Nevertheless, the *in vitro*, animal, and epidemiologic studies on the topic of green tea consumption and GC risks offered insightful results worthy of future research on potential cancer prevention in human.

This review has several limitations. First of all, this review only analyzed studies of the Japanese and Chinese population. Green tea is consumed mainly in Asian countries, such as China, Japan, and South Korea, it is not appropriate to generalize the findings in the study and apply it to all populations. The findings and explanations should be explored in further clinical research on a more diverse population. Second, as with most literature reviews, the results should be interpreted with caution because the highest consumption of green tea, lengths of follow-up, and questionnaire were not uniform. Although all publications included in this review adjusted for the consumption of dietary items other than green tea as much as possible, the possibility of residual confounding factors cannot be excluded. Lastly, 15 of the 17 studies investigated did not incorporate *H. pylori* infection, a strong risk factor for GC, as a confounding factor. The subjects with chronic gastritis caused by *H. pylori* infection might have limited their consumption of foods and beverages, including green tea. On the other hand, several studies have indicated positive interactions between green tea and *H. pylori*. EGCG, one of the green tea catechins, has been shown to possess significant protective effect against *H. pylori*-induced cytotoxicity in gastric epithelial cells *via* interference of the toll-like receptor 4 signaling induced by *H. pylori*<sup>48]</sup>. Green tea or GTPs have exhibited bactericidal and/or bacteriostatic effects against *H. pylori*<sup>49,50]</sup>. Various components of green tea have been reported to inhibit *H. pylori* infection as well as *H. pylori*-induced gastritis and gastric epithelial cell proliferation in animal models<sup>51-53]</sup>. Chronic atrophic gastritis (CAG) represents a precancerous lesion of the stomach, and *H. pylori* infection is known to increase the risk of CAG. Shibata *et al.*<sup>54]</sup> conducted a cross-sectional study on 636 subjects living in a farming village in Japan to examine the relationship among green tea consumption, *H. pylori* infection, and CAG. *H. pylori* infection was positively associated with the risk of CAG (OR = 3.73; 95%CI: 2.59-5.36). High green tea consumption (> 10 cups/d) was negatively associated with the risk of CAG, even after adjustment for *H. pylori* and lifestyle factors associated with green tea consumption (OR = 0.63; 95%CI: 0.43-0.93). The results support the hypothesis that green tea consumption prevents gastric preneoplasia. Hence, future epidemiological studies on green tea and GC risk should consider multivariate analysis on relationship among *H. pylori* infection sustained by cytotoxin-associated gene A-positive strains, other risk factors, and green tea consumption.

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**P- Reviewers** Franceschi F, Shibata T, Yen HH  
**S- Editor** Huang XZ **L- Editor** A **E- Editor** Xiong L



## Hepatocellular carcinoma and food contamination: Ochratoxin A as a great prompter

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Received: February 26, 2013 Revised: April 18, 2013

Accepted: May 8, 2013

Published online: June 28, 2013

**Key words:** Ochratoxin A; Mycotoxin; Food contamination; Hepatocellular carcinoma; Liver cancer; Egypt

**Core tip:** This manuscript is a short commentary to the paper which considered ochratoxin A, a mycotoxin produced by fungi, as an important carcinogen and etiological agent able to induce hepatocellular carcinoma. Based on a recent study, we try to link food contamination with a possible increased incidence on hepatocellular carcinoma cases at eastern populations.

Felizardo RJF, Câmara NOS. Hepatocellular carcinoma and food contamination: Ochratoxin A as a great prompter. *World J Gastroenterol* 2013; 19(24): 3723-3725 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3723.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3723>

### Abstract

Ochratoxin A (OTA) is a secondary metabolite of *Aspergillus* and *Penicillium*, microorganisms that can be hazardous to health when present as food contaminants. OTA is a potent member of a group of mycotoxins. Prolonged exposure to mycotoxins in the diet is related to cancer, among other diseases. Hepatocellular carcinoma (HCC) accounts for 70%-90% of primary liver cancers and is the third leading cause of cancer-related deaths worldwide. In a recent study, Ibrahim *et al* proposed a correlation between the incidence of HCC and contamination with OTA. Analysis of OTA in serum samples showed that HCC patients had the highest incidence of OTA of the subjects examined (5-fold higher than that of the control group). OTA levels were significantly increased in HCC patients. This study demonstrates that chronic exposure to high levels of OTA may be associated with a high risk of liver cancer development. Future epidemiologic studies of HCC should focus on good practices in food preparation, food storage and the consumption of OTA-containing foods.

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### COMMENTARY ON HOT TOPICS

Food contamination is a public health problem that is monitored worldwide by the Food and Agriculture Organization of the United Nations and by the World Health Organization. Care in food preparation and storage are extremely important to avoid ingestion of various microorganisms and their toxins. High temperatures and humidity during harvest, storage and processing of grains, nuts and other crops, are appropriate conditions for fungal and mold development, especially when food is stocked in an inappropriate manner. Species of *Aspergillus* and *Penicillium* are the major producers of aflatoxins and ochratoxins, mycotoxins that have been classified as potent carcinogens by the International Agency for Research on Cancer and that are well known for liver toxicity.

Hepatocellular carcinoma (HCC) is a major health problem with a rising incidence in western countries<sup>[1]</sup>, though most HCC cases (> 80%) occur in sub-Saharan Africa or eastern Asia. China alone accounts for more than 50% of the world's cases<sup>[2]</sup>. HCC accounts for 70%-90% of primary liver cancers, making it the third leading cause

of cancer-related deaths worldwide<sup>[3-5]</sup>. Hepatitis B virus, hepatitis C virus infections and alcohol intake are widely recognized as the main causes of HCC<sup>[6,7]</sup>.

Aflatoxins (AFT) are secondary metabolites produced by some *Aspergillus* species that contaminate food during storage, production and processing. Due to their high toxicity and mutagenic, teratogenic and carcinogenic effects<sup>[8]</sup>, they have long been suggested as possible an etiologic agent of HCC. Mycotoxin poisoning occurring in the presence of hepatitis B virus infection is related to an increased risk of HCC development. AFT are metabolized by hepatic enzymes, generating reactive epoxide species that are able to form a covalent bond with guanine<sup>[9]</sup>. The resulting adducts can promote cellular and macromolecule damage and have already been described as biomarkers for aflatoxin contamination<sup>[10,11]</sup>.

Though food contamination by certain AFTs is correlated with HCC occurrence, it is unknown if ochratoxin A (OTA) has a role in HCC pathogenesis, as proposed recently by Ibrahim *et al.*<sup>[12]</sup>. Though a possible relationship between OTA and HCC has been statistically analyzed in several regions, there is as yet no consensus.

OTA is an isocoumarin-derived mycotoxin that is most commonly produced by *Aspergillus ochraceus*<sup>[13]</sup> growing on stored barley, corn or wheat. OTA can be found in a wide range of human foods such as cereals, beer, wine, cocoa, coffee, dried vine fruit and spices, as well as in some meat products and milk. It is a potent, thermostable, immunosuppressive and carcinogenic toxin<sup>[14]</sup>, and its effects are attributable to its ability to interfere with protein synthesis. After being absorbed, OTA is metabolized by the liver and excreted as both bile and in the renal proximal tubules<sup>[15]</sup>. In experimental animals, OTA induces tumors in the kidney as well as in the liver<sup>[15,16]</sup>. Although several lines of evidence derived from animal experiments implicate OTA in hepatic carcinogenesis<sup>[17,18]</sup>, no epidemiological data are available to evaluate such relationship. Biomarkers such as reduced glutathione-conjugates, N-acetylcysteine-conjugates and DNA-OTA adducts have been detected as products of OTA metabolism by the liver<sup>[19,20]</sup>, but no association between HCC and OTA has been reported.

According to Ibrahim *et al.*<sup>[12]</sup>, Egyptians diagnosed with HCC (and defined by high serum levels of alpha-fetoprotein and altered liver enzyme levels) have significantly higher levels of OTA in their sera than control subjects; the increase is approximately 5-fold. The authors also investigated the strength of the association between OTA and HCC and found that HCC was 9.8 times as frequent in an OTA-exposed group of subjects. This evaluation essentially aimed to determine the contribution of OTA exposure to HCC pathogenesis and suggested that OTA is a plausible etiologic factor for HCC.

Ibrahim *et al.*<sup>[12]</sup> encourage the search for new possibilities linking OTA to HCC or other diseases in regions around the world. For instance, OTA is frequently related to Balkan nephropathy, which is characterized by a discrete form of tubulointerstitial nephropathy with insidious presentation and slow progression and high levels of

OTA in the urine<sup>[15]</sup>. Not only humans but also some ruminant and non-ruminant animals can be contaminated by OTA. Metabolic studies show that OTA can persist in the body of pigs; the hazard thus arises due to contamination of animal feed and constitutes an additional source of OTA contamination in human food<sup>[21]</sup>.

This study demonstrated that chronic exposure to high levels of OTA could increase the risk of liver cancer. Future epidemiologic studies of HCC should focus on good practices in food preparation, food storage and the consumption of OTA-containing foods such as cereals and milk by liver-diseased patients. Ibrahim *et al.*<sup>[12]</sup> opened the discussion of this new possible cause of HCC, which should not be ignored.

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P-Reviewer Jin B S-Editor Gou SX  
L-Editor A E-Editor Xiong L



## Small-bowel capsule endoscopy: A ten-point contemporary review

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Received: March 29, 2013 Revised: May 27, 2013

Accepted: June 1, 2013

Published online: June 28, 2013

### Abstract

The introduction of capsule endoscopy (CE) in clinical practice increased the interest for the study of the small-bowel. Consequently, in about 10 years, an impressive quantity of literature on indications, diagnostic yield (DY), safety profile and technical evolution of CE has been published as well as several reviews. At present time, there are 5 small-bowel capsule enteroscopy (SBCE) models in the worldwide market. Head-to-head trials have showed in the great majority of studies comparable results in terms of DY, image quality and completion rate. CE meta-analyses formed the basis of national/international guidelines; these guidelines place CE in a prime position for the diagnostic work-up of patients with obscure gastrointestinal bleeding, known and/or suspected Crohn's disease and possible small-bowel neoplasia. A 2-L polyethylene glycol-based purge, administered the day before the procedure,

is the most widely practiced preparation regimen. Whether this regimen can be further improved (*i.e.*, by further decreasing its volume, changing the timing of administration, coupling it with prokinetics and/or other factors) or if it can really affect the DY, is still under discussion. Faecal calprotectin has been used in SBCE studies in two settings: in patients taking non-steroidal anti-inflammatory drugs, to evaluate the type and extent of mucosal damage and, more importantly from a clinical point of view, in patients with known or suspected Crohn's disease for assessment of inflammation activity. Although there is still a lot of debate around the exact reasons of SBCE poor performance in various small-bowel segments, it is worth to remember that the capsule progress is non-steerable, hence more rapid in the proximal than in lower segments of the small-bowel. Capsule aspiration, a relatively unexpected complication, has been reported with increasing frequency. This is probably related with the increase in the mean age of patients undergoing CE. CE video review is a time-consuming procedure. Therefore, several attempts have been made to develop technical software features, in order to make CE video analysis easier and shorter (without jeopardizing its accuracy). Suspected Blood Indicator, QuickView and Fujinon Intelligent Chromo Endoscopy are some of the software tools that have been checked in various clinical studies to date.

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**Key words:** Capsule endoscopy; Calprotectin; Meta-analysis; Review; Preparation; Reading software; Complication; Indications

**Core tip:** This innovative, concise and "unique" review (structured as Q and A with several tables that make this paper very easy to read and hopefully enjoyable), keeps narrative text to the necessary minimum, in order to guide the reader to consult the wealth of information included in tabulated form. These tables are the outcome of the authors' personal endeavor to compile in a detailed, yet easy to refer way, informa-

tion that has often been overlooked by the plethora of similar reviews and/or info on contentious issues in capsule enteroscopy. We believe that this document can be used as reference for study, in reference lists of future manuscript and as important guide for future clinical research on the field.

Koulaouzidis A, Rondonotti E, Karargyris A. Small-bowel capsule endoscopy: A ten-point contemporary review. *World J Gastroenterol* 2013; 19(24): 3726-3746 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3726.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3726>

## INTRODUCTION

An early conceptual abstract on capsule endoscopy (CE), entitled “an endorobot for flexible endoscopy, a feasibility study”, was published in 1994<sup>[1]</sup>. Then, in 1997 two groups of pioneers, initially working independently in Israel and London, joined forces to achieve wireless endoscopy<sup>[2]</sup>. Three years later, in the Digestive Disease Week meeting of the millennium and almost concurrently in *Nature*<sup>[3]</sup>, Professor Swain presented the world’s first wireless capsule endoscope.

Indeed, the brainchild of Iddan<sup>[4]</sup> has revolutionised the field of gastrointestinal (GI) diagnostics, turning into reality the concept of painless and wireless endoscopy. Furthermore, the introduction of CE in clinical practice increased the interest for the study of the small-bowel. Consequently, in about 10 years, an impressive quantity of literature on indications, diagnostic yield (DY), safety profile and technical evolution of CE has been published as well as several reviews. Therefore, we aim to focus readers’ attention on contemporary and contentious issues, often missed from similar reviews on the field. We herein present (in a comprehensive yet user-friendly manner) a systematic review of the current literature in a form of question-and-answer. We expect CE readers, of all experience levels, will find this review useful source of further reading and reference.

## WHICH ARE THE DIFFERENCES AMONG THE CURRENT COMMERCIALY AVAILABLE CAPSULES?

Since 2001, the year of approval by the Food and Drug Administration of the first video capsule with the prophetic, yet slightly unfortunate, brand name mouth-to-anus (M2A<sup>®</sup>; Given<sup>®</sup>Imaging, Yoqneam, Israel), a total of more than 2000000 capsules have been ingested world-wide<sup>[5]</sup>. Furthermore, over the last decade, technology has improved in the field of CE as competition has become quite stiff. At present time, there are 5 small-bowel capsule enteroscopy (SBCE) models in the market world-wide (Table 1)<sup>[5,6]</sup>. Although similar in size and shape, they differ on several technical aspects. Of the 5 SBCE, four

are in widespread use, although most of the published literature studies are with PillCam<sup>®</sup>. Nevertheless, head-to-head trials have showed in the great majority of studies comparable results in terms of DY, image quality and completion rate (Table 2)<sup>[7-11]</sup>.

## DO HIGH-GRADE EVIDENCE SUPPORT THE USE OF CE IN CLINICAL PRACTICE?

In recent years, many authors<sup>[12-14]</sup> reviewed systematically the validity of SBCE in clinical practice. Out of this evidence base, it clearly emerges that in daily practice the leading indications for CE are: Obscure gastrointestinal bleeding (OGIB accounts for 60%-70% of all SBCE examinations world-wide), and Crohn’s disease (CD; known and/or suspected). Other clinical indications, although less common, are coeliac disease, small-bowel polyposis syndromes and clinical suspicion of small-bowel neoplasia<sup>[15,16]</sup>. Therefore, we decided to summarize (Table 3)<sup>[17-32]</sup>, the results of the more robust - from a methodological point of view - publications which addressed the role of CE in the field of small-bowel coeliac disease. These meta-analyses have formed the basis of national/international guidelines, which place CE in a prime position for the diagnostic work-up of patients with OGIB, known and/or suspected CD and possible small-bowel neoplasia<sup>[33-36]</sup>.

## WHICH IS THE BEST PREPARATION REGIMEN FOR SMALL-BOWEL CAPSULE ENDOSCOPY?

This certainly is one of the most contentious issues in CE. Since the introduction of CE in clinical practice, it was clear that small-bowel cleanliness is one of the key factors (as in fact is often the case for endoscopic examinations) to guarantee high diagnostic performance. Thus far, several studies have been performed in order to test whether the administration of different purgatives and/or prokinetics would impact on small-bowel cleanliness. It is noteworthy that these studies are rather heterogeneous in terms of type of laxatives administered, dosages and/or administration schedule (Table 3)<sup>[22,25,30]</sup>. Furthermore, in some studies laxatives and prokinetics were administered concurrently, which is probably a further source of bias. Essentially, the current evidence base suggests that a preparation regimen based on laxatives [more specifically polyethylene glycol (PEG)] is more effective -than fasting alone- in improving the small-bowel mucosa visualization. Among the PEG-based laxatives, a low volume schedule seems to be at least equally effective than high volume regimens<sup>[25,30]</sup>. Therefore, a 2-L PEG-based purge, administered the day before the procedure, is the most widely practiced preparation regimen. Whether this regimen can be further improved (*i.e.*, by further decreasing its volume, changing the timing of administration, coupling it with prokinetics and/or other pharmaceutical factors) or if it can really affect the DY, is still under discussion<sup>[37]</sup>.

**Table 1 Available types of small-bowel capsule endoscopes and operating characteristics**

| Capsule device                   | Company   | Country       | Field of view (°) | Lens          | LEDs | Image sensor | Transmission                      | Frames per second (fps) | Dimensions (mm) | Weight (g) | Battery life (h)     | Real-time imager | FDA approval | Reviewing software            | Optical enhancements |
|----------------------------------|---|---------------|-------------------|---------------|------|--------------|-----------------------------------|-------------------------|-----------------|------------|----------------------|------------------|--------------|-------------------------------|----------------------|
| PillCam <sup>®</sup> SB2         | Given <sup>®</sup> Imaging, Yokneam                   | Israel        | 156               | Multi-element | 4    | CMOS         | Radiofrequency                    | 2-4 <sup>1</sup>        | 11 × 26         | 3.45       | 9->11.5 <sup>2</sup> | Yes              | Yes          | Rapid <sup>®</sup> v7         | Blue-mode FICE 1,2,3 |
| MiroCam <sup>®</sup> v2          | IntraMedic <sup>®</sup> Co., Seoul                    | South Korea   | 170               | N/A           | 4    | CMOS         | EFP                               | 3                       | ø11 × 24        | 3.2        | 12                   | Yes              | Yes          | MiroView <sup>®</sup> v2      | ALICE colour-mode    |
| EndoCapsule <sup>®</sup>         | Olympus <sup>®</sup> Co., Tokyo                       | Japan         | 145               | N/A           | 4    | CCD          | Radiofrequency                    | 2                       | ø11 × 26        | 3.45       | 10                   | Yes              | Yes          | OLYMPUS <sup>®</sup> WS-1     | Contrast imaging     |
| OMOM <sup>®</sup> (SmartCapsule) | Chongding Jinshan Science and Technology Co., Beijing | China         | 140               | N/A           | 4    | CCD          | Radiofrequency                    | 2 (variable)            | 13 × 27.9       | 6          | 8                    | Yes              | No           | OMOM <sup>®</sup> workstation | N/A                  |
| CapsoCam <sup>®</sup> SV1        | CapsoVision <sup>®</sup> Inc., Saratoga               | United States | 360               | N/A           | 16   | N/A          | On-board EPROM flash memory (USB) | 16 (4 per camera)       | 11 × 31         | N/A        | 15                   | No               | No           | CapsoView <sup>®</sup>        | N/A                  |

<sup>1</sup>PillCam<sup>®</sup>SB2 (L) captures 2 fps - PillCam<sup>®</sup>SB2-4 captures 4 fps; <sup>2</sup>PillCam<sup>®</sup>SB2 (L) battery life > 11.5 h - PillCam<sup>®</sup>SB2-4 battery life > 11.5 h. LED: Light emitting diode; N/A: Not available; CMOS: Complementary metal-oxide-semiconductor; CCD: Charge-coupled device; EFP: Electric field propagation; EPROM: Erasable programmable read-only memory; USB: Universal Serial Bus; FDA: Food and Drug Administration; FICE: Fujinon Intelligent Chromo Endoscopy; ALICE: A Large Ion Collider Experiment.

## IS THERE A ROLE FOR FAECAL TESTING (CALPROTECTIN) AS "SELECTION TOOL" FOR CAPSULE ENDOSCOPY

Due to its high DY and its negative predictive value (NPV), CE has shown considerable cost-effectiveness<sup>[38]</sup>. However, CE still remains less widely available and likely more expensive, when compared to other diagnostic modalities for the small-bowel<sup>[39]</sup>. Furthermore, although CE is generally considered overall a safe modality, it can lead to severe complications (capsule retention in some patients' subgroups is reported as high as 15%<sup>[13-15,40]</sup>. Consequently, any tool or methods that allows selection of candidates, hence a more targeted and/or smooth "delivery" of SBCE, is a welcome approach. However, any pre-CE selection tool should be easy to perform, safe, inexpensive and fast<sup>[41]</sup>. In light of all these issues, faecal inflammation tests [of which, faecal calprotectin (FC) is the more widely available] have been proposed. In fact, FC has been used in SBCE studies in two settings: in patients taking non-steroidal anti-inflammatory drugs, to evaluate the type and extent of mucosal damage (Table 4)<sup>[41-44]</sup> and, more importantly from a clinical point of view, in patients with known or suspected CD for assessment of inflammation activity (Table 4)<sup>[45-48]</sup>. In these patients, although there is no clear agreement on a cut-off level, FC seems to be a cost-effective "screening test", able to identify those with higher possibility to present small-bowel lesions.

## HAS CE THE SAME DIAGNOSTIC CAPABILITY ALONG THE SMALL BOWEL?

There are several papers, mostly case presentations and/or case series, reporting patients in whom CE failed to identify small-bowel lesions which were subsequently diagnosed by other modalities<sup>[49-52]</sup>. Such missed lesions (including neoplastic pathology) were occasionally large and often located in the proximal small-bowel<sup>[50,51]</sup>. Although there is still a lot of debate about the reasons of poor SBCE performance<sup>[53]</sup>, it is worth remembering that for any non-steerable capsule progress is more rapid in the proximal than in lower segments of the small-bowel<sup>[53]</sup>; furthermore, opaque bile secretions and/or intra-luminal content might consequently hamper/prevent detailed mucosa visualization. Table 5 summarises all studies reporting the number of exams in which one of the few small-bowel landmarks, the ampulla of Vater (AoV), was visible during CE<sup>[54-66]</sup>. Hence, this evidence base provides an indirect confirmation of the limitations of SBCE in evaluating the proximal small-bowel. Interestingly, even in earlier studies<sup>[54]</sup> which have not been confirmed since by other investigators, the AoV was missed in > 50% of SBCE examinations. This is obviously an important drawback, especially when SBCE is used as surveillance tool, in patients with small-bowel polyposis syndromes.

Table 2 Head-to-head trials of small-bowel capsule endoscopy systems

| Ref.                                 | Country       | Centre                   | Objective(s)   | Study type  | Design  | CE type  | Outcome(s)  | Conclusion   |
|--------------------------------------|---------------|--------------------------|--|-------------|---|--|---|--|
| Hartmann <i>et al</i> <sup>[7]</sup> | Germany       | Single centre            | Head-to-head evaluation of technical performance and DY of two CE systems (PillCam <sup>®</sup> SB <i>vs</i> EndoCapsule <sup>®</sup> )  | Prospective | <ul style="list-style-type: none"> <li>▶ OGI<sup>®</sup> pts;</li> <li>▶ Pts randomized to undergo 2 CEs using different CE in random order</li> </ul>  | <ul style="list-style-type: none"> <li>▶ PillCam<sup>®</sup>SB (Given Imaging, Yoqneam, Israel)</li> </ul>   | <ul style="list-style-type: none"> <li>▶ Pts enrolled: 40;</li> <li>▶ CR: PillCam<sup>®</sup>SB 33/40 (82%); EndoCapsule<sup>®</sup> 40/40 (100%); <i>P</i> = NS;</li> <li>▶ Overall DY: PillCam<sup>®</sup>SB 26/50 (52%); EndoCapsule<sup>®</sup> 29/50 (58%); <i>P</i> = NS;</li> <li>▶ DY (SB P2): PillCam<sup>®</sup>SB 22/50 (44%); EndoCapsule<sup>®</sup> 25/50 (50%); <i>P</i> = NS;</li> <li>▶ In all discordant SB P2 findings (not detected by the PillCam<sup>®</sup>SB but detected by EndoCapsule<sup>®</sup>), PillCam<sup>®</sup>SB examinations were incomplete</li> </ul>  | <ul style="list-style-type: none"> <li>▶ Statistically non-significant trend for EndoCapsule<sup>®</sup> to detect more bleeding sources in pts with suspected small-bowel bleeding than PillCam<sup>®</sup>SB;</li> <li>▶ This is (likely) due to the longer recording time with EndoCapsule<sup>®</sup></li> </ul> |
| Cave <i>et al</i> <sup>[8]</sup>     | United States | Multi-centre (4 centres) | Comparison of performance (DY in pts with OGI <sup>®</sup> ); EndoCapsule <sup>®</sup> <i>vs</i> PillCam <sup>®</sup> SB                 | Prospective | <ul style="list-style-type: none"> <li>▶ OGI<sup>®</sup> pts;</li> <li>▶ EndoCapsule<sup>®</sup> and PillCam<sup>®</sup>SB swallowed by each participant 40 min apart;</li> <li>▶ Ingestion of CEs in randomized order;</li> <li>▶ Head-to-head comparison of CEs</li> </ul>  | <ul style="list-style-type: none"> <li>▶ EndoCapsule<sup>®</sup> (Olympus<sup>®</sup> America, Allentown, PA);</li> <li>▶ PillCam<sup>®</sup>SB (Given Imaging, Yoqneam, Israel)</li> </ul>      | <ul style="list-style-type: none"> <li>▶ Pts with OGI<sup>®</sup> (transfused or with haematocrit &lt; 31% (males) or &lt; 28% (females): 63;</li> <li>▶ Available data 51/63; 9 pts excluded for technical reasons + 3 pts for protocol violation;</li> <li>▶ 24 videos read as normal, 14 as abnormal (from both CEs). Disagreement occurred in 13;</li> <li>▶ No adverse events reported for either CE. Overall agreement: 38/51 (74.5%), <math>\kappa = 0.48</math>, <i>P</i> = 0.008;</li> <li>▶ Lack of electromechanical interference between 2 different CE</li> </ul>  | <ul style="list-style-type: none"> <li>▶ Both devices are safe and have comparable DY within the previously reported range;</li> <li>▶ Subjective difference in image quality favouring the EndoCapsule<sup>®</sup>;</li> </ul>  |
| Kim <i>et al</i> <sup>[9]</sup>      | South Korea   | Single centre            | Head-to-head evaluation of technical performance DY and of two capsule systems (PillCam <sup>®</sup> SB <i>vs</i> MiroCam <sup>®</sup> ) | Prospective | <ul style="list-style-type: none"> <li>▶ Pts referred to CE for various indications;</li> <li>▶ Each pt was randomly assigned to swallow 1 of 2 CEs, the second CE was swallowed once fluoroscopy indicated that first CE had reached the SB</li> </ul>   | <ul style="list-style-type: none"> <li>▶ MiroCam<sup>®</sup> (IntroMedic Co. Ltd., Seoul, South Korea);</li> <li>▶ PillCam<sup>®</sup>SB (Given<sup>®</sup> Imaging, Yoqneam, Israel)</li> </ul> | <ul style="list-style-type: none"> <li>▶ Pts enrolled: 24;</li> <li>▶ Mean operating time: MiroCam<sup>®</sup> 702 min; PillCam<sup>®</sup>SB 446 min, <i>P</i> &lt; 0.001;</li> <li>▶ CR: MiroCam<sup>®</sup> 20/24 (83%); PillCam<sup>®</sup>SB 14/24 (59%), <i>P</i> = 0.031;</li> <li>▶ DY: MiroCam<sup>®</sup> 11/24 (45.8%); PillCam<sup>®</sup>SB 10/24 (41.7%), <i>P</i> = 1.0;</li> <li>▶ DY (additive of both capsules): 12/24 (50%);</li> <li>▶ Concordance of findings among the two capsule systems 87.5%, <math>\kappa = 0.74</math></li> </ul>   | <ul style="list-style-type: none"> <li>▶ MiroCam shows a longer operating time and a higher CR;</li> <li>▶ Nevertheless, the 2 capsule systems showed comparable efficiency;</li> <li>▶ Sequential capsule endoscopy with the MiroCam and PillCam SB produced slight (but NS) increase in DY</li> </ul>              |
| Poche <i>et al</i> <sup>[10]</sup>   | France        | Multi-centre             | Head-to-head evaluation of the diagnostic concordance ( $\kappa$ value); PillCam <sup>®</sup> SB SB2 <i>vs</i> MiroCam <sup>®</sup>      | Prospective | <ul style="list-style-type: none"> <li>▶ OGI<sup>®</sup> pts;</li> <li>▶ Each pt ingested 2 CEs at a 1 h interval in a random order;</li> <li>▶ Videos read in a random order by 2 experienced (&gt; 200 CEs) readers;</li> <li>▶ Image-by-image review of cases of disagreement between the readers was performed by 3 expert readers</li> </ul> | <ul style="list-style-type: none"> <li>▶ MiroCam<sup>®</sup> (IntroMedic Co. Ltd., Seoul, South Korea);</li> <li>▶ PillCamSB2 (Given<sup>®</sup> Imaging, Yoqneam, Israel)</li> </ul>            | <ul style="list-style-type: none"> <li>▶ 83 pts; drop-outs explained (10 technical issues), 73 pts/ videos analysed;</li> <li>▶ 31 concordant (+) and 30 concordant (-) <i>vs</i> cases (42.4%) and 30 concordant (+) <i>vs</i> cases (41.1%);</li> <li>▶ Satisfactory diagnostic concordance between the 2 systems (<math>\kappa = 0.66</math>);</li> <li>▶ DY similar among the 2 CE systems (PillCam<sup>®</sup>SB 2 <i>vs</i> MiroCam<sup>®</sup>: 46.6% <i>vs</i> 56.2%, respectively; <i>P</i> = 0.02);</li> <li>▶ SBT longer with MiroCam<sup>®</sup> <i>vs</i> PillCam<sup>®</sup>SB (mean SBT: 268 <i>vs</i> 234 min, <i>P</i> &lt; 0.05);</li> <li>▶ Reading time longer with MiroCam<sup>®</sup> <i>vs</i> PillCam<sup>®</sup>SB (mean reading time 40 <i>vs</i> 23 min, <i>P</i> &lt; 0.05);</li> <li>▶ (+) <i>vs</i> diagnosis obtained in 46.6% <i>vs</i> 56.2% of pts with PillCam<sup>®</sup>SB2 <i>vs</i> MiroCam<sup>®</sup>, respectively;</li> <li>▶ PillCam<sup>®</sup>SB2 <i>vs</i> MiroCam<sup>®</sup> CEs identified 78.6% <i>vs</i> 95.2% of (+) <i>vs</i> cases, respectively, <i>P</i> = 0.02</li> </ul> | <ul style="list-style-type: none"> <li>▶ MiroCam<sup>®</sup> showed a slightly higher DY, difference not statistically significant;</li> <li>▶ The 2 CE systems showed comparable efficiency for the diagnosis of OGI<sup>®</sup></li> </ul>   |
| Dolak <i>et al</i> <sup>[11]</sup>   | Austria       | Single centre            | Head-to-head comparison (MiroCam <sup>®</sup> <i>vs</i> EndoCapsule <sup>®</sup> ) of CR of SB examinations, DY in SB disease            | Prospective | <ul style="list-style-type: none"> <li>▶ Pts referred to CE for various indications;</li> <li>▶ Each pt was randomly assigned to swallow either MiroCam<sup>®</sup> first, followed by the EndoCapsule<sup>®</sup> 2 h later, or vice versa;</li> <li>▶ All videos analysed by two investigators independently</li> </ul>                         | <ul style="list-style-type: none"> <li>▶ MiroCam<sup>®</sup> (IntroMedic Co. Ltd., Seoul, South Korea);</li> <li>▶ EndoCapsule<sup>®</sup> (Olympus America, Allentown, PA)</li> </ul>           | <ul style="list-style-type: none"> <li>▶ CR: MiroCam<sup>®</sup> 48/50 (96%) <i>vs</i> EndoCapsule<sup>®</sup> 45/50 (90%); <i>P</i> = 0.38;</li> <li>▶ DY in SB: MiroCam<sup>®</sup> 25/50 (50%) <i>vs</i> EndoCapsule<sup>®</sup> 24/50 (48%); <i>P</i> &gt; 0.99;</li> <li>▶ Concordance of findings among the two CE systems: 68%, <math>\kappa = 0.50</math></li> </ul>  | <ul style="list-style-type: none"> <li>▶ The two capsule endoscopy systems were not statistically different with regards to CR and DY;</li> <li>▶ Moderate concordance, mainly caused by missed pathological findings (which affected both devices), needs consideration in clinical practice</li> </ul>             |

DY: Diagnostic yield; CE: Capsule endoscopy; OGI<sup>®</sup>: Obscure gastrointestinal bleeding; pts: Patients; CR: Completion rate; NS: Not significant (statistically); SB: Small-bowel; P2: Refers to grading of angioectasias; SBT: Small-bowel transit time.

Table 3 Available meta-analyses and systematic reviews in the field of small-bowel capsule endoscopy

| Ref.                                  | Title  | Search (start - end date) | Type                                      | Subject   | Data extra-actors found | Total titles entered | Titles meta-analysis | Individuals included          | Outcome/conclusion   |
|---------------------------------------|--|---------------------------|---|---|-------------------------|----------------------|----------------------|-------------------------------|--|
| Liao <i>et al</i> <sup>[3]</sup>      | Indications, detection, completion, and retention rates of SBCE: A systematic review   | 2000 - Jan 2009           | Systematic review of evidence base        | Indications, DR, CR and RR of SBCE  | 2                       | 227                  | 227                  | 22753 Pts; 22840 CE           | <ul style="list-style-type: none"> <li>► Most common indications: OGIB (66.0%); investigation of clinical symptoms (10.6%); definite/suspected CD (10.4%);</li> <li>► Pooled DRs for overall, OGIB, CD, neoplasia: 59.4%, 60.5%, 55.3%, 55.9%, respectively;</li> <li>► Commonest cause for OGIB: angiodysplasia (50.0%);</li> <li>► Pooled CRs (overall): 83.5%; breakdown 83.6% (OGIB), 85.4% (clinical symptoms), 84.2% (CD);</li> <li>► Pooled RRs (overall): 1.4%; breakdown 1.2% (OGIB), 2.6% (clinical symptoms), 2.1% (CD);</li> <li>► Hence, most common indication for SBCE is OGIB, with high DR and low RR;</li> <li>► A relatively high RR is associated with definite/suspected CD and neoplasms</li> <li>► 17 studies (526 patients) met inclusion criteria;</li> <li>► Overall, the rate difference for SB disease (<i>i.e.</i>, the absolute pooled difference in the rate of positive findings) of SBCE vs alternative modalities was 41% (95%CI: 35.6-45.9);</li> <li>► For OGIB, 37% (95%CI: 29.6-44.1) for Crohn's disease; 45% (95%CI: 30.9-58.0);</li> <li>► Incomplete SBCE occurred in 13%, more often in OGIB (17%) than in pts with CD (8%) (<math>P &lt; 0.006</math>);</li> <li>► Adverse events: 29 pts (6%);</li> <li>► Capsule retention more frequent in pts with CD (3% vs 1%, OR 4.37)</li> <li>► 14 studies (<math>n = 396</math>) compared DY CE vs PE in OGIB, 63% vs 28%, respectively (Y = 35%, <math>P &lt; 0.00001</math>, 95%CI: 26%-43%);</li> <li>► For clinically significant findings (<math>n = 376</math>) DY was 56% (CE) vs 26% (PE), Y = 30%, <math>P &lt; 0.00001</math>, 95%CI: 21%-38%;</li> <li>► 3 studies (<math>n = 88</math>) compared DY of CE vs SBBaR, 67% vs 8%, respectively (Y = 59%, <math>P &lt; 0.00001</math>, 95%CI: 48%-70%);</li> <li>► For clinically significant findings DY was 42% (CE) vs 6% (SBBaR); Y = 36%, <math>P &lt; 0.00001</math>, 95%CI: 25%-48%;</li> <li>► NNT to yield one additional clinically significant finding with CE over either modality: 3 (95%CI: 2-4);</li> <li>► 1 study compared DY (significant findings) of CE vs intraoperative enteroscopy (<math>n = 42</math>, Y = 0%, <math>P = 1.0</math>, 95%CI: -16%-16%);</li> <li>► 1 study compared DY (significant findings) of CE vs CT enteroclysis (<math>n = 8</math>, Y = 38%, <math>P = 0.08</math>, 95%CI: -4%-79%);</li> <li>► 1 study compared DY (significant findings) of CE vs mesenteric angiogram (<math>n = 17</math>, Y = -6%, <math>P = 0.73</math>, 95%CI: -39%-28%);</li> <li>► 1 study compared DY (significant findings) of CE vs SB MRI (<math>n = 14</math>, Y = 36%, <math>P = 0.007</math>, 95%CI: 10%-62%);</li> <li>► CE-DY vs PE (vascular lesions): 36% vs 20% (Y = 16%, <math>P &lt; 0.00001</math>, 95%CI: 9%-23%);</li> <li>► CE-DY vs PE (inflammatory lesions): 11% vs 2% (Y = 9%, <math>P = 0.0001</math>, 95%CI: 5%-13%);</li> <li>► CE-DY vs PE (tumours or "other" findings): no difference</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► 9 studies (<math>n = 250</math>) compared DY CE vs SBBaR in CD: 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 4 studies (<math>n = 114</math>) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 3 studies (<math>n = 93</math>) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul> |
| Marmo <i>et al</i> <sup>[7]</sup>     | Meta-analysis: Capsule enteroscopy vs conventional modalities in diagnosis of SB diseases                                      | Jan 1966 - Mar 2005       | Meta-analysis of diagnostic test accuracy | DY/safety of SBCE vs alternative modalities (PE, SBBaR or enteroclysis) in SB disease     | 2                       | 187                  | 17                   | 526 pts (289 OGIB and 237 CD) | <ul style="list-style-type: none"> <li>► 17 studies (526 patients) met inclusion criteria;</li> <li>► Overall, the rate difference for SB disease (<i>i.e.</i>, the absolute pooled difference in the rate of positive findings) of SBCE vs alternative modalities was 41% (95%CI: 35.6-45.9);</li> <li>► For OGIB, 37% (95%CI: 29.6-44.1) for Crohn's disease; 45% (95%CI: 30.9-58.0);</li> <li>► Incomplete SBCE occurred in 13%, more often in OGIB (17%) than in pts with CD (8%) (<math>P &lt; 0.006</math>);</li> <li>► Adverse events: 29 pts (6%);</li> <li>► Capsule retention more frequent in pts with CD (3% vs 1%, OR 4.37)</li> <li>► 14 studies (<math>n = 396</math>) compared DY CE vs PE in OGIB, 63% vs 28%, respectively (Y = 35%, <math>P &lt; 0.00001</math>, 95%CI: 26%-43%);</li> <li>► For clinically significant findings (<math>n = 376</math>) DY was 56% (CE) vs 26% (PE), Y = 30%, <math>P &lt; 0.00001</math>, 95%CI: 21%-38%;</li> <li>► 3 studies (<math>n = 88</math>) compared DY of CE vs SBBaR, 67% vs 8%, respectively (Y = 59%, <math>P &lt; 0.00001</math>, 95%CI: 48%-70%);</li> <li>► For clinically significant findings DY was 42% (CE) vs 6% (SBBaR); Y = 36%, <math>P &lt; 0.00001</math>, 95%CI: 25%-48%;</li> <li>► NNT to yield one additional clinically significant finding with CE over either modality: 3 (95%CI: 2-4);</li> <li>► 1 study compared DY (significant findings) of CE vs intraoperative enteroscopy (<math>n = 42</math>, Y = 0%, <math>P = 1.0</math>, 95%CI: -16%-16%);</li> <li>► 1 study compared DY (significant findings) of CE vs CT enteroclysis (<math>n = 8</math>, Y = 38%, <math>P = 0.08</math>, 95%CI: -4%-79%);</li> <li>► 1 study compared DY (significant findings) of CE vs mesenteric angiogram (<math>n = 17</math>, Y = -6%, <math>P = 0.73</math>, 95%CI: -39%-28%);</li> <li>► 1 study compared DY (significant findings) of CE vs SB MRI (<math>n = 14</math>, Y = 36%, <math>P = 0.007</math>, 95%CI: 10%-62%);</li> <li>► CE-DY vs PE (vascular lesions): 36% vs 20% (Y = 16%, <math>P &lt; 0.00001</math>, 95%CI: 9%-23%);</li> <li>► CE-DY vs PE (inflammatory lesions): 11% vs 2% (Y = 9%, <math>P = 0.0001</math>, 95%CI: 5%-13%);</li> <li>► CE-DY vs PE (tumours or "other" findings): no difference</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► 9 studies (<math>n = 250</math>) compared DY CE vs SBBaR in CD: 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 4 studies (<math>n = 114</math>) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 3 studies (<math>n = 93</math>) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul>  |
| Triester <i>et al</i> <sup>[6]</sup>  | A meta-analysis of the yield of CE compared to other diagnostic modalities in patients with OGIB                               | N/A - April 2005          | Meta-analysis of diagnostic test accuracy | Y (yield of CE-yield of comparative modality) and 95%CI of CE over comparative modalities | 2                       | 80                   | 14                   | 396 CE-PE; 88 CE-SBBaR        | <ul style="list-style-type: none"> <li>► 14 studies (<math>n = 396</math>) compared DY CE vs PE in OGIB, 63% vs 28%, respectively (Y = 35%, <math>P &lt; 0.00001</math>, 95%CI: 26%-43%);</li> <li>► For clinically significant findings (<math>n = 376</math>) DY was 56% (CE) vs 26% (PE), Y = 30%, <math>P &lt; 0.00001</math>, 95%CI: 21%-38%;</li> <li>► 3 studies (<math>n = 88</math>) compared DY of CE vs SBBaR, 67% vs 8%, respectively (Y = 59%, <math>P &lt; 0.00001</math>, 95%CI: 48%-70%);</li> <li>► For clinically significant findings DY was 42% (CE) vs 6% (SBBaR); Y = 36%, <math>P &lt; 0.00001</math>, 95%CI: 25%-48%;</li> <li>► NNT to yield one additional clinically significant finding with CE over either modality: 3 (95%CI: 2-4);</li> <li>► 1 study compared DY (significant findings) of CE vs intraoperative enteroscopy (<math>n = 42</math>, Y = 0%, <math>P = 1.0</math>, 95%CI: -16%-16%);</li> <li>► 1 study compared DY (significant findings) of CE vs CT enteroclysis (<math>n = 8</math>, Y = 38%, <math>P = 0.08</math>, 95%CI: -4%-79%);</li> <li>► 1 study compared DY (significant findings) of CE vs mesenteric angiogram (<math>n = 17</math>, Y = -6%, <math>P = 0.73</math>, 95%CI: -39%-28%);</li> <li>► 1 study compared DY (significant findings) of CE vs SB MRI (<math>n = 14</math>, Y = 36%, <math>P = 0.007</math>, 95%CI: 10%-62%);</li> <li>► CE-DY vs PE (vascular lesions): 36% vs 20% (Y = 16%, <math>P &lt; 0.00001</math>, 95%CI: 9%-23%);</li> <li>► CE-DY vs PE (inflammatory lesions): 11% vs 2% (Y = 9%, <math>P = 0.0001</math>, 95%CI: 5%-13%);</li> <li>► CE-DY vs PE (tumours or "other" findings): no difference</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► CE superior to PE/SB radiography for diagnosing SB pathology in pts with OGIB (yield comparable to intraoperative endoscopy);</li> <li>► Incremental yield of CE over PE/SB radiography is &gt; 30% for clinically significant findings, due to visualization of additional vascular, inflammatory lesions by CE;</li> <li>► CE was also superior to SB radiography, C + IL, CT enterography, PE for diagnosing non-stricturing SBCE;</li> <li>► Marked improvement in yield with the use of CE over all other methods in pts who had established CD and were evaluated for SB recurrence;</li> <li>► Unknown whether these results will translate into improved pt outcomes with the use of CE vs alternate methods</li> <li>► 9 studies (<math>n = 250</math>) compared DY CE vs SBBaR in CD: 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 4 studies (<math>n = 114</math>) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 3 studies (<math>n = 93</math>) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul>   |
| Leighton <i>et al</i> <sup>[9]</sup>  | Capsule endoscopy: A meta-analysis for use with OGIB and CD  | N/A - April 2005          | Meta-analysis of diagnostic test accuracy | DY and safety of SBCE vs alternative modalities (PE, SBBaR or enteroclysis) in SB disease | 2                       | 80                   | 20                   | 537 pts                       | <ul style="list-style-type: none"> <li>► 20 studies (537 pts) compared DY CE vs PE in OGIB, 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 10 studies (314 pts) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 5 studies (159 pts) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul>   |
| Leighton <i>et al</i> <sup>[9]</sup>  | Capsule endoscopy: A meta-analysis for use with OGIB and CD  | N/A - April 2005          | Meta-analysis of diagnostic test accuracy | DY and safety of SBCE vs alternative modalities (PE, SBBaR or enteroclysis) in SB disease | 2                       | 80                   | 20                   | 537 pts                       | <ul style="list-style-type: none"> <li>► 20 studies (537 pts) compared DY CE vs PE in OGIB, 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 10 studies (314 pts) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 5 studies (159 pts) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul>   |
| Triester <i>et al</i> <sup>[20]</sup> | A meta-analysis of the yield of CE compared to other diagnostic modalities in patients with non-stricturing SB Crohn's disease | N/A - Aug 2005            | Meta-analysis of diagnostic test accuracy | DY and safety of SBCE vs alternative modalities (PE, SBBaR or enteroclysis) in SB disease | 2                       | 82                   | 9                    | 250 pts                       | <ul style="list-style-type: none"> <li>► 9 studies (250 pts) compared DY CE vs SBBaR in CD: 63% vs 23%, respectively (Y = 40%, <math>P &lt; 0.001</math>, 95%CI: 28%-51%);</li> <li>► 4 studies (114 pts) compared DY CE vs C + IL in CD: 61% vs 46%, respectively (Y = 15%, <math>P = 0.02</math>, 95%CI: 2%-27%);</li> <li>► 3 studies (93 pts) compared DY CE vs CT enterography/enteroclysis: 69% vs 30%, respectively (Y = 38%, <math>P = 0.001</math>, 95%CI: 15%-60%);</li> <li>► 2 studies compared DY CE vs PE: (Y = 38%, <math>P &lt; 0.001</math>, 95%CI: 26%-50%);</li> <li>► 1 study compared DY CE vs SBMRI (Y = 22%, <math>P = 0.16</math>, 95%CI: -9%-53%);</li> <li>► Sub-analysis (pts with suspected CD): no difference in DY CE vs SBBaR (<math>P = 0.09</math>), C + IL (<math>P = 0.48</math>), CT enterography (<math>P = 0.07</math>) or PE (<math>P = 0.51</math>);</li> <li>► Sub-analysis (pts with established CD): difference in DY CE vs SBBaR (<math>P &lt; 0.001</math>), C + IL (<math>P = 0.002</math>), CT enterography (<math>P &lt; 0.001</math>) and PE (<math>P &lt; 0.001</math>)</li> </ul>   |

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| Pasha <i>et al</i> <sup>[21]</sup>     | DBE and CE have comparable DY in SB disease. A meta-analysis  | N/A - Dec 2006  | Meta-analysis of diagnostic test accuracy | Comparison of DY of CE vs DBE                      | 2 | 113 | 11 | 397 | pts                         | <ul style="list-style-type: none"> <li>► Pooled DY CE vs DBE: 60% vs 57% (IYw = 3%, 95%CI: -4%-10%, P = 0.42, FEM);</li> <li>► Pooled DY CE vs DBE (vascular findings, 10 studies): 24% vs 24% (IYw = 0%, 95%CI: -5%-6%, P = 0.88, REM);</li> <li>► Pooled DY CE vs DBE (inflammatory findings, 9 studies): 18% vs 16% (IYw = 0%, 95%CI: -5%-6%, P = 0.89, FEM);</li> <li>► Pooled DY CE vs DBE (polyps/tumours, 9 studies): 11% vs 11% (IYw = -1%, 95%CI: -5%-4%, P = 0.76, FEM);</li> <li>► SB disease: CE vs DBE have comparable DY, including OGIB, CE should be the initial diagnostic test for determining the insertion route of DBE</li> <li>► 237 pts, 130 with and 107 without preparation;</li> <li>► Seven out of 8 studies included a comparison of GTT, SBTT and CR;</li> <li>► SBCE CR: 76% in pts with preparation vs 68% without prep (difference did not reach statistical significance);</li> <li>► No statistically significant difference between CEs performed with or without preparation in GTT (pooled effect size, -0.054; 95%CI: -0.418-0.308) or SBTT (pooled effect size, -0.327; 95%CI: -1.419 - -0.765)</li> </ul>  |
| Niv <sup>[22]</sup>                    | Efficiency of bowel preparation for capsule endoscopy examination: A meta-analysis                                | N/A - July 2007 | Meta-analysis of RCTs and cohort studies  | Purgative use vs fasting alone for SBCE            | 1 | 6   | 8  | 130 | bowel prep; 107 fasting     | <ul style="list-style-type: none"> <li>► 3 studies (n = 107, 63 pts with CD/44 without) met inclusion criteria;</li> <li>► Pooled SBCE (overall) Sens and Spec: 83% (95%CI: 71%-90%) and 98% (95%CI: 88%-99.6%), respectively;</li> <li>► No major complications reported;</li> <li>► Costs mentioned only in 1 study. Overall, diagnostic characteristics of SBCE, could not justify the routine use of SBCE as alternative to biopsy</li> </ul>  |
| El-Matary <i>et al</i> <sup>[23]</sup> | Diagnostic characteristics of given video capsule endoscopy in diagnosis of celiac disease: A meta-analysis       | N/A - Feb 2007  | Meta-analysis of diagnostic test accuracy | Coeliac and CE                                     | 2 | N/A | 3  | 107 | pts                         | <ul style="list-style-type: none"> <li>► 8 studies (n = 277 pts) prospectively compared the yield of CE and DBE were included;</li> <li>► No difference between the yield of CE and DBE (170/277 vs 156/277, OR 1.21, 95%CI: 0.64-2.29);</li> <li>► Sub analysis: yield of CE significantly higher than that of DBE without combination of oral+anal insertion approaches (137/219 vs 110/219, OR 1.67, 95%CI: 1.14-2.44, P &lt; 0.01), but not superior to the yield of DBE with combination of the two insertion approaches (26/48 vs 37/48, OR 0.33, 95%CI: 0.05-2.21, P &lt; 0.05);</li> <li>► Focused meta-analysis of the fully published articles concerning OGIB showed similar results wherein the yield of CE was significantly higher than that of DBE without combination of oral + anal insertion approaches (118/191 vs 96/191, fixed model: OR 1.61, 95%CI: 1.07-2.43, P &lt; 0.05) and the yield of CE was significantly lower than that of DBE by oral+anal combinatory approaches (11/24 vs 21/24, fixed model: OR 0.12, 95%CI: 0.03-0.52, P &lt; 0.01)</li> </ul>   |
| Chen <i>et al</i> <sup>[24]</sup>      | A meta-analysis of the yield of CE compared to DBE in pts with SB diseases  | N/A - Feb 2007  | Meta-analysis of diagnostic test accuracy | Comparison of DY of CE vs DBE                      | 2 | 163 | 8  | 277 | pts                         | <ul style="list-style-type: none"> <li>► 12 eligible studies (6 prospective/6 retrospective), including 16 sets of data;</li> <li>► Significant difference in DY between pts prepared with purgatives (n = 263) vs pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002);</li> <li>► Significant difference in SBVQ between pts prepared with purgatives (n = 404) vs pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005);</li> <li>► There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE/GTT or VCESBTT</li> <li>► 8 studies (n = 236 pts) compared CE vs C+IL, 4 (n = 119 pts) CE vs CTE, 2 (n = 102 pts) vs PE, 4 (n = 123 pts) vs MRE;</li> <li>► For suspected CD, several comparisons met statistical significance: Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, P &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, P &lt; 0.00001, 95%CI: 31%-63%), CE vs C+IL: 47% vs 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%);</li> <li>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, P &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, P &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, P ≤ 0.0001, 95%CI: 16%-47%)</li> <li>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</li> <li>► Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I<sup>2</sup> = 38.8%) or publication bias (P = 0.251);</li> <li>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I<sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I<sup>2</sup> = 38.9%</li> </ul> |
| Rokkas <i>et al</i> <sup>[25]</sup>    | Does purgative preparation influence the diagnostic yield of small bowel video capsule endoscopy? A meta-analysis | N/A - Feb 2008  | Meta-analysis of RCTs and cohort studies  | Purgative use vs fasting alone for SBCE            | 2 | 194 | 12 | 718 | pts purgative; 444 controls | <ul style="list-style-type: none"> <li>► 12 eligible studies (6 prospective/6 retrospective), including 16 sets of data;</li> <li>► Significant difference in DY between pts prepared with purgatives (n = 263) vs pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002);</li> <li>► Significant difference in SBVQ between pts prepared with purgatives (n = 404) vs pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005);</li> <li>► There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE/GTT or VCESBTT</li> <li>► 8 studies (n = 236 pts) compared CE vs C+IL, 4 (n = 119 pts) CE vs CTE, 2 (n = 102 pts) vs PE, 4 (n = 123 pts) vs MRE;</li> <li>► For suspected CD, several comparisons met statistical significance: Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, P &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, P &lt; 0.00001, 95%CI: 31%-63%), CE vs C+IL: 47% vs 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%);</li> <li>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, P &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, P &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, P ≤ 0.0001, 95%CI: 16%-47%)</li> <li>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</li> <li>► Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I<sup>2</sup> = 38.8%) or publication bias (P = 0.251);</li> <li>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I<sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I<sup>2</sup> = 38.9%</li> </ul> |
| Dionisio <i>et al</i> <sup>[26]</sup>  | CE has a significantly higher DY in patients with suspected and established small-bowel CD: A meta-analysis       | 2000 - May 2009 | Meta-analysis of diagnostic test accuracy | DY of CE vs modalities in suspected/established CD | 2 | 291 | 12 | 428 | pts                         | <ul style="list-style-type: none"> <li>► 12 eligible studies (6 prospective/6 retrospective), including 16 sets of data;</li> <li>► Significant difference in DY between pts prepared with purgatives (n = 263) vs pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002);</li> <li>► Significant difference in SBVQ between pts prepared with purgatives (n = 404) vs pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005);</li> <li>► There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE/GTT or VCESBTT</li> <li>► 8 studies (n = 236 pts) compared CE vs C+IL, 4 (n = 119 pts) CE vs CTE, 2 (n = 102 pts) vs PE, 4 (n = 123 pts) vs MRE;</li> <li>► For suspected CD, several comparisons met statistical significance: Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, P &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, P &lt; 0.00001, 95%CI: 31%-63%), CE vs C+IL: 47% vs 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%);</li> <li>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, P &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, P &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, P ≤ 0.0001, 95%CI: 16%-47%)</li> <li>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</li> <li>► Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I<sup>2</sup> = 38.8%) or publication bias (P = 0.251);</li> <li>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I<sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I<sup>2</sup> = 38.9%</li> </ul> |
| Wu <i>et al</i> <sup>[27]</sup>        | Systematic review and meta-analysis of RCTs of Simethicone for GI endoscopic visibility                           | N/A - Nov 2009  | Meta-analysis of RCTs                     | Simethicone and CE                                 | 2 | 128 | 4  | 121 | pts                         | <ul style="list-style-type: none"> <li>► 12 eligible studies (6 prospective/6 retrospective), including 16 sets of data;</li> <li>► Significant difference in DY between pts prepared with purgatives (n = 263) vs pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002);</li> <li>► Significant difference in SBVQ between pts prepared with purgatives (n = 404) vs pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005);</li> <li>► There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE/GTT or VCESBTT</li> <li>► 8 studies (n = 236 pts) compared CE vs C+IL, 4 (n = 119 pts) CE vs CTE, 2 (n = 102 pts) vs PE, 4 (n = 123 pts) vs MRE;</li> <li>► For suspected CD, several comparisons met statistical significance: Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, P &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, P &lt; 0.00001, 95%CI: 31%-63%), CE vs C+IL: 47% vs 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%);</li> <li>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, P &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, P &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, P ≤ 0.0001, 95%CI: 16%-47%)</li> <li>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</li> <li>► Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I<sup>2</sup> = 38.8%) or publication bias (P = 0.251);</li> <li>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I<sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I<sup>2</sup> = 38.9%</li> </ul> |
| Rokkas <i>et al</i> <sup>[25]</sup>    | Does purgative preparation influence the diagnostic yield of small bowel video capsule endoscopy? A meta-analysis | N/A - Feb 2008  | Meta-analysis of RCTs and cohort studies  | Purgative use vs fasting alone for SBCE            | 2 | 194 | 12 | 718 | pts purgative; 444 controls | <ul style="list-style-type: none"> <li>► 12 eligible studies (6 prospective/6 retrospective), including 16 sets of data;</li> <li>► Significant difference in DY between pts prepared with purgatives (n = 263) vs pts prepared with clear liquids (n = 213): OR = 1.813 (95%CI: 1.251-2.628, P = 0.002);</li> <li>► Significant difference in SBVQ between pts prepared with purgatives (n = 404) vs pts prepared with clear liquids (n = 249): OR = 2.113 (95%CI: 1.252-3.566, P = 0.005);</li> <li>► There was no statistically significant difference regarding CR rate. Purgatives did not affect VCE/GTT or VCESBTT</li> <li>► 8 studies (n = 236 pts) compared CE vs C+IL, 4 (n = 119 pts) CE vs CTE, 2 (n = 102 pts) vs PE, 4 (n = 123 pts) vs MRE;</li> <li>► For suspected CD, several comparisons met statistical significance: Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, P &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, P &lt; 0.00001, 95%CI: 31%-63%), CE vs C+IL: 47% vs 25% (IYw = 22%, P = 0.009, 95%CI: 5%-39%);</li> <li>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, P &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, P &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, P ≤ 0.0001, 95%CI: 16%-47%)</li> <li>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</li> <li>► Pooled OR = 2.84 (95%CI: 1.74-4.65, P = 0.00); no significant heterogeneity (P = 0.16, I<sup>2</sup> = 38.8%) or publication bias (P = 0.251);</li> <li>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting): Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, P = 0.00) with P = 0.78, I<sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, P = 0.203) with P = 0.20, I<sup>2</sup> = 38.9%</li> </ul> |

|  |   |                     |   |   |      |    |                               |   |   |
|--|---|---------------------|---|---|------|----|-------------------------------|---|---|
| Dionisio <i>et al.</i> <sup>[26]</sup>     | CE has a significantly higher DY in patients with suspected and established small-bowel CD: A meta-analysis | 2000 - May 2009     | Meta-analysis of diagnostic test accuracy | 2 | 291  | 12 | 428 pts                       | DY of CE vs modalities in patients with suspected/ established CD                       | <p>► 8 studies (<i>n</i> = 236 pts) compared CE vs C + IL, 4 (<i>n</i> = 119 pts) CE vs CTE, 2 (<i>n</i> = 102 pts) vs PE, 4 (<i>n</i> = 123 pts) vs MRE;</p> <p>► For suspected CD, several comparisons met statistical significance; Yields in this subgroup were: CE vs SBR: 52% vs 16% (IYw = 32%, <i>P</i> &lt; 0.0001, 95%CI: 16%-48%), CE vs CTE: 68% vs 21% (IYw = 47%, <i>P</i> &lt; 0.00001, 95%CI: 31%-63%), CE vs C + IL: 47% vs 25% (IYw = 22%, <i>P</i> = 0.009, 95%CI: 5%-39%);</p> <p>► For established CD, statistically significant yields for CE vs an alternate diagnostic modality in patients were seen: CE vs PE: 66% vs 9% (IYw = 57%, <i>P</i> &lt; 0.00001, 95%CI: 43-71%), CE vs SBR: 71% vs 36% (IYw = 38%, <i>P</i> &lt; 0.00001, 95%CI: 22%-54%), CE vs CTE: 71% vs 39% (IYw = 32%, <i>P</i> ≤ 0.0001, 95%CI: 16%-47%)</p> <p>► Adequate or excellent/good SB mucosa visualization in pts receiving Simethicone vs those who did not (66.1% vs 37.2%);</p> <p>► Pooled OR = 2.84 (95%CI: 1.74-4.65, <i>P</i> = 0.00); no significant heterogeneity (<i>P</i> = 0.16, <i>I</i><sup>2</sup> = 38.8%) or publication bias (<i>P</i> = 0.251);</p> <p>► Sens analysis: studies stratified by factors such as bowel preparation (purgative vs fasting); Significant results for bowel preparation + fasting (OR = 4.43, 95%CI: 1.82-10.76, <i>P</i> = 0.00) with <i>P</i> = 0.78, <i>I</i><sup>2</sup> = 0.0%, No significant results for bowel preparation + purgative (OR = 1.59, 95%CI: 0.78-3.27, <i>P</i> = 0.203) with <i>P</i> = 0.20, <i>I</i><sup>2</sup> = 38.9%</p> <p>► Most common indication for CE (in pts &lt; 18 yr): suspicion or evaluation of IBD (overall 54%). Breakdown: suspected CD (34%), known CD (16%), UC (1%), indeterminate colitis (3%)</p> <p>► CR and RR: 86.2% (95%CI: 81.5-90.3%) and 2.6% (95%CI: 1.5-4.0%), respectively;</p> <p>► CE RR (gastric and SB): 0.5% and 1.9%, respectively, similar to those of adults, by indication;</p> <p>► CE with positive findings: 65.4% (95%CI: 54.8%-75.2%);</p> <p>► CE resulting in new diagnosis: 69.4% (95%CI: 46.9%-87.9%); CE leading to change in therapy: 68.3% (95%CI: 43.6%-88.5%)</p> |
| Wu <i>et al.</i> <sup>[27]</sup>           | Systematic review and meta-analysis of RCTs of Simethicone for GI endoscopic visibility                     | N/A - Nov 2009      | Meta-analysis of RCTs                     | 2 | 128  | 4  | 121 pts                       | Simethicone and CE  | <p>► Pooled DY for CE: 62% (95%CI: 47.3%-76.1%)</p> <p>► Pooled DY for DBE: 56% (95%CI: 48.9%-62.1%); OR for CE vs DBE of 1.39 (95%CI: 0.88-2.20, <i>P</i> = 0.16); Subgroup analyses</p> <p>► DBE-DY after (+)ve CE: 75.0% (95%CI: 60.1%-90.0%)</p> <p>► DBE-DY after (-)ve CE: 27.5% (95%CI: 16.7%-37.8%)</p> <p>► DBE-OR (for successful diagnosis after (+)ve CE) compared with DBE: 1.79 (95%CI: 1.09-2.96, <i>P</i> = 0.02)</p> <p>► In OGIB CE and DBE have similar DY, DBE-DY significantly higher when performed in pts with prior positive CE</p> <p>► Studies, using PEG or NaP-based bowel cleansing regimens;</p> <p>► Any form of purgative significantly better visibility than fasting alone (OR = 2.31; 95%CI: 1.46-3.63, <i>P</i> &lt; 0.0001);</p> <p>► Similar results on DY (OR = 1.88; 95%CI: 1.24-2.84; <i>P</i> = 0.023);</p> <p>Subgroup analyses (per cleansing regimen used):</p> <p>► PEG-based regimens showed benefit (OR = 3.11; 95%CI: 1.96-4.94, <i>P</i> &lt; 0.0001);</p> <p>► NaP-based regimens no significant difference from fasting alone (OR = 1.32; 95%CI: 0.59-2.96, <i>P</i> &lt; 0.0001);</p> <p>► Use of purgatives (alongside fasting) is recommended in SBCE; PEG-based regimens offer a clear advantage over NaP;</p> <p>► Lower volume PEG regimens as efficacious as higher volumes traditionally used for colonoscopy preparation</p> <p>► Pooled CE Sens: 89% (95%CI: 82%-94%) and Spec: 95% (95%CI: 89%-98%), AuROC: 0.9584;</p> <p>► Although not as accurate as pathology, CE a reasonable alternative method of diagnosing coeliac disease</p>   |
| Cohen <i>et al.</i> <sup>[28]</sup>        | Use of CE in diagnosis and management of pediatric patients, based on meta-analysis                         | Jan 2001 - May 2010 | Systematic review of evidence base        | 2 | N/A  | 15 | 740 examinations; 723 pts     | Systematic compilation of data on indications and outcomes of CE in paediatric patients | <p>► Pooled SBCE-DY in IDA: 47% (95%CI: 42%-52%), with significant heterogeneity among included studies (<i>I</i><sup>2</sup> = 78.8%, <i>P</i> &lt; 0.0001);</p> <p>► Pooled SBCE-DY (subgroup 1: 4 studies focused solely on IDA pts): 66.6% (95%CI: 61.0%-72.3%, <i>I</i><sup>2</sup> = 44.3%);</p> <p>► Pooled SBCE-DY (subgroup 2: 20 studies not focusing only on IDA pts): 44% (95%CI: 39%-48%, <i>I</i><sup>2</sup> = 64.9%);</p> <p>► SBCE in subgroup 1: more vascular (31% vs 22.6%, <i>P</i> = 0.007), inflammatory (17.8% vs 11.3%, <i>P</i> = 0.009), neoplastic (7.95% vs 2.25%, <i>P</i> &lt; 0.0001) lesions detected</p>  |
| Teshima <i>et al.</i> <sup>[29]</sup>      | DBE and CE for OGIB: An updated meta-analysis   | N/A - June 2010     | Meta-analysis of diagnostic test accuracy | 2 | 147  | 10 | 651 CE; 642 DBE               | OGIB; CE or DBE   | <p>► Meta-analysis of SB preparation for SBCE</p> <p>► Meta-analysis of RCTs of RCTs</p> <p>► Purgative use vs fasting alone for SBCE</p>   |
| Belsey <i>et al.</i> <sup>[30]</sup>       | Meta-analysis: efficacy of SB preparation for SBCE  | Jan 2000 - Dec 2010 | Meta-analysis of RCTs                     | 2 | 33   | 8  | 291 PEG; 133 NaP; 322 fasting | Purgative use vs fasting alone for SBCE   | <p>The role of video CE in the diagnosis of coeliac disease: A meta-analysis</p> <p>► Diagnostic yield of SBCE in patients with IDA: A systematic review</p>  |
| Rokkas <i>et al.</i> <sup>[31]</sup>       | The role of video CE in the diagnosis of coeliac disease: A meta-analysis                                   | N/A - April 2011    | Meta-analysis of diagnostic test accuracy | 2 | 461  | 6  | 166 pts                       | Coeliac and CE  |   |
| Koulaouzidis <i>et al.</i> <sup>[32]</sup> | Diagnostic yield of SBCE in patients with IDA: A systematic review  | Jan 2001 - Nov 2011 | Systematic review of evidence base        | 2 | 1225 | 24 | 1960 pts                      | IDA and CE  |   |

CE: Capsule endoscopy; N/A: Not available or not applicable; Sens: Sensitivity; Spec: Specificity; AuROC: Area under Receiver operation characteristics curve; DBE: Double-balloon enteroscopy; OGIB: Obscure gastrointestinal bleeding; DY: Diagnostic Yield; pts: Patients; IY: Incremental yield; GTT: Gastric transit time; SBCT: Small bowel transit time; SBCE: Small-bowel capsule endoscopy; OR: Odds ratio; RR: Relative risk; C + IL: Colonoscopy with ileoscopy; PE: Push enteroscopy; SBCE: Small bowel Crohn's disease; CT: Computed tomography; IDA: Iron deficiency anemia; FEM: Fixed effect model.

**Table 4** Studies evaluating the clinical application of faecal calprotectin in the setting of small-bowel capsule endoscopy

| Ref.                                      | Country                               | Centre        | Study type    | Design   | Participants  | FC   | CE   | Objective(s)  | Outcome(s)   |
|---|---------------------------------------|---------------|---------------|--|---|--|--|---|--|
| Goldstein <i>et al</i> <sup>[41]</sup>    | United States                         | Multi-centre  | Prospective   | Double-blind, triple-dummy, placebo controlled | 334 healthy subjects  | N/A  | M2A®; Given® Imaging, Yokneam, Israel  | Evaluate incidence of SB injury and correlation with FC in healthy subjects on celecoxib or ibuprofen + omeprazole                                    | <ul style="list-style-type: none"> <li>► Mean increase in FC higher in subjects on ibuprofen+omeprazole compared with celecoxib alone (<math>P &lt; 0.001</math>);</li> <li>► No correlation between FC and SB mucosal breaks</li> </ul>   |
| Hawkey <i>et al</i> <sup>[42]</sup>       | Germany, United Kingdom               | Multi-centre  | Prospective   | Double-blind, double-dummy, placebo controlled | 139 healthy subjects  | Phical Calprotectin Test Kit NovaTec Immunodiagnostica, GmbH Dietzenbac, Germany | M2A®; Given® Imaging, Yokneam, Israel  | Investigate SB injury lumiracoxib reduces <i>vs</i> naproxen + omeprazole   | <ul style="list-style-type: none"> <li>► More SB mucosal breaks on naproxen+omeprazole (77.8% <i>vs</i> 40.4%, <math>P &lt; 0.001</math>);</li> <li>► Furthermore, higher FC <i>vs</i> placebo (96.8 <i>vs</i> 14.5 µg/g, <math>P &lt; 0.001</math>);</li> <li>► 27.7% on lumiracoxib had SB mucosal breaks (<i>vs</i> placebo, <math>P = 0.196</math>; <i>vs</i> naproxen, <math>P &lt; 0.001</math>)</li> <li>► No increase in FC (-5.7 µg/g; <i>vs</i> placebo, <math>P = 0.377</math>; <i>vs</i> naproxen, <math>P &lt; 0.001</math>)</li> </ul> |
| Smecuol <i>et al</i> <sup>[43]</sup>      | Argentina, Spain, Canada              | Multi-centre  | Prospective   | Non-blinded study                              | 20 healthy subjects   | Calprest® Eurospital SpA, Trieste, Italy   | M2A®; Given® Imaging, Yokneam, Israel  | Determine SB damage by low-dose ASA (on a short-term basis)   | <ul style="list-style-type: none"> <li>► Short-term administration of low-dose ASA associated with mucosal abnormalities of the SB mucosa;</li> <li>► Median baseline FC (6.05 µg/g; range: 1.9-79.2 µg/g) increased significantly after ASA use</li> </ul>  |
| Werlin <i>et al</i> <sup>[44]</sup>       | United States, Israel, United Kingdom | Multi-centre  | Prospective   | N/A  | 42 pts with CF* (aged 10-36 yr); 29 had pancreatic insufficiency    | Calprest® Eurospital SpA, Trieste, Italy   | PillCam®SB; Given® Imaging, Yokneam, Israel  | Examine the SB of pts with CF without overt evidence of GI disease using CE   | <ul style="list-style-type: none"> <li>► Varying degrees of diffuse areas of inflammatory findings in the SB: oedema, erythema, mucosal breaks and frank ulcerations;</li> <li>► No adverse events recorded; FC markedly high in pts with pancreatic insufficiency, 258 µg/g (normal &lt; 50)</li> </ul>   |
| Koulaouzidis <i>et al</i> <sup>[45]</sup> | United Kingdom                        | Single centre | Retrospective | Chart review                                   | 70 pts with suspected CD and (-) <i>ve</i> bi-directional endoscopy | CALPRO NovaTec Immunodiagnostica GmbH, Dietzenbac, Germany                       | (1) PillCam® SB; Given® Imaging, Yokneam, Israel; (2) MiroCam®; IntroMedic Co., Seoul, South Korea | Value of FC as selection tool for further investigation of the SB with SBCE, in a cohort of pts with suspected CD                                     | <ul style="list-style-type: none"> <li>► FC = 50-100 µg/g; normal SBCE, despite symptoms suggestive of IBD;</li> <li>► FC &gt; 100 µg/g; good predictor of positive SBCE;</li> <li>► FC &gt; 200 µg/g; associated with higher SBCE DY (65%); confirmed CD in 50%;</li> <li>► Measurement of FC prior SBCE: useful tool to select patients for referral. If FC &lt; 100 µg/g; SBCE is not indicated (NPV 1.0)</li> </ul>  |
| Jensen <i>et al</i> <sup>[46]</sup>       | Denmark                               | Single centre | Prospective   | Blinded study                                  | 83 pts from GI OPD clinics with suspected CD                        | Calprotectin ELISA, BÜHLMANN Laboratories AG, Basel, Switzerland                 | PillCam®SB; Given® Imaging, Yokneam, Israel  | Determine FC levels in CD restricted to SB compared to colonic CD, in pts on first diagnostic work-up; Assess the Sens and Spec of FC in suspected CD | <ul style="list-style-type: none"> <li>► In pts with SB or colonic CD FC is equal: median 890 µg/g <i>vs</i> 830 mg/kg, respectively (<math>P = 1.0</math>);</li> <li>► FC cut-off = 50 µg/g: 92% and 94% Sens for SB and colonic CD, respectively;</li> <li>► Overall, Sens and Spec for FC: 95% and 56%;</li> <li>► CD was ruled out with NPV of 92%;</li> <li>► In suspected CD, FC is effective marker to <i>r/o</i> CD and select patients for endoscopy</li> </ul>   |

|   |                |               |                |               |                               |   |   |  |   |
|---|----------------|---------------|----------------|---------------|-------------------------------|---|---|--|---|
| Koulaouzidis <i>et al</i> <sup>[47]</sup> | United Kingdom | Single centre | Retro-spective | Chart review  | 49 pts; known or suspected CD | CALPRO NovaTec Immuno-diagnostica GmbH, Dietzenbac, Germany | PillCam®; Given® Imaging, Yokneam, Israel; MiroCam®; IntroMedic Co., Seoul, South Korea | Assess performance of 2 SBCE inflammation scoring systems (LS and CECDAI) correlating them with FC; Define threshold levels for CECDAI | <ul style="list-style-type: none"> <li>▶ LS performs better than CECDAI in describing SB inflammation, especially at FC &lt; 100 µg/g</li> <li>▶ CECDAI levels of 3.8 and 5.8 correspond to LS thresholds of 135 and 790, respectively</li> </ul>   |
| Sipponen <i>et al</i> <sup>[48]</sup>     | Finland        | Single centre | Pro-spective   | Blinded study | 84 pts; known or suspected CD | Calprest® Eurospital SpA, Trieste, Italy                    | PillCam®; Given® Imaging, Yokneam, Israel; MiroCam®; IntroMedic Co., Seoul, South Korea | Study the role of FC and S100A12 in predicting SB inflammatory lesions   | <ul style="list-style-type: none"> <li>▶ CE abnormal in 35/84 (42%) pts: 14 CD, 8 NSAID-enteropathy, 8 angioectasias, 4 polyps/tumours, 1 ischemic stricture</li> <li>▶ Median FC/S100A12: 22 µg/g (range: 2-342 µg/g)/0.048 µg/g (range: 0.003-1.215 µg/g)</li> <li>▶ FC significantly higher in CD pts (median 91, range: 2-312) compared with pts with normal CE or other abnormalities (<i>P</i> = 0.008)</li> <li>▶ Faecal S100A12 (0.087 µg/g, range: 0.008-0.896 µg/g): no difference between the groups (<i>P</i> = 0.166)</li> <li>▶ Sens, Spec, PPV, NPV in detecting SB inflammation; FC (cut-off 50 µg/g): 59%, 71%, 42%, 83%; S100A12 (cut-off 0.06 µg/g): 59%, 66%, 38%, 82%, respectively</li> </ul> |

CF: Cystic Fibrosis; CD: Crohn’s disease; GI: Gastrointestinal; OPD: Out-Patient Department; SB: Small-bowel; FC: Faecal calprotectin; ASA: Acetylsalicylic acid; CE: Capsule endoscopy/e; Pts: Patients; Sens: Sensitivity; Spec: Specificity; SBCE: Small-bowel capsule endoscopy; LS: Lewis score; CECDAI: Capsule endoscopy Crohn’s disease activity index; NPV: Negative predictive value; PPV: Positive predictive value; N/A: Not available or not applicable; NSAID: Non-steroidal anti-inflammatory drug.

**Table 5 Studies looking at the identification rate of the ampulla in capsule endoscopy**

| Ref.                                      | CE  | Type of CE model; Company          | AoV seen, n (%) | Reviewers | Reviewing speed (fps) | Frames AoV visible <sup>2</sup> | Comments                                 |
|---|-----|------------------------------------|-----------------|-----------|-----------------------|---------------------------------|--|
| Wijeratne <i>et al</i> <sup>[53]</sup>    | 138 | NS                                 | 9 (6.0)         | 1         | NS                    | NS                              | 4 FAP patients (AoV not seen)            |
| Kong <i>et al</i> <sup>[54]</sup>         | 110 | M2A®; Given® Imaging Ltd.          | 48 (43.6)       | 2         | 15                    | 3.5 ± 2.5                       |  |
| Clarke <i>et al</i> <sup>[55]</sup>       | 125 | M2A®; Given® Imaging Ltd.          | 13 (10.4)       | 2         | 5                     | NS                              |  |
| Iaquinto <i>et al</i> <sup>[56]</sup>     | 23  | PillCam®SB; Given® Imaging Ltd.    | 0 (0.0)         | 2         | NS                    | N/A                             | FAP patients (11/23 had duodenal polyps) |
| Metzger <i>et al</i> <sup>[57]</sup>      | 20  | PillCam®SB1; Given® Imaging Ltd.   | 1 (5.0)         | NS        | NS                    | NS                              | Repeat examinations                      |
|   |     | PillCam®SB2; Given® Imaging Ltd.   | 5 (25.0)        | NS        | NS                    | NS                              |  |
| Katsinelos <i>et al</i> <sup>[58]</sup>   | 14  | NS                                 | 0 (0.0)         | 1         | NS                    | N/A                             | FAP patients                             |
| Nakamura <i>et al</i> <sup>[59]</sup>     | 96  | PillCam®SB1; Given® Imaging Ltd.   | 18 (18.0)       | 2         | 10                    | NS                              |  |
| Karagiannis <i>et al</i> <sup>[60]</sup>  | 10  | PillCam®Colon; Given® Imaging Ltd. | 6 (60.0)        | NS        | NS                    | NS                              | Two-headed PillCam®                      |
| Lee <i>et al</i> <sup>[61]</sup>          | 30  | PillCam®SB; Given® Imaging Ltd.    | 13 (43.3)       | NS        | NS                    | NS                              |  |
|   | 30  | PillCam®SB2; Given® Imaging Ltd.   | 15 (50.0)       | NS        | NS                    | NS                              |  |
|   | 50  | PillCam®SB1; Given® Imaging Ltd.   | 0 (0.0)         | 2         | NS                    | N/A                             |  |
| Selby <i>et al</i> <sup>[62]</sup>        | 50  | PillCam®SB2; Given® Imaging Ltd.   | 9 (18.0)        | 2         | NS                    | NS                              |  |
|   | 8   | PillCam®ESO1; Given® Imaging Ltd.  | 0 (0.0)         | 2         | NS                    | N/A                             | Two-headed PillCam®                      |
|   | 12  | PillCam®ESO2; Given® Imaging Ltd.  | 1 (8.0)         | 2         | NS                    | NS                              | Two-headed PillCam®                      |
| Koulaouzidis <i>et al</i> <sup>[63]</sup> | 11  | PillCam®ESO1; Given® Imaging Ltd.  | 4 (36.4)        | 1         | 7                     | NS                              | Two-headed PillCam®                      |
|   | 7   | PillCam®ESO2; Given® Imaging Ltd.  | 1 (14.3)        | 1         | 9                     | NS                              | Two-headed PillCam®                      |
| Park <i>et al</i> <sup>[64]</sup>         | 30  | PillCam®SB; Given® Imaging Ltd.    | 13 (43.3)       | 6         | 7                     | 3.1 ± 1.1                       |  |
|   | 30  | PillCam®SB2; Given® Imaging Ltd.   | 15 (50.0)       | 6         | 9                     | 3.1 ± 1.5                       |  |
|   | 262 | PillCam®SB1; Given® Imaging Ltd.   | 28 (10.7)       | 1         | 6                     | 36.35 ± 73.24                   |  |
| Koulaouzidis <i>et al</i> <sup>[65]</sup> | 148 | PillCam®SB2; Given® Imaging Ltd.   | 13 (8.8)        | 1         | 6                     | 42.46 ± 69.3                    |  |
|   | 209 | MiroCam®; IntroMedic Ltd.          | 18 (8.6)        | 1         | 6                     | 87.20 ± 248.4                   |  |
| Friedrich <i>et al</i> <sup>[66]</sup>    | 25  | CapsCam®SV1; Capsovision Ltd.      | 22 (71)         | 3         | NS                    | 3.1 ± 1.8                       |  |

<sup>1</sup>Published only as abstracts; <sup>2</sup>mean ± SD. CE: Capsule endoscopy; NS: Not stated; N/A: Not available or not applicable; AoV: Ampulla of Vater; fps: Frames per second; FAP: Familial adenomatous polyposis syndrome.

## CAPSULE ENDOSCOPE ASPIRATION; HOW COMMON IS THIS?

Capsule enteroscopy is generally considered safe, having an overall complication rate of about 1%-3%<sup>[13,14]</sup>. Undoubtedly, the most feared complication of CE is

capsule retention in the small bowel (overall retention rate 1.5%-2%), which seems directly related with the clinical indication for SBCE<sup>[13,14,40]</sup>. Interestingly enough, other possible complications - which were postulated at the time of CE introduction (*i.e.*, retention inside colonic diverticula, interaction with pacemakers, *etc.*) to represent

potential hurdles for the method, were shown to be very infrequent and/or without clinically relevant consequences<sup>[67-71]</sup>. Conversely, capsule aspiration - an unexpected complication - has been reported with increasing frequency (Table 6)<sup>[72-93]</sup>. Overall, this is probably related to the increase in the mean age of patients undergoing CE. In fact, capsule aspiration occurs in 1 out of 800-1000 procedures<sup>[88]</sup>, mostly in elderly male patients with comorbidities and/or swallowing disorders. In the majority of cases capsule aspiration resolves quickly, because patients expectorate the capsule. However, in selected cases, emergency bronchoscopy is required. Thus far, only one fatality-directly associated with capsule aspiration- has been reported<sup>[90]</sup>.

## CAN WE SHORTEN OUR READING TIME IN CAPSULE ENDOSCOPY?

Few will disagree with the notion that CE is a time-consuming procedure. In fact, although capsule administration and swallowing requires only a couple of minutes, SBCE transit through the small bowel, although variable, on average lasts about 2-5 h<sup>[94]</sup>. This results in 14400-72000 frames, depending on capsule frame rate (Table 1). This large amount of visual information requires careful evaluation by the CE reader. In addition, any small-bowel lesion may only be visible in just a few or even in a single frame<sup>[95]</sup>. Therefore, focused and undivided attention is required for the entire duration of each CE video evaluation. In light of all that, several attempts have been made to develop technical software features, in order to make CE video analysis easier and shorter (without jeopardising its accuracy). The first software feature designed for this purpose was the Suspected Blood Indicator (SBI), an automatic system able to pick up, in a completely automatic fashion, frames containing several red pixels and, therefore (theoretically), to detect blood and or other red-coloured lesions. Nevertheless, the accuracy profile of this tool (Table 7) is suboptimal and, at present time<sup>[96-102]</sup>, it can be used only as supportive tool<sup>[102]</sup>.

Given<sup>®</sup>Imaging Ltd. has also introduced another software tool, which aims specifically at shortening the CE reading time, the QuickView. This sampling tool is able to select one frame every X CE frames (the sampling rate can be set by the reader) and therefore present, with the click of a tag-button, a shortened CE video which can be reviewed in a few minutes. Although the sampling method of the QuickView system is only quantitative, it has showed a promising sensitivity and specificity in identifying small-bowel lesions (Table 8), and reveals promising potential when coupled with other image enhancing systems<sup>[104-112]</sup>. Olympus has similar software function (express mode) and we are aware of a single relevant study with very similar results<sup>[113]</sup>.

In the last few years, Given<sup>®</sup>Imaging Ltd., through a collaboration with Fujinon Inc., Japan introduced the electronic chromo-endoscopy (Fujinon Intelligent Chromo Endoscopy, FICE) in the field of capsule enteroscopy.

Data available thus far, show that application of FICE in SBCE videos, leads to improved image quality and definition of the surface texture of small-bowel lesions (Table 9)<sup>[114-120]</sup>. Although this seems to facilitate the detectability of small-bowel findings, it is still under question whether it proves to be clinically significant<sup>[121]</sup>. Similar function from Olympus Inc., shows promising results<sup>[122]</sup>.

## WHAT'S NEW ON THE FIELD OF SMALL-BOWEL CAPSULE ENDOSCOPY?

As aforementioned, there are differences among different capsule models (Table 1). Since its introduction in clinical practice in 2001, CE technology has been significantly. For instance, battery life is longer, image capture frame rate has increased, angle of view is now wider, light control has been optimized, and many real time viewing systems are now available. Nevertheless, these impressive advancements, do not allow overcoming the main current limitation of CE, *i.e.*, uncontrolled propulsion; CE relies totally on natural bowel peristalsis, *i.e.*, it still remains a rather "passive" diagnostic technique.

Several research groups are working to design brand new capsules able to actively move or to be remotely manoeuvred through their descent in the small bowel<sup>[123]</sup>. These new capsules would allow not only recognizing a small bowel lesion but also, in a near future, to collect targeted tissue samples or to deliver drugs (Table 10)<sup>[124-141]</sup>.

## CONCLUSION

Since CE introduction in clinical practice in 2001, over 1500 papers, focused on SBCE, have been published (PubMed search 17/03/2012; keyword term: "small bowel capsule endoscopy"; available from: <http://www.ncbi.nlm.nih.gov/pubmed/?term=small+bowel+capsule+endoscopy>).

Out of those, < 20% are clinical trials; case reports and reviews account for about 40% of published evidence. As the amount of information has increased exponentially, and in fact continues to do so<sup>[12]</sup>, it is often difficult for the busy clinician to retrieve and filter data or extract answers to questions arising from the daily clinical practice. In the present review, we opted to answer certain pertinent questions on contentious and important issues in CE through comprehensive tables. Essentially, we aim to present an easy-to-read review with all the necessary evidence to support opinions expressed herein.

The analysis of the publications listed in the tables clearly demonstrates how SBCE, although much "younger" than other endoscopic techniques, has found a definite role in the diagnostic work-up of certain patient-subgroups. Further success of this modality depends not only on continuous technological progress (*i.e.*, introduction of new capsule models, improved battery life and/or development of new reading software features)<sup>[142]</sup> but also on the search for new diagnostic strategies, aiming to select for SBCE those patients with higher potential for positive DY<sup>[32,45,81,111,117]</sup>.

Table 6 Case reports of aspiration of capsule endoscopes

| Ref.                                      | Case (age/gender) | Comorbidities  | CE model/company                | Swallowing difficulties                | No. of attempts to swallow CE/ gagging or coughing                                     | Aspiration time/where in bronchial tree CE seen    | Capsule removal (if employed)  | Final diagnosis  |
|---|-------------------|--|---------------------------------|--|--|--|--|--|
| Schneider <i>et al</i> <sup>[72]</sup>    | 64/male           | Mechanical MV on phenprocoumon, BMI 15.5                               | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | 4/gagging and spitting capsule - last attempt recurrent coughing (aspiration presumed) | 2 min/ trachea-bronchi                             | Spontaneous resolution   | NS   |
| Fleischer <i>et al</i> <sup>[73]</sup>    | 76/male           | HHT  | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | 1/lopped in his throat - no respiratory difficulty, could talk, vital signs normal     | 60 min/ cricopharyngeus                            | Endoscopy-Roth net; 6 d post-dilation, patient ingested capsule with no problem                    | Spasticity, prominence of cricopharyngeus; endoscopy and oesophageal dilation 1 wk later |
| Sinn <i>et al</i> <sup>[74]</sup>         | 69/female         | On phenprocoumon   | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | 1/coughed several times  | 50 s/bifurcation of the trachea                    | Spontaneous resolution   | NS   |
| Tabib <i>et al</i> <sup>[75]</sup>        | 87/female         | Recent onset IDA, CHF, IHD, AF, bladder cancer, CRF                    | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia, pre-CE barium meal | 2/choking, dyspnoea, CE felt lodged in the throat                                      | NS/right main-stem bronchus - bronchus intermedius | Rigid bronchoscopy   | NS   |
| Buchkremer <i>et al</i> <sup>[76]</sup>   | 74/male           | Recent diagnosis of coeliac disease; past Hx of ankylosing spondylitis | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | NS/dyspnoea started after CE ingestion   | NS/right main-stem bronchus                        | Flexible bronchoscopy  | NS   |
| Rondonotti <i>et al</i> <sup>[77]</sup>   | NS                | NS   | MZA®; Given® Imaging Ltd.       | NS                                     | NS/coughed several times   | NS/NS  | Spontaneous resolution   | NS   |
| Nathan <i>et al</i> <sup>[78]</sup>       | 93/male           | No significant past medical Hx   | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | 1/coughed hours post-ingestion   | Approximately 8 h/ bronchial tree                  | Spontaneous resolution   | NS   |
| Shiff <i>et al</i> <sup>[79]</sup>        | 75/male           | NS   | MZA®; Given® Imaging Ltd.       | No Hx of dysphagia                     | 2/some coughing  | NS/bronchi   | Spontaneous resolution   | NS   |
| Sepehr <i>et al</i> <sup>[80]</sup>       | 67/male           | HTN, DM, CVA   | NS                              | Hx of dysphagia (intermittent)         | 1/coughing, tachypnoea, and tachycardia  | NS/trachea   | Eventually, CE endoscopic placement  | NS   |
| Koulaouzidis <i>et al</i> <sup>[81]</sup> | 76/male           | NS   | PillCam®SB; Given® Imaging Ltd. | No Hx of dysphagia                     | 1/coughed weakly   | 15 s/ trachea                                      | Spontaneous resolution   | NS   |
| Guy <i>et al</i> <sup>[82]</sup>          | 90/male           | Ischaemic CVA  | NS                              | No Hx of dysphagia                     | NS/no symptoms   | NS/ bronchial tree                                 | Rigid bronchoscopy - stone retrieval basket  | NS   |
| Leeds <i>et al</i> <sup>[83]</sup>        | 85/male           | NS   | NS                              | No Hx of dysphagia                     | NS/difficulty swallowing CE, slightly painful  | 8 h/lobar bronchus                                 | Spontaneous resolution   | NS   |
| Bredenoord <i>et al</i> <sup>[84]</sup>   | 65/male           | Sigmoid colectomy for diverticulae; Ileal carcinoid resected           | NS                              | Hx of dysphagia                        | Lengthy swallowing attempt/ coughing noted   | NS/right main bronchus                             | Spontaneous resolution, eventually, CE was swallowed on same session                               | Normal small-bowel   |
| Choi <i>et al</i> <sup>[85]</sup>         | 75/male           | Prior CVA  | PillCam®SB; Given® Imaging Ltd. | No Hx of dysphagia                     | NS/coughed several times   | 2 h/left main bronchus                             | Flexible Bronchoscopy-Roth net and bronchial wall irrigation to induce cough                       | NS, patient declined further investigations  |
| Depriest <i>et al</i> <sup>[86]</sup>     | 90/male           | IHD, AF, PVD (warfarin + clopidogrel)                                  | PillCam®SB; Given® Imaging Ltd. | No Hx of dysphagia                     | NS/some cough  | NS/left main bronchus, then right main bronchus    | Chest percussive therapy + postural drainage; Flexible bronchoscopy + extraction basket + Roth net | NS   |
| Depriest <i>et al</i> <sup>[86]</sup>     | 90/male           | IHD, AF, PVD (warfarin + clopidogrel)                                  | PillCam®SB; Given® Imaging Ltd. | No Hx of dysphagia                     | NS/some cough  | NS/left main bronchus, then right main bronchus    | Chest percussive therapy + postural drainage; flexible bronchoscopy + extraction basket + Roth net | NS   |

|                                      |            |   |  |                    |   |   |  |   |
|--------------------------------------|------------|---|--|--------------------|---|---|--|---|
| Kurtz <i>et al</i> <sup>[87]</sup>   | 73/ male   | Renal cell cancer, MV (bovine), hyperlipidaemia, melana | NS   | No Hx of dysphagia | Sips of water, 1 <sup>st</sup> attempt, 2 min later non-productive cough (20 s)             | Level of carina; then right main stem bronchus    | Bronchoscopy-retrieval basket (multiple spontaneous ejections from trachea prior bronchoscopy) | NS  |
| Lucendo <i>et al</i> <sup>[88]</sup> | 80/ male   | Advanced PD, DM, walking + speech difficulties          | PillCam <sup>®</sup> SB; Given <sup>®</sup> Imaging Ltd. | No Hx of dysphagia | Several attempts/persistent coughing and some dyspnoea                                      | 20 s/tracheobronchial tree                        | Spontaneous resolution   | Oesophageal ulcer + ileal ulcer                           |
| Pezzoli <i>et al</i> <sup>[89]</sup> | 82/ male   | Unexplained anemia, HTN                                 | NS   | No Hx of dysphagia | NS/asymptomatic (minimal cough)   | 3 d/in the right bronchus                         | Spontaneous resolution   | NS  |
| Parker <i>et al</i> <sup>[90]</sup>  | 77/ female | Hysterectomy  | NS   | No Hx of dysphagia | Initial attempt unsuccessful/chocking episode, CE coughed-up                                | NS/NS   | Spontaneous resolution, endoscopic placement with AdvanCE <sup>®</sup> device                  | Patient suffered intracranial bleed, eventually succumbed |
| Despott <i>et al</i> <sup>[91]</sup> | 65/ male   | COPD, cirrhosis, pancreatitis                           | NS   | No Hx of dysphagia | NS/asymptomatic   | NS/right main bronchus                            | Rigid bronchoscopy-Roth net  | Endoscopic placement with AdvanCE <sup>®</sup> device     |
|                                      | 73/ male   | COPD  | NS   | NS                 | NS/brief coughing   | NS/left main bronchus                             | Bronchoscopy-snare + Roth net  | Endoscopic placement with AdvanCE <sup>®</sup> device     |
| Girdhar <i>et al</i> <sup>[92]</sup> | 81/ male   | NS  | NS   | NS                 | NS/fleeting choking sensation   | NS/right main bronchus                            | Rigid bronchoscopy-crocodile grasping forceps  | NS  |
|                                      | 83/ male   | COPD, GORD  | PillCam <sup>®</sup> SB; Given <sup>®</sup> Imaging Ltd. | No Hx of dysphagia | Difficult, requiring multiple sips of water/ some cough, after 1 h mild shortness of breath | NS/left main bronchus                             | Flexible bronchoscopy + rat-tooth alligator forceps + stiff-wire basket with a pin-wise handle | NS  |
| Poudel <i>et al</i> <sup>[93]</sup>  | 80/ male   | AF, IHD, CVA on anti-coagulants, anaemia + melana       | M2A <sup>®</sup> ; Given <sup>®</sup> Imaging Ltd.       | NS                 | NS  | 24 h/left main stem bronchus; then right bronchus | Flexible bronchoscopy + net + snare forceps + tripod; eventually, grasped with basket          | NS  |

MV: Mitral valve; BMI: Body mass index; HHT: Hereditary haemorrhagic telangiectasia; IDA: Iron deficiency anaemia; CHF: Chronic heart failure; IHD: Ischaemic heart disease; AF: Atrial fibrillation; CRF: Chronic renal failure; Hx: History; NS: Not stated; HTN: Hypertension; DM: Diabetes mellitus; CVA: Cerebrovascular accident; PVD: Peripheral vascular disease; COPD: Chronic obstructive pulmonary disease; GORD: Gastro-oesophageal reflux disease; CE: Capsule endoscopy.

| Table 7 Studies looking at the clinical validity of Suspected Blood Indicator, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy |               |               |   |               |   |                                      |   |  |
|--|---------------|---------------|---|---------------|---|--------------------------------------|---|--|
| Ref.   | Country       | Centre        | Objective(s)                                    | Study type    | Design  | CE type                              | Outcome(s)  | Conclusions  |
| Gross <i>et al</i> <sup>[94]</sup>   | United States | Single centre | Accuracy of SBI to number of blood transfusions | Retrospective | ► Gold standard for lesions detected by experienced CE reviewer   | M2A; Given <sup>®</sup> Imaging Ltd. | ► Gold standard: 72 pts; ► pts received blood transfusions ranging between 0-16 units; ► Overall: A total of 17 pts had positive SBI. Active bleeding in 16 pts, who were transfused an average of 8 units before the study; ► 55 pts had a negative SBI and no active bleeding was seen on their capsule studies. In this group, the average number of PRBC transfused was 1 unit. There was one patient who had a false positive SBI with no active bleeding seen in the capsule study review | Pts receiving blood transfusions are more likely to have a positive SBI correlating with the localization of active bleeding |
| Liangpunsakul <i>et al</i> <sup>[95]</sup>   | United States | Single centre | Assess accuracy of SBI                          | Retrospective | ► Gold standard for lesions detected by experienced CE reviewer; ► Significant lesions considered AVMs, ulcers, erosions, active bleeding; ► Reviewing speed: 15fps | M2A; Given <sup>®</sup> Imaging Ltd. | ► Gold standard: 109 lesions; ► SBI: 31 potential areas of blood; correctly identified lesions: 28; ► Overall: SBI (Sens, PPV, accuracy): 25.7%, 90%, 34.8%, respectively; ► For actively bleeding SB lesions only: SBI (Sens, PPV, accuracy): 81.2%, 81.3%, 83.3%, respectively  | SBI has good Sens and PPV for actively bleeding SB lesions   |

|   |               |                          |  |                |  |                          |  |  |
|---|---------------|--------------------------|--|----------------|--|--------------------------|--|--|
| D'Halluin <i>et al.</i> <sup>[68]</sup>   | France        | Multi-centre (7 centres) | Assess Sens/Spec of SBI (in OGIB)  | Retro-spective | <ul style="list-style-type: none"> <li>► Gold standard for lesions detected by experienced CE reviewer, SBI tags marked by another investigator;</li> <li>► Significant lesions considered Bleeding or having a bleeding potential: high (P2), low (P1), or absent (P0);</li> <li>► Concordance: same time code in frames selected by expert reader and those tagged by SBI;</li> <li>► Reviewing speed: NS</li> </ul> | M2A; Given® Imaging Ltd. | <ul style="list-style-type: none"> <li>► 156 SBCE recordings evaluated: in 83 (normal): either no lesion (<i>n</i> = 71) or P0 lesion (<i>n</i> = 12); in 73 abnormal: P2 (<i>n</i> = 114) and P1 (<i>n</i> = 92) lesions;</li> <li>► 154 red tags analysed: SBI (Sens, Spec, PPV, NPV) for P2 or P1: 37%, 59%, 50%, 46%, respectively</li> </ul>  | ► SBI-based detection of SB lesions (with bleeding potential) is of limited clinical value   |
| Singnorelli <i>et al.</i> <sup>[69]</sup> | Italy         | Single centre            | Assess Sens/Spec of SBI per lesion, overall, according to red findings (identified by the reader), and per patient | Retro-spective | <ul style="list-style-type: none"> <li>► Gold standard for lesions detected by four experienced CE reviewers;</li> <li>► Outcomes: Sens, Spec and accuracy calculated both per lesion/patient;</li> <li>► Reviewing speed: NS</li> </ul>   | M2A; Given® Imaging Ltd. | <ul style="list-style-type: none"> <li>► 95 patients; 209 red findings;</li> <li>► Overall Sens: 28%;</li> <li>► Sens higher for identification of blood (61%) than for nonbleeding "red" findings, <i>e.g.</i>, AVMs (26%);</li> <li>► Per-patient Sens, Spec: 41%, 70%, respectively</li> </ul>  | <ul style="list-style-type: none"> <li>► SBI has low Sens/Spec in per-lesion and per-patient SBCE evaluation;</li> <li>► Complementary/rapid screening tool;</li> <li>► Complete review of the recordings is still necessary</li> </ul>          |
| Ponferrada <i>et al.</i> <sup>[100]</sup> | Spain         | Single centre            | Assess accuracy/performance of SBI   | Prospective    | <ul style="list-style-type: none"> <li>► Gold standard for lesions detected by experienced CE reviewers</li> </ul>   | M2A; Given® Imaging Ltd. | <ul style="list-style-type: none"> <li>► 57 consecutive patients;</li> <li>► Indications: OGIB (64.9%), CD (14%), malabsorption (14%), suspicion of SB tumour (7.1%);</li> <li>► SBI Sens, Spec, PPV, NPV: 58.3%, 75.5%, 38.8%, 87.2%, respectively</li> <li>► CE indications: OGIB (<i>n</i> = 112), suspected CD (<i>n</i> = 122), anaemia of unknown origin (<i>n</i> = 53), other (<i>n</i> = 4);</li> <li>► 221 lesions with bleeding potential;</li> <li>► Overall: SBI (Sens, Spec, PPV, NPV): 56.4%, 33.5%, 24.0%, 67.3%, respectively;</li> <li>► For actively bleeding lesions: SBI (Sens, PPV): 58.3%, 70%, respectively;</li> <li>► For suspected CD: SBI (Sens, NPV): 64%, 80.4%, respectively;</li> <li>► For OGIB: SBI Sens 58.3%;</li> <li>► For anaemia: SBI Sens 41.3%;</li> </ul> | <ul style="list-style-type: none"> <li>► SBI performance characteristics suboptimal/insufficient to screen for SB lesions with bleeding potential;</li> <li>► Even in pts with active intestinal bleeding, SBI Sens was only &lt; 60%</li> </ul> |
| Buscaglia <i>et al.</i> <sup>[101]</sup>  | United States | Single centre            | Assess accuracy/performance of SBI according to CE indications   | Retro-spective | <ul style="list-style-type: none"> <li>► Gold standard for lesions detected by experienced CE reviewer;</li> <li>► Significant lesions:AVMs, varices, venous ectasias, red spots, ulcers, erosions, blood, blood clots</li> <li>► Concordant and discordant findings between CE reviewer and SBI;</li> <li>► Reviewing speed: 8-15 fps</li> </ul>  | M2A; Given® Imaging Ltd. | <ul style="list-style-type: none"> <li>► 221 lesions with bleeding potential;</li> <li>► Overall: SBI (Sens, Spec, PPV, NPV): 56.4%, 33.5%, 24.0%, 67.3%, respectively;</li> <li>► For actively bleeding lesions: SBI (Sens, PPV): 58.3%, 70%, respectively;</li> <li>► For suspected CD: SBI (Sens, NPV): 64%, 80.4%, respectively;</li> <li>► For OGIB: SBI Sens 58.3%;</li> <li>► For anaemia: SBI Sens 41.3%;</li> </ul>   | <ul style="list-style-type: none"> <li>► SBI performance characteristics suboptimal/insufficient to screen for SB lesions with bleeding potential;</li> <li>► Even in pts with active intestinal bleeding, SBI Sens was only &lt; 60%</li> </ul> |
| Park <i>et al.</i> <sup>[102]</sup>       | South Korea   | Single centre            | Investigate whether SBI is affected by background colour and CE velocity   | Experimental   | <ul style="list-style-type: none"> <li>► Paper-made phantom SB models in a variety of colours to simulate the background colours observed in CE;</li> <li>► Red spots were attached inside them;</li> <li>► CE manually passed through models;</li> <li>► SBI red spots detection rate was evaluated based on colours of SB models and CE velocities (0.5, 1, 2 cm/s)</li> </ul>                                       | M2A; Given® Imaging Ltd. | <ul style="list-style-type: none"> <li>► SBI red spots detection rate differed significantly per background colour of SB model, <i>P</i> &lt; 0.001;</li> <li>► SBI red spots detection rate highest for very pale magenta, burnt sienna, yellow background;</li> <li>► SBI red spots detection rate lowest for dark brown, very pale yellow background</li> <li>► SBI red spots detection rate decreases at rapid CE passage (1-2 cm/s) compared to slower (0.5 cm/s) for very pale yellow (<i>P</i> = 0.042), yellow (<i>P</i> = 0.001), very pale magenta (<i>P</i> = 0.002), burnt sienna (<i>P</i> = 0.001) background;</li> <li>► Red spots detection rate no different according to velocity for light greyish pink (<i>P</i> = 0.643) or dark brown (<i>P</i> = 0.396) background</li> </ul> | <ul style="list-style-type: none"> <li>► SBI Sens affected by background colour and capsule passage velocity in the models</li> </ul>  |

PRBC: Pack red blood cells; fps: Frames per second; SBI: Suspected Blood Indicator; CE: Capsule endoscopy; AVM: Arterio-venous malformations; SB: Small-bowel; Sens: Sensitivity; Spec: Specificity; PPV: Positive predictive value; NPV: Negative predictive value; OGIB: Obscure gastrointestinal bleeding; CD: Crohn's disease.

**Table 8 Studies looking at the clinical validity of QuickView, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy**

| Ref.                                       | QuickView sampling rate | QuickView reading frame mode/reading speed (fps) | Average reading time (mean) | Comparison with reading frame mode/reading speed used (fps) | Rapid Reader version | Reviewers |      | Cases |          |       | QuickView       |                     | Lesions missed      |
|--|-------------------------|--|-----------------------------|---|----------------------|-----------|------|-------|----------|-------|-----------------|---------------------|---------------------|
|  |                         |  |                             |   |                      | Total     | OGIB | CD    | Polypsis | Other | Sensitivity (%) | Specificity (%)     |                     |
| Ponferrada <i>et al</i> <sup>[100]</sup>   | NS                      | 25, 15, 5  | NS                          | Conventional/NS/15, 15, 5                                   |                      | 2         | 57   | 37    | 8        | N/A   | 12              | NS                  | NS                  |
| Schmelkin <sup>[104]</sup>                 | NS                      | NS   | NS                          | NS  | 4.0                  | 1         | 47   | 47    | N/A      | N/A   | N/A             | 100                 | N/A                 |
| Appalane <i>et al</i> <sup>[105]</sup>     | NS                      | Single frame, 25                                 | 3 min                       | NS  | NS                   | 2         | 50   | NS    | NS       | NS    | NS              | NS                  | 2                   |
| Westerhof <i>et al</i> <sup>[106]</sup>    | High (17)               | NS   | 4.4 min (median)            | Conventional/dual view/18                                   | 4.0                  | 2         | 100  | 56    | 30       | 2     | 12              | NS                  | 13                  |
| Shiotani <i>et al</i> <sup>[107]</sup>     | High (17)               | Single, 6  | 17.9 min                    | NS  | 5.0                  | 3         | 44   | NS    | NS       | NS    | 14              | NS                  | 10                  |
| Hosoe <i>et al</i> <sup>[108]</sup>        | Normal                  | NS   | NS                          | NS  | 5.0                  | 3         | 45   | NS    | NS       | NS    | 14              | NS                  | NS                  |
| Saurin <i>et al</i> <sup>[109]</sup>       | NS                      | NS   | 11.6 min                    | Conventional/NS/NS  | 5.0                  | 12        | 106  | 106   | N/A      | N/A   | N/A             | 89.2                | Jul-84              |
| Shiotani <i>et al</i> <sup>[110]</sup>     | 5, 15, 25, 35           | Single, NS                                       | NS                          | NS  | 6.5                  | 4         | 87   | NS    | NS       | NS    | NS              | NS                  | NS                  |
| Koulaouzidis <i>et al</i> <sup>[111]</sup> | 35                      | Dual view (WL + BM)                              | 475 s (QuickView WL)        | Conventional/   | 7.0                  | 1         | 200  | 106   | 81       | 4     | 9               | 92.3 (QVWL P1 + P2) | 96.3 (QVWL P1 + P2) |
|  |                         | 18   | 450 s (QuickView BM)        | single or dual view/12-20                                   |                      |           |      |       |          |       |                 | 91 (QVBM P1+P2)     | 96 (QVBM P1 + P2)   |
| Kyriakos <i>et al</i> <sup>[112]</sup>     | NS                      | NS, 3  | 16.3 min (6.7)              | Conventional/NS/NS  | 5.0                  | 2         | 100  | 55    | 22       | 3     | 20              | NS                  | NS                  |

NS: Not stated; NA: Not applicable; fps: Frames per second; QVWL, QuickView with white light; QVBM: QuickView with blue mode; OGIB: Obscure gastrointestinal bleeding; CD: Crohn's disease; P1, P2: Classification as per probability of bleeding.

**Table 9 Studies looking at the clinical validity of Fujinon intelligent chromoendoscopy enhancement/Blue mode, feature of capsule endoscopy reading software, in small-bowel capsule endoscopy**

| Ref.                                  | Country | Centre        | Study type    | Objective(s)  | Design  | Images   | FICE       | CE                              | Outcome(s)  |
|---------------------------------------|---------|---------------|---------------|---|---|--|------------|---------------------------------|---|
| Imagawa <i>et al</i> <sup>[114]</sup> | Japan   | Single centre | Retrospective | Assess whether visualization of SB lesions improves with FICE   | ► 5 experienced readers compared CE-WL images to their FICE counterparts            | ► Angiectasis (n = 23);<br>► Erosion/ulcers (n = 47);<br>► Tumour (n = 75) | FICE 1,2,3 | PillCam®SBI; Given®Imaging Ltd. | ► FICE 1: AVMs: improvement in 87% (20/23) cases; erosion/ulceration: improvement 53.3% (26/47) cases; tumour images: improvement 25.3% (19/75) cases;<br>► FICE 2: AVMs: improvement in 87% (20/23) cases; erosion/ulceration: improvement in 25.5% (12/47) cases; tumour images: improvement in 20.0% (15/75) cases;<br>► FICE 3: All images groups: only equivalence achieved in all cases; intra-observer agreement: good to satisfactory (5.4 or higher) |
| Imagawa <i>et al</i> <sup>[115]</sup> | Japan   | Single centre | Prospective   | Assess whether FICE improves detection rate of SB lesions in CE | ► A CE reader reviewed CE-WL videos;<br>► Another reader, reviewed SB lesions in CE | 50 pts   | FICE 1,2,3 | PillCam®SBI; Given®Imaging Ltd. | ► Angioectasias detection: CE-WL: 17 AVMs; CE-FICE 1: 48 AVMs; CE-FICE 2: 45 AVMs; CE-FICE 3: 24 AVMs; significant CE-FICE 1 and 2 (P = 0.0003 and P < 0.0001, respectively)<br>► Detection rate for erosion, ulceration and tumour did not differ statistically between CE-WL and CE-FICE 1,2,3;<br>► Similar interpretation time (CE-WL: 36 ± 6.9 min; CE-FICE 1: 36 ± 6.4 min; FICE 2: 38 ± 5.8 min; FICE 3: 35 ± 6.7 min)                                 |

|   |                |               |               |  |  |  |  |  |   |
|---|----------------|---------------|---------------|--|--|--|--|--|---|
| Gupta <i>et al</i> <sup>[16]</sup>      | Belgium        | Single centre | Retrospective | Assess potential benefit of FICE for SB lesion detection in patients with OGIB   | CE videos analysed by 2 GI fellows with and without FICE 1,2,3;<br>Reference standard: Senior consultant described findings as P0, P1 and P2 lesions   | 60 pts with OGIB   | FICE 1,2,3<br>Given <sup>®</sup> Imaging Ltd.            | FillCam <sup>®</sup> SBI;<br>Given <sup>®</sup> Imaging Ltd.     | <ul style="list-style-type: none"> <li>Overall, 157 lesions diagnosed with CE-FICE vs 114 with CE-WL (<math>P = 0.15</math>);</li> <li>For P2 lesions; CE-FICE Sens/Spec: 94%/95% vs CE-WL Sens/Spec: 97%/96%, respectively; 5/55 AVMs better characterized with CE-FICE than CE-WL</li> <li>More P0 diagnosed by CE-FICE than CE-WL (39 vs 8, <math>P &lt; 0.001</math>);</li> <li>Intra-class kappa correlations between fellows and reference: CE-FICE vs CE-WL for P2 lesions: 0.88 vs 0.92; CE-FICE vs CE-WL for P1 lesions: 0.61 vs 0.79</li> <li>Total of 167 images; for all lesion categories: <ul style="list-style-type: none"> <li>Blue mode vs WL: image improvement in 83%; <math>\kappa = 0.786</math></li> <li>FICE 1 vs WL: image improvement in 34%; <math>\kappa = 0.646</math></li> <li>FICE 2 vs WL: image improvement in 8.6%; <math>\kappa = 0.617</math></li> <li>FICE 3 vs WL: image improvement in 7.7%; <math>\kappa = 0.669</math></li> </ul> </li> <li>Concordance between the 4 gastroenterologists: 0.650;</li> <li>CE-WL identified 75 findings and the CE-FICE 95;</li> <li>CE-FICE did not miss any lesions identified by CE-WL and allowed the identification of a higher number of AVMs (35 vs 32) and erosions (41 vs 24)</li> </ul> |
| Krystallis <i>et al</i> <sup>[17]</sup> | United Kingdom | Single centre | Retrospective | Assess FICE and Blue mode visualisation of SB lesions in CE  | <ul style="list-style-type: none"> <li>2 experienced reviewers CE-WL images to FICE/Blue mode counterparts</li> <li>Villi oedema (<math>n = 11</math>);</li> <li>Cobblestone (<math>n = 15</math>);</li> <li>Blood lumen (<math>n = 15</math>);</li> <li>LICS/other (<math>n = 46</math>)</li> </ul> | <ul style="list-style-type: none"> <li>Angioectasias (<math>n = 18</math>);</li> <li>Erosion/ulcers (<math>n = 60</math>);</li> <li>Villi oedema (<math>n = 17</math>);</li> <li>Cobblestone (<math>n = 11</math>);</li> <li>Blood lumen (<math>n = 15</math>);</li> <li>LICS/other (<math>n = 46</math>)</li> </ul> | Blue mode; FICE 1,2,3<br>Given <sup>®</sup> Imaging Ltd. | FillCam <sup>®</sup> SBI/SB2;<br>Given <sup>®</sup> Imaging Ltd. | <ul style="list-style-type: none"> <li>Total of 167 images; for all lesion categories: <ul style="list-style-type: none"> <li>Blue mode vs WL: image improvement in 83%; <math>\kappa = 0.786</math></li> <li>FICE 1 vs WL: image improvement in 34%; <math>\kappa = 0.646</math></li> <li>FICE 2 vs WL: image improvement in 8.6%; <math>\kappa = 0.617</math></li> <li>FICE 3 vs WL: image improvement in 7.7%; <math>\kappa = 0.669</math></li> </ul> </li> <li>Concordance between the 4 gastroenterologists: 0.650;</li> <li>CE-WL identified 75 findings and the CE-FICE 95;</li> <li>CE-FICE did not miss any lesions identified by CE-WL and allowed the identification of a higher number of AVMs (35 vs 32) and erosions (41 vs 24)</li> </ul>  |
| Duque <i>et al</i> <sup>[18]</sup>      | Portugal       | Single centre | Prospective   | Assess reproducibility and diagnostic accuracy of CE-FICE  | <ul style="list-style-type: none"> <li>4 physicians reviewed 150 FICE CE-WL; the other, CE-FICE videos</li> <li>2 experienced physicians analysed 20 CE; 1 interpreted CE-WL; the other, CE-FICE videos</li> </ul>   | 20 patients with OGIB  | Blue mode; FICE 1,2,3<br>Given <sup>®</sup> Imaging Ltd. | FillCam <sup>®</sup> SBI;<br>Given <sup>®</sup> Imaging Ltd.     | <ul style="list-style-type: none"> <li>Concordance between the 4 gastroenterologists: 0.650;</li> <li>CE-WL identified 75 findings and the CE-FICE 95;</li> <li>CE-FICE did not miss any lesions identified by CE-WL and allowed the identification of a higher number of AVMs (35 vs 32) and erosions (41 vs 24)</li> </ul>  |
| Nakamura <i>et al</i> <sup>[19]</sup>   | Japan          | Single centre | Prospective   | Assess preview of angioectasias by CE-FICE preview (compared to CE-WL)   | <ul style="list-style-type: none"> <li>One experienced physician analysed CEs in QuickView mode</li> <li>Mean reading time, sensitivity and specificity for angiodysplasia detection were evaluated including SBI</li> </ul>   | 50 pts with erosion/ulcers; angiodysplasia were randomly assigned to 2 equally sized groups of CE-WL reading and CE-FICE reading   | SBI;<br>Blue mode; FICE 1,2,3                            | FillCam <sup>®</sup> SBI;<br>Given <sup>®</sup> Imaging Ltd.     | <ul style="list-style-type: none"> <li>Mean reading time: 14min for both CE-WL and CE-FICE reading;</li> <li>The two previews for angiodysplasia were significantly superior to the function of SBI (<math>P &lt; 0.01</math>);</li> <li>Sens and Spec of CE-WL: 80% and 100%, respectively;</li> <li>Sens and Spec of CE-FICE: 91% and 86%, respectively;</li> <li>FICE reading was superior in Sens, while it resulted in more false (+) ve lesion findings and lower Spec</li> </ul>   |
| Sakai <i>et al</i> <sup>[20]</sup>      | Japan          | Single centre | Prospective   | Assess whether CE-FICE improves detectability of SB lesions by CE trainees and if it contributes to reducing the bile-pigment effect; Evaluate whether poor bowel preparing affects the accuracy of lesion recognition by FICE | <ul style="list-style-type: none"> <li>4 gastroenterology trainees interpreted 12 CE videos with WL and FICE 1,2,3;</li> <li>Lesion detection rate under each of the three FICE settings was compared with that by conventional CE-WL</li> </ul>   | <ul style="list-style-type: none"> <li>60 AVMs;</li> <li>82 erosions/ulcers</li> </ul>   | FICE 1,2,3<br>Given <sup>®</sup> Imaging Ltd.            | FillCam <sup>®</sup> SBI;<br>Given <sup>®</sup> Imaging Ltd.     | <ul style="list-style-type: none"> <li>60 angioectasias; CE trainees identified: 26 by CE-WL, 40 by CE-FICE1, 38 by CE-FICE2, 31 by CE-FICE3;</li> <li>82 erosions/ulcers, CE trainees identified: 38 by CE-WL, 62 by CE-FICE1, 60 CE-FICE2, 20 by CE-FICE3;</li> <li>CE-FICE 1 and 2 significantly improved detectability of angioectasias (<math>P = 0.0017</math> and <math>P = 0.014</math>, respectively) and erosions/ulcers (<math>P = 0.0012</math> and <math>P = 0.0094</math>, respectively);</li> <li>Detectability of SB lesions by CE-FICE1 was not affected (<math>P = 0.59</math>) by the presence of bile-pigments;</li> <li>Detectability of SB lesions by CE-WL (<math>P = 0.020</math>) and CE-FICE2 (<math>P = 0.0023</math>) was reduced by the presence of bile-pigments;</li> <li>In poor bowel visibility conditions, CE-FICE yielded a high rate of false-positive findings</li> </ul>   |

FICE: Fujinon<sup>®</sup> Intelligent chromoendoscopy enhancement; CE: Capsule endoscopy; SB: Small bowel; WL: White light; OGIB: Obscure gastrointestinal bleeding; SBI: Suspected Blood Indicator; AVM: arterio-venous malformation;  $\kappa$ : Inter-observer agreement; LICS: Lesions of indeterminate clinical significance; Sens: Sensitivity; Spec: Specificity.

**Table 10** Experimental and models in development for capsule-endoscopy the future?

| Ref.                                      | Project  | Status    | Active actuation | Magnetic propulsion | Therapeutic capabilities |
|---|--|-----------|------------------|---------------------|--------------------------|
| Johannessen <i>et al</i> <sup>[124]</sup> | IDEAS: A miniature lab-in-a-pill multi-Sens or microsystem   | Prototype | No               | Yes                 | Yes                      |
| Karagozler <i>et al</i> <sup>[125]</sup>  | Miniature endoscopy capsule robot using biomimetic micro-patterned adhesives   | Prototype | Yes              | No                  | No                       |
| Quirini <i>et al</i> <sup>[126]</sup>     | An approach to capsular endoscopy with active motion   | Prototype | Yes              | No                  | No                       |
| Valdastrì <i>et al</i> <sup>[127]</sup>   | Wireless therapeutic endoscopic capsule: <i>in vivo</i> experiment   | Prototype | No               | Yes                 | Yes                      |
| Glass <i>et al</i> <sup>[128]</sup>       | A legged anchoring mechanism for capsule endoscopes using micro-patterned adhesives                                    | Prototype | Yes              | No                  | No                       |
| Valdastrì <i>et al</i> <sup>[129]</sup>   | An endoscopic capsule robot: a meso-scale engineering case study   | Concept   | Yes              | No                  | No                       |
| Tortora <i>et al</i> <sup>[130]</sup>     | Propeller-based wireless device for active capsular endoscopy in the gastric district                                  | Prototype | Yes              | No                  | No                       |
| Valdastrì <i>et al</i> <sup>[131]</sup>   | A magnetic internal mechanism for precise orientation of the camera in wireless endoluminal applications               | Prototype | No               | Yes                 | No                       |
| Ciuti <i>et al</i> <sup>[132]</sup>       | Robotic magnetic steering and locomotion of capsule endoscope for diagnostic and surgical endoluminal procedures       | Prototype | No               | Yes                 | Yes                      |
| Bourbakis <i>et al</i> <sup>[133]</sup>   | Design of new-generation robotic capsules for therapeutic and diagnostic endoscopy                                     | Concept   | Yes              | No                  | Yes                      |
| Gao <i>et al</i> <sup>[134]</sup>         | Design and fabrication of a magnetic propulsion system for self-propelled capsule endoscope                            | Concept   | No               | Yes                 | No                       |
| Simi <i>et al</i> <sup>[135]</sup>        | Design, fabrication, and testing of a capsule with hybrid locomotion for gastrointestinal tract exploration            | Concept   | No               | Yes                 | No                       |
| Morita <i>et al</i> <sup>[136]</sup>      | A further step beyond wireless capsule endoscopy   | Concept   | No               | Yes                 | No                       |
| Yang <i>et al</i> <sup>[137]</sup>        | Autonomous locomotion of capsule endoscope in gastrointestinal   | Concept   | Yes              | No                  | No                       |
| Filip <i>et al</i> <sup>[138]</sup>       | Electronic stool (e-Stool): A novel self-stabilizing video capsule endoscope for reliable non-invasive colonic imaging | Prototype | Yes              | No                  | No                       |
| Yim <i>et al</i> <sup>[139]</sup>         | Design and rolling locomotion of a magnetically actuated soft capsule endoscope  | Prototype | Yes              | No                  | No                       |
| Kong <i>et al</i> <sup>[140]</sup>        | A robotic biopsy device for capsule endoscopy  | Prototype | Yes              | No                  | Yes                      |
| Woods <i>et al</i> <sup>[141]</sup>       | Wireless capsule endoscope for targeted drug delivery: Mechanics and design considerations                             | Prototype | Yes              | No                  | Yes                      |

Certain issues (*i.e.*, best small-bowel preparation for CE<sup>[143,144]</sup>, occurrence of some potentially life-threatening complications, visualisation quality of the proximal small-bowel) remain open and they will surely be the target of further clinical studies and technical improvements.

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P- Reviewer Driscoll D S- Editor Wen LL  
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## Predominant mucosal expression of 5-HT<sub>4(+h)</sub> receptor splice variants in pig stomach and colon

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Supported by Grant G.0061.08 from the Fund for Scientific Research Flanders

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Received: August 17, 2012 Revised: December 3, 2012

Accepted: December 15, 2012

Published online: June 28, 2013

### Abstract

**AIM:** To investigate cellular 5-HT<sub>4(+h)</sub> receptor distribution, particularly in the epithelial layer, by laser microdissection and polymerase chain reaction (PCR) in porcine gastrointestinal (GI) tissues.

**METHODS:** A stepwise approach was used to evaluate RNA quality and to study cell-specific 5-HT<sub>4</sub> receptor mRNA expression in the porcine gastric fundus and colon descendens. After freezing, staining and laser microdissection and pressure catapulting (LMPC), RNA quality was evaluated by the Experion automated electrophoresis system. 5-HT<sub>4</sub> receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expressions were examined by endpoint reverse transcription (RT)-PCR in mucosal and muscle-myenteric plexus (MMP) tissue fractions, in mucosal and MMP parts of hematoxylin and eosin (HE) stained tissue sections and

in microdissected patches of the epithelial and circular smooth muscle cell layer in these sections. Pig gastric fundus tissue sections were also stained immunohistochemically (IHC) for enterochromaffin cells (EC cells; MAB352); these cells were isolated by LMPC and examined by endpoint RT-PCR.

**RESULTS:** After HE staining, the epithelial and circular smooth muscle cell layer of pig colon descendens and the epithelial cell layer of gastric fundus were identified morphologically and isolated by LMPC. EC cells of pig gastric fundus were successfully stained by IHC and isolated by LMPC. Freezing, HE and IHC staining, and LMPC had no influence on RNA quality. 5-HT<sub>4</sub> receptor and GAPDH mRNA expressions were detected in mucosa and MMP tissue fractions, and in mucosal and MMP parts of HE stained tissue sections of pig colon descendens and gastric fundus. In the mucosa tissue fractions of both GI regions, the expression of h-exon containing receptor [5-HT<sub>4(+h)</sub> receptor] mRNA was significantly higher ( $P < 0.01$ ) compared to 5-HT<sub>4(+h)</sub> receptor expression, and a similar trend was obtained in the mucosal part of HE stained tissue sections. Large microdissected patches of the epithelial and circular smooth muscle cell layer of pig colon descendens and of the epithelial cell layer of pig gastric fundus, also showed 5-HT<sub>4</sub> receptor and GAPDH mRNA expression. No 5-HT<sub>4</sub> receptor mRNA expression was detected in gastric LMPC-isolated EC cells from IHC stained tissues, which cells were positive for GAPDH.

**CONCLUSION:** Porcine GI mucosa predominantly expresses 5-HT<sub>4(+h)</sub> receptor splice variants, suggesting their contribution to the 5-HT<sub>4</sub> receptor-mediated mucosal effects of 5-HT.

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**Key words:** 5-hydroxytryptamine 4 receptors; Pig; Gastric fundus; Colon descendens; Epithelium; Smooth muscle; Laser microdissection and pressure catapulting

Priem EKV, De Maeyer JH, Vandewoestyne M, Deforce D, Lefebvre RA. Predominant mucosal expression of 5-HT<sub>4(hb)</sub> receptor splice variants in pig stomach and colon. *World J Gastroenterol* 2013; 19(24): 3747-3760 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i24/3747.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3747>

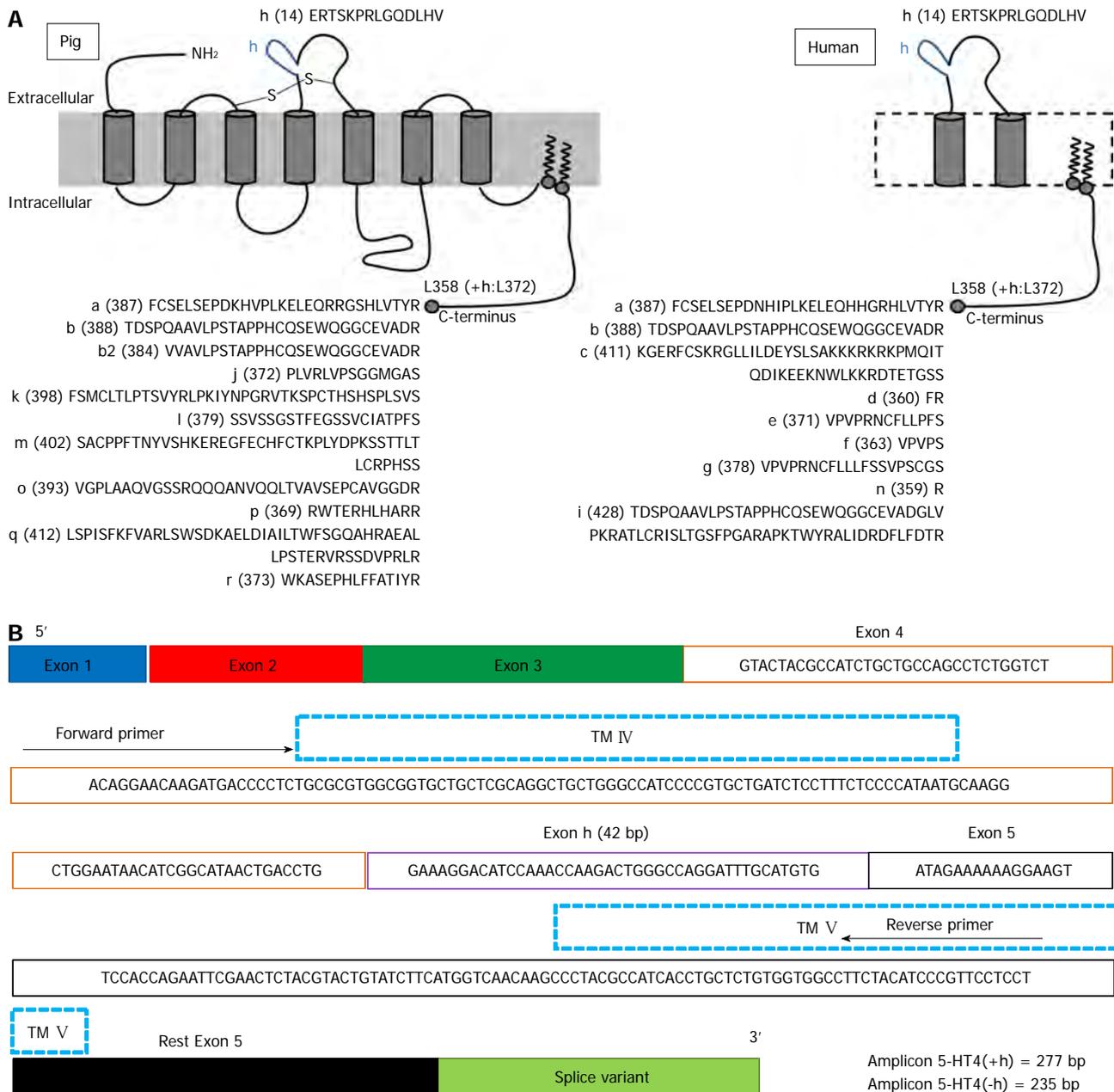
## INTRODUCTION

The 5-HT<sub>4</sub> receptor is a G-protein coupled receptor (GPCR) that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-HT). The 5-HT<sub>4</sub> receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates gastrointestinal (GI) motility<sup>[1-3]</sup>. This presynaptic facilitation is thought to be the principal mechanism for the prokinetic action of 5-HT<sub>4</sub> receptor agonists, explaining their therapeutic use in GI dysmotility-related disorders, such as chronic constipation, gastroparesis and gastroesophageal reflux disease<sup>[4]</sup>. The selective 5-HT<sub>4</sub> receptor agonist prucalopride is now used in patients with chronic laxative-resistant constipation; indeed, it facilitates acetylcholine release from cholinergic neurons towards human colonic circular<sup>[5]</sup>, as well as longitudinal<sup>[6]</sup>, smooth muscle. The non-selective 5-HT<sub>4</sub> receptor agonist cisapride was, until it was withdrawn because of non-specific cardiac side effects, used for increasing gastric emptying in patients with gastroparesis<sup>[7]</sup>. In addition, prucalopride accelerates gastric emptying in humans<sup>[8]</sup>, corresponding with its facilitating effect on acetylcholine release from cholinergic nerves towards human gastric circular muscle<sup>[9]</sup>. Our group has previously shown that the pig is a good model for human 5-HT<sub>4</sub> receptors on GI cholinergic neurons; the presence of facilitatory 5-HT<sub>4</sub> receptors on cholinergic neurons innervating pig gastric circular<sup>[10]</sup> and longitudinal<sup>[11]</sup> muscle and colonic circular muscle<sup>[12]</sup> was illustrated in functional assays.

However, apart from cholinergic neurons, other locations for the 5-HT<sub>4</sub> receptor in the colon and stomach have been proposed. In the human colon, 5-HT<sub>4</sub> receptors were reported to be present on circular smooth muscle cells, inducing relaxation<sup>[13]</sup>. A functional study by Borman *et al*<sup>[14]</sup> in 1996 reported that 5-HT-induced secretion in the human sigmoid colon is mediated *via* 5-HT<sub>2A</sub> receptors; however, in the ascending colon, a combination of 5-HT<sub>2A</sub> and 5-HT<sub>4</sub> receptors appears to be involved. Nevertheless, a recent study showed the presence of mRNA of several 5-HT<sub>4</sub> receptor splice variants in the mucosal layer of the human sigmoid colon<sup>[15]</sup>; 5-HT<sub>4</sub> receptor mRNA was also reported in the pig colonic mucosa<sup>[16]</sup>. In the rat colon, it has been suggested that 5-HT-induced mucosal ion transport and Cl<sup>-</sup> secretion is mediated by 5-HT<sub>4</sub> receptors<sup>[17-20]</sup>. Immunohistochemical and functional assays showed the presence of 5-HT<sub>4</sub> receptors in mouse colonic epithelial cells, including enterochromaffin (EC) cells and goblet cells, inducing mucosal 5-HT release and Cl<sup>-</sup> secretion<sup>[20]</sup>. The presence

of 5-HT<sub>4</sub> receptor transcripts, detected by reverse transcription polymerase chain reaction (RT-PCR), has also been reported in the gastric mucosa of humans<sup>[21,22]</sup> and pigs<sup>[16]</sup>, but cellular distribution within the epithelial layer has not yet been investigated.

More detailed information on the expression and localization of GPCRs, with special attention to the 5-HT<sub>4</sub> receptor, is needed in human enteric neuronal subpopulations, mast cells and epithelial cells, to provide a better understanding of function and activity of 5-HT<sub>4</sub> receptors in the GI wall, which may offer new therapeutic perspectives<sup>[23]</sup>. To date, the majority of information on 5-HT<sub>4</sub> receptor distribution is based on functional studies<sup>[12]</sup> or on 5-HT<sub>4</sub> receptor expression studies using homogenates of tissues<sup>[15,16,21,22]</sup>. However, homogenates of tissues limit the potential of expression studies: important cell-specific transcript information is lost because of the heterogeneity of tissues, such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues<sup>[24]</sup> and laser microdissection has already been used in previously reported gene expression studies to investigate site-specific gene expression. In the laser microdissected enteric ganglia of the human intestine, 5-HT<sub>3A</sub> receptor mRNA expression was described<sup>[25]</sup> and in microdissected human colonic mucosal epithelium, transcripts encoding 5-HT<sub>3A</sub>, 5-HT<sub>3C</sub>, 5-HT<sub>3D</sub> and 5-HT<sub>3E</sub> subunits were detected<sup>[26]</sup>. In different species, 5-HT<sub>4</sub> receptors show splice variation in the intracellular C-terminus starting after the common amino acid structure L358. In humans, nine splice variants have been described (Figure 1A). In pig, at least another nine different splice variants, not described in humans, have been reported (Figure 1A), as well as unique splice variation, with variants composed of duplicated exons<sup>[16]</sup>. Splice variants in the extracellular loops of GPCRs are rare<sup>[27]</sup>; however, the 5-HT<sub>4</sub> receptor can have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (Figure 1A). In humans, this H variant has been described in combination with the b-terminal exon [5-HT<sub>4(hb)</sub>]<sup>[28]</sup>. When comparing the pharmacology of the 5-HT<sub>4(hb)</sub> splice variant, when transiently expressed in cells being CV-1 (simian) in origin, and carrying the SV40 genetic material (COS)-7 cells, with that of the 5-HT<sub>4(b)</sub> and 5-HT<sub>4(a)</sub> splice variant, it showed a smaller fraction of receptors coupled to G-proteins and the 5-HT<sub>4</sub> receptor antagonist GR113808 behaved as a partial agonist<sup>[28]</sup>. In the human GI tract, the h exon could be amplified in combination with the b exon only from the lower esophageal sphincter; however, h exon-carrying 5-HT<sub>4</sub> transcripts were also obtained from other parts of the GI tract, suggesting that the h-exon might be expressed in combination with other C-terminal exons<sup>[28]</sup>. In pigs, De Maeyer *et al*<sup>[16]</sup> showed that the 5-HT<sub>4(h)</sub> splice variant also exists in combination with C-terminal



**Figure 1 Schematic representation.** A: The porcine and human 5-HT<sub>4</sub> receptor splice variants; B: cDNA of the porcine 5-HT<sub>4(hb)</sub> receptor based on gene browsing (transcript ID: ENSSSCT0000015770 on [http://www.ensembl.org/Sus\\_scrofa/Gene](http://www.ensembl.org/Sus_scrofa/Gene)). The 5-HT<sub>4</sub> receptor variants have identical sequences up to Leu<sup>358</sup>, or Leu<sup>372</sup> when exon h is included, and differ by the length and composition of their C-terminal domain. The presence of the h sequence of 14 amino acids in the second extracellular loop depends on the all or none inclusion of exon h between exon 4 and 5. The positions of the boundaries in between exons, the transmembrane (TM) domains IV and V and the positions of primers used in this study are indicated. The primers will detect all 5-HT<sub>4</sub> receptor splice variants. Amplification will result in a 277 bp amplicon, when containing the 42 bp h-exon or a 235 bp amplicon, not containing the h-exon.

exons other than 5-HT<sub>4(b)</sub>, namely 5-HT<sub>4(ha)</sub>, 5-HT<sub>4(hm)</sub> and 5-HT<sub>4(hr)</sub>. H-exon containing 5-HT<sub>4</sub> transcripts were also found along the porcine GI tract, with predominant expression in the mucosal layer. Therefore, the aim of the present study was to develop and validate an experimental protocol for the assessment of 5-HT<sub>4</sub> receptor distribution (with and without the h exon) at the cellular level in laser microdissected porcine GI tissues, paying special attention to the mucosal layer of pig colon descendens and gastric fundus.

## MATERIALS AND METHODS

### Tissue preparation and tissue processing

Young male pigs (10-12 wk, 15-25 kg-breed Line 36) were obtained from Rattlerow Seghers, Lokeren Belgium. The Ethical Committee for Animal Experiments from the Faculty of Medicine and Health Sciences at Ghent University approved all the experimental procedures.

The pigs were anaesthetized with an intramuscular injection of 5 mL Zoletil 100 (containing 50 mg/mL ti-

letamine and 50 mg/mL zolazepam; Virbac Belgium SA, Heverlee, Belgium). After exsanguination, the stomach and the colon descendens, prelevated 10 cm above the anus to the transverse colon, were removed and thoroughly washed in ice cold aerated (5% CO<sub>2</sub>/95% O<sub>2</sub>) phosphate buffered saline (PBS) at pH 7.4 (Life Technologies Europe, Ghent, Belgium). The gastric fundus was cut open along the lesser curvature and small pieces of tissue were cut in the direction of the circular muscle layer from the ventral side. The colon descendens was opened along the mesenteric border, fat tissue was removed and tissues were cut in the direction of the circular muscle layer.

#### Freezing tissue fractions for direct RNA processing:

The GI tissues were divided by blunt dissection into a mucosal-submucosal (mucosa) fraction and a muscular-myenteric plexus (MMP) fraction. The fractions were cut into small pieces, put in an RNase-free vial (Life Technologies Europe), rapidly frozen in liquid N<sub>2</sub> and stored at -80 °C. After frozen tissue homogenization and before RNA extraction, MMP samples were treated with proteinase K (Qiagen, Antwerp, Belgium) to increase the total RNA output. Proteinase K removes proteins such as the contractile proteins, connective tissue and collagen, which define a fibrous tissue such as the smooth muscle layer (Rneasy fibrous tissue handbook, Qiagen). RNA from mucosa and MMP fractions was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's guidelines and RNA samples were stored at -80 °C.

#### Freezing tissues for section preparation and laser microdissection:

Whole tissues, containing the mucosal and the smooth muscle layers were cut into full-thickness small pieces with a sterile scalpel, placed in tissue embedding medium PELCO CryO-Z-T (Pelco International, CA, United States), rapidly frozen in liquid N<sub>2</sub> containing cold isopentane and stored at -80 °C. The frozen tissue samples were cut into 8 µm-thick sections using a cryostat (Leica CM 1950; Leica Microsystems, Diegem, Belgium) with disposable RNase-free knives. Sections of 8 µm thickness are considered to represent a monolayer of cells<sup>129,301</sup>. The sections were placed on chilled (-20 °C) nuclease free polyethylene naphthalate-covered membrane slides (Carl Zeiss, Oberkochen, Germany) and immediately stored at -80 °C until the staining procedure. The membrane slides used for immunohistochemistry were extra coated with poly-L-Lysine (Sigma, Bornem, Belgium), which was diluted with 0.1% diethylpyrocarbonate (DEPC)-treated water. All materials (pincers, brushes) were treated with RNase ZAP (Sigma) and glassware and pincers were heated for 6 h at 200 °C, to remove all exogenous RNases.

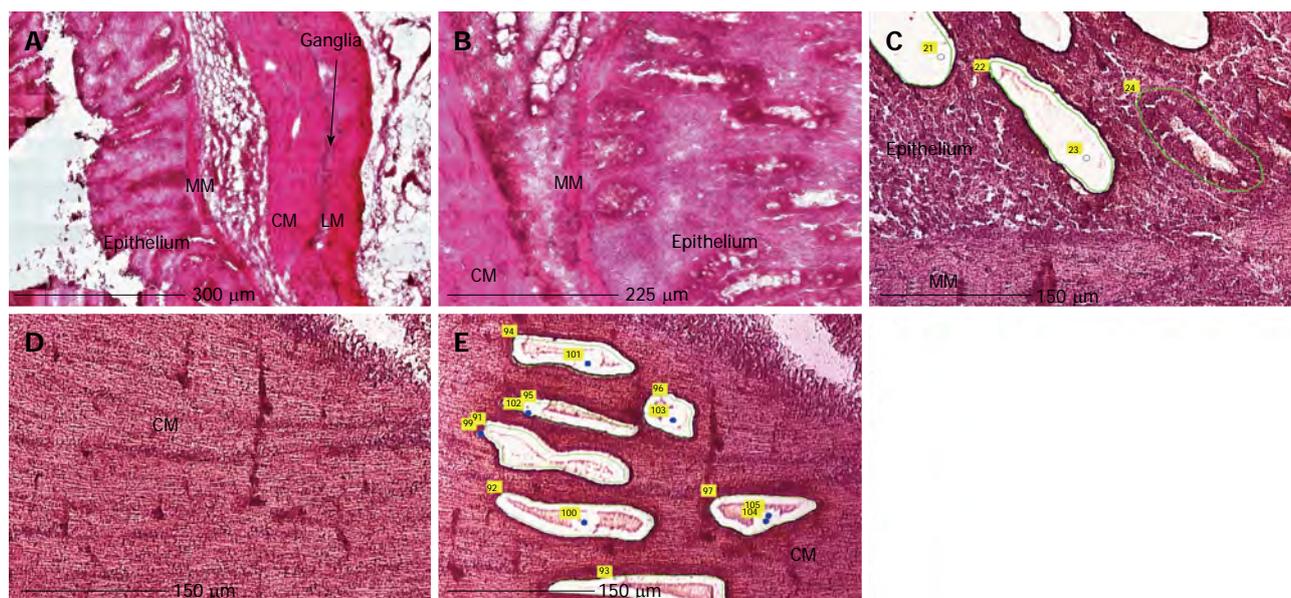
To distinguish morphologically the different layers of the tissue sections for laser microdissection, the frozen tissue sections were stained with hematoxylin and eosin (Sigma) in RNase-free conditions. Hematoxylin and eosin (HE) staining started with fixing the slides in 70%

ethanol for 1 min, followed by dipping the slides for 15 s in DEPC-treated water to remove PELCO CryO-Z-T embedding medium. Hematoxylin staining was carried out by placing the slides for 1 min in the hematoxylin solution (0.1%), followed by dipping the slides for 15 s in DEPC-treated water and 15 s in 70% ethanol. Slides were then placed for 1 min in eosin solution (0.25%), followed by dehydrating the slides for 15 s in the following order: DEPC-treated water, 70% ethanol, 100% ethanol. The staining procedure was finished with a 3 min xylene treatment and the slides were air dried for 10 min at room temperature, before scraping off the whole tissue section, or the mucosal and MMP part of the tissue section separately, or applying laser microdissection. Staining solutions based on ethanol and xylene were pre-cooled at -20 °C; aqueous solutions were pre-cooled at 4 °C. All solutions were diluted with 0.1% DEPC-treated water, kept in 50 mL RNase-free conical tubes (Life Technologies Europe) and kept on ice during the staining procedure.

**Immunohistochemistry:** To distinguish and isolate EC cells using the laser microdissection and pressure catapulting (LMPC) technique, visualization with cell-specific antibodies of these cells is required. To extract intact RNA of the cell samples, an immunohistochemically (IHC) protocol under RNase-free conditions was developed according to the staining procedure reported by Brown *et al.*<sup>241</sup>. Cryosections were rinsed for 15 s with cold (4 °C) PBS (pH 7.4; Life Technologies Europe) and then fixed for 5 min in ice-cold (-20 °C) acetone. Acetone was removed by a cold PBS rinse (15 s) and slides were incubated for 30 min at 4 °C with blocking buffer (0.25% Triton X-100, 1% bovine serum albumin, 10% goat serum) supplemented with 1 mol/L NaCl. Then, sections were briefly rinsed with cold PBS and incubated overnight at 4 °C with the rat anti-serotonin primary antibody MAB352 (Milipore, Overijse, Belgium), used as a marker for EC cells. MAB352 was diluted 1:200 in PBS supplemented with 1 mol/L NaCl. Unbound primary antibody was removed by rinsing three times with cold PBS supplemented with 1 mol/L NaCl. Sections were then incubated with chicken anti-rat secondary antibody Alexa Fluor 488 (Life Technologies Europe) diluted 1:100 in PBS with 1 mol/L NaCl for 2 h at 4 °C. Unbound secondary antibody was removed by rinsing three times with cold PBS with 1 mol/L NaCl and excess NaCl was removed by a PBS rinse (5 s). Sections were dehydrated in 70% and then 100% ethanol (3 min each) and air dried for 10 min at room temperature before laser microdissection.

#### Laser microdissection and pressure catapulting

LMPC was performed using the laser microdissection system from PALM Technologies (Carl Zeiss) containing a PALM Microbeam, RoboStage and a PALM RoboMover (PALM RoboSoftware version 4). Under direct microscopic visualization, LMPC permits the procurement of histologically or immunohistologically defined



**Figure 2** Photomicrographs of hematoxylin and eosin stained tissue sections of colon descendens: epithelium, muscularis mucosae, circular muscle layer, longitudinal muscle layer and ganglion. A: Overview of all layers in colon descendens; B: Detail of the epithelium; C: Epithelium with large patches microdissected by LMPC; D: Details of CM; E: CM with large patches of smooth muscle cells microdissected by LMPC. MM: Muscularis mucosae; CM: Circular muscle layer; LM: Longitudinal muscle layer; LMPC: Laser microdissection and pressure catapulting.

tissue and cell samples (Figure 2). Approximately 15 large patches of cells from the epithelium or circular smooth muscle layer in HE stained sections, or 70 EC cells in IHC stained sections were laser-dissected and pressure-catapulted in 50  $\mu$ L RLT lysis buffer (RNeasy kit, Qiagen). The cell collecting time was limited to 2 h per slide and after 2 h of cell sampling, the remaining tissue on the membrane slide was scraped off and RNA was extracted to determine if RNA integrity was preserved after 2 h. The samples were homogenized by vortexing, centrifuged and then placed at  $-80^{\circ}\text{C}$  for later use. Seven EC cell collections were pooled into one sample with a final volume of 350  $\mu$ L, resulting in a collection of approximately 500 cells per sample. Total RNA from the cell samples was extracted using the RNeasy Micro kit (Qiagen), according to the manufacturer's instructions.

#### Endpoint RT-PCR

Quantification of RNA was determined using a Nanodrop ND-1000 spectrophotometer (Isogen Life Science, Temse, Belgium) and the quality of RNA extracted from tissue fractions and tissue sections was assessed using the Experion automated electrophoresis system (BioRad, Nazareth Eke, Belgium).

cDNA of tissue fractions was prepared from 1  $\mu$ g total RNA, whereas cDNA of whole tissue sections, parts of tissue sections and LMPC samples was prepared from the maximal input of total RNA as possible, as the amount of total RNA was less than 1  $\mu$ g. The production of cDNA from sample RNA by RT was carried out according to manufacturer's instructions, using SuperScript III Reverse Transcriptase SuperMix (Life Technologies Europe) containing random hexamers and oligo (dT)<sub>20</sub>. The obtained cDNA was stored at  $-20^{\circ}\text{C}$  before PCR.

cDNA amplification reactions were carried out using the AccuPrime Pfx SuperMix (Life Technologies Europe). The template cDNA of mucosa and MMP tissue fractions for amplification was diluted 1:10. Expression of the 5-HT<sub>4</sub> receptor within the samples was analysed using 5-HT<sub>4</sub> receptor-specific primers spanning exon-intron junctions: an exon 4-specific forward primer and an exon 5-specific reverse primer (Figure 1B). These primers will detect alternative splicing of the h-exon because the h-exon is located between exon 4 and exon 5. The quality of cDNA produced was assessed by amplifying cDNA for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To amplify cDNA of tissue fractions and tissue sections, PCR reactions were performed using the following protocol: 5 min at  $95^{\circ}\text{C}$ , followed by 36 cycles with annealing temperature of  $54^{\circ}\text{C}$ . The LMPC samples had a low RNA output; therefore, PCR reactions to amplify the cDNA for both 5-HT<sub>4</sub> receptor and GAPDH, were performed using two rounds of PCR<sup>[15]</sup>, according to the following protocol: a first reaction of 5 min at  $95^{\circ}\text{C}$  followed by 36 cycles at  $54^{\circ}\text{C}$  annealing temperature, followed by a second reaction using 1.5  $\mu$ L of the product of the first reaction as a template for a second round of PCR (5 min at  $95^{\circ}\text{C}$  followed by 36 cycles) with the same primers, but with a higher annealing temperature of  $56^{\circ}\text{C}$  to increase specificity. RT and endpoint PCR reactions were processed on a C1000 Thermal Cycler (BioRad). PCR products were separated by 2% agarose gel electrophoresis and visualised by ethidium bromide staining. The primers (Eurogentec, Seraing, Belgium) used were published previously by De Maeyer *et al.*<sup>[16]</sup>: 5-HT<sub>4</sub>R forward primer (5'-ACAGGAA-CAAGATGACCCCT-3'); 5-HT<sub>4</sub>R reverse primer (5'-AG-GAGGAACGGGATGTAGAA-3'); GAPDH forward

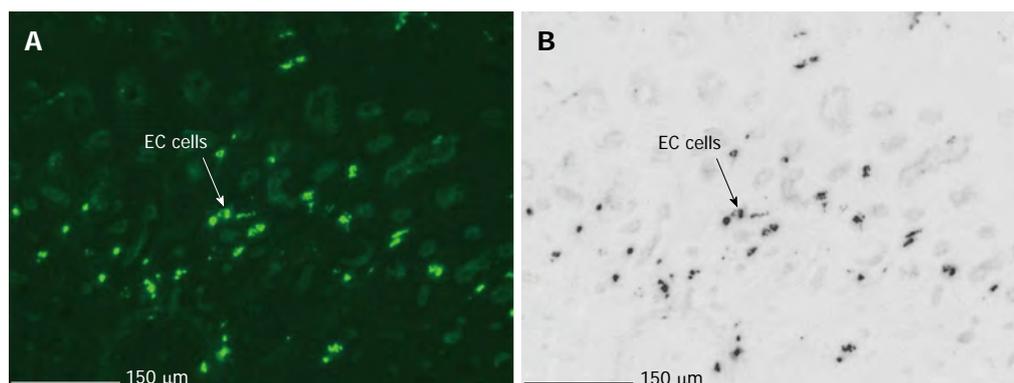


Figure 3 Photomicrographs of immunohistologically stained 8  $\mu\text{m}$  sections of pig gastric fundus showing. A: MAB352 (1:200) immunofluorescent enterochromaffin cells (EC) in the epithelium ( $\times 20$ ); B: Invert color visualization of MAB352 (1:200) immunofluorescent EC cells.

primer (5'-ACCACAGTCCATGCCATCAC-3'); GAPDH reverse primer (5'-TCCACCTGTTGCTGTA-3').

### Statistical analysis

Semi-quantification of PCR products was determined by the intensity of PCR bands on the agarose gels using Image J 1.45 software. Band intensity was expressed as relative absorbance units, and the background of the image was determined and subtracted from the gel image. The ratio between the 5-HT<sub>4</sub> receptor and GAPDH RNA was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Data presented are mean  $\pm$  SE of the mean for animals ( $n$ ). Statistical analyses were performed using Graphpad Prism software v.5.01 (United States). Differences in intensity were determined by an unpaired  $t$  test;  $P < 0.05$  was considered statistically significant.

## RESULTS

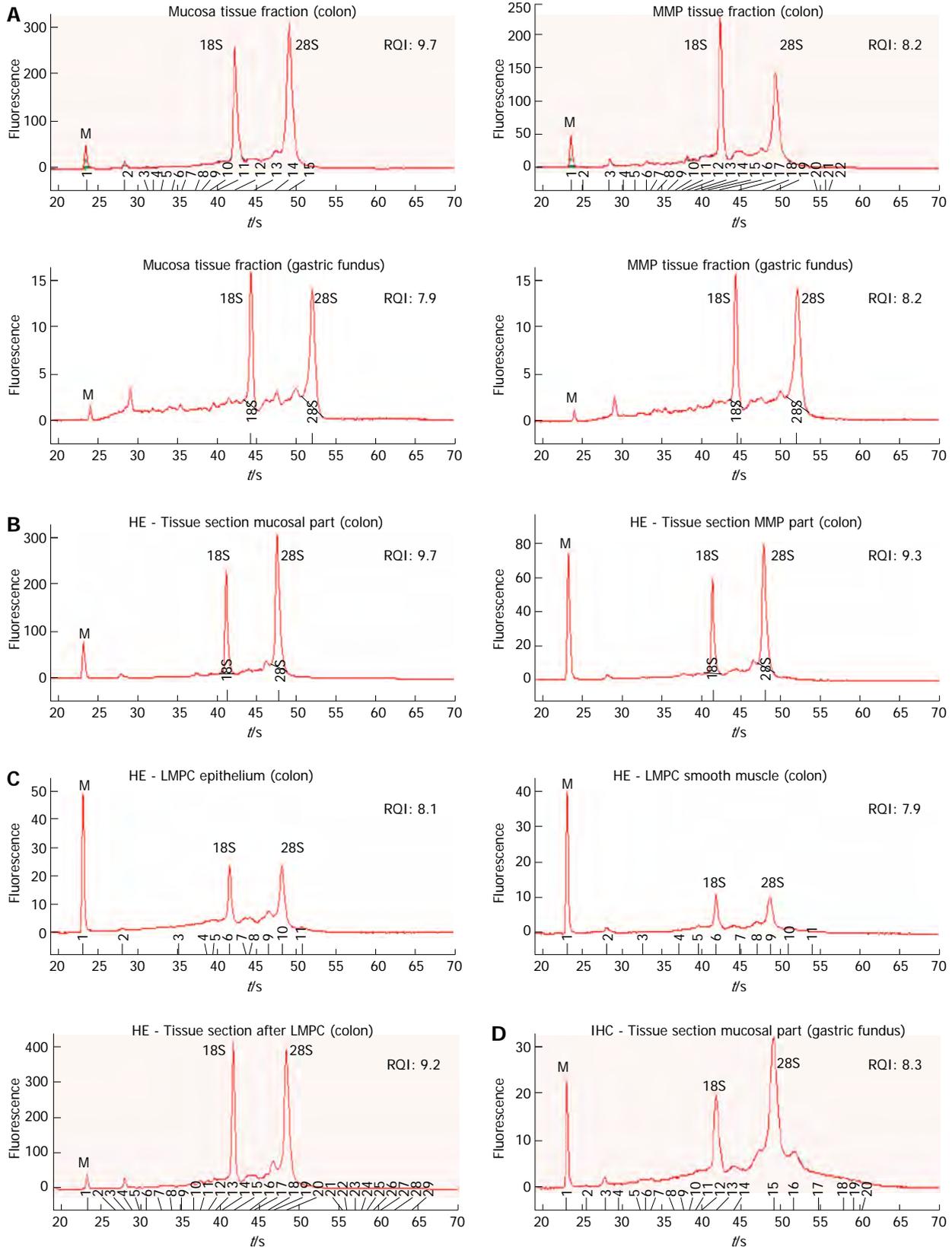
### Evaluating cell-specific visualization and RNA integrity

The main difficulties, when using LMPC to analyse gene expression of a specific cell type, are efficient and selective isolation of the desired cells, and obtaining RNA of good quality. Therefore, optimization of the LMPC experimental design was needed for the pig GI tissues, which are highly heterogeneous and rich in endogenous RNase and other enzymes. First, to select the desired cells from the heterogeneous GI tissues, a good visualization of the tissue layers and cells under direct microscopy was necessary. This requires that the morphology of the tissue be preserved, because fractures and air bubbles within the specimen will hamper the view. Tissues for section preparation and LMPC were therefore frozen in liquid N<sub>2</sub> containing isopentane. After HE staining, the different layers of colon descendens (Figure 2) and gastric fundus (not shown) could be identified based on their morphological characteristics. After cell-specific IHC staining, MAB352-immunoreactive EC cells (Figure 3) were present in the crypts, villi and epithelial lining of the mucous membrane of the gastric fundus and were isolated by LMPC.

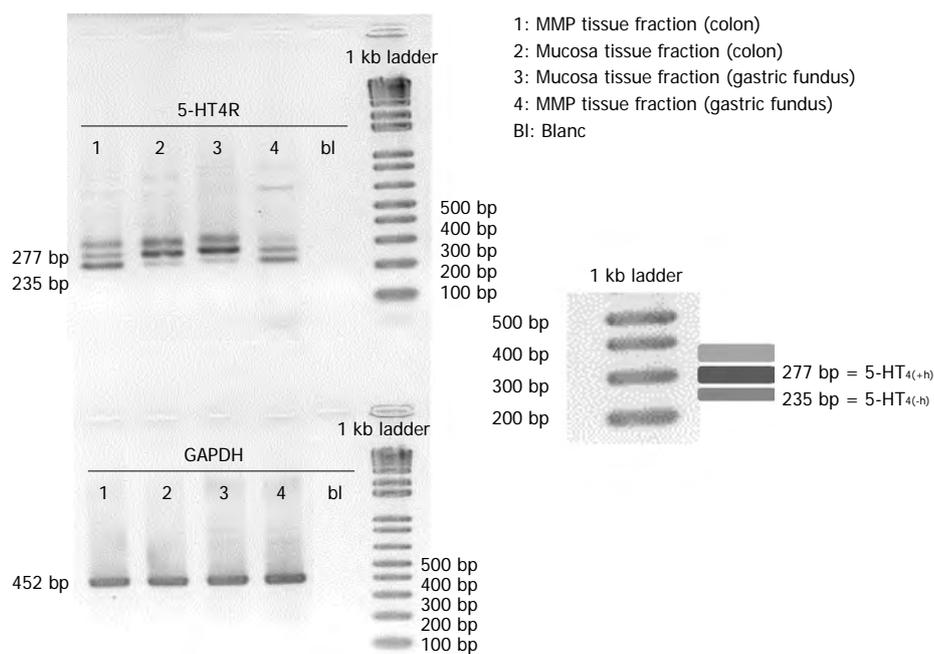
To evaluate the impact of the different protocol steps on RNA integrity, a systematic approach was followed by evaluating RNA yield and quality after each protocol step. RNA quality was assessed by comparing 28S and 18S and pre-18S ribosomal peaks to a set of degradation standards using the Experion automated electrophoresis system, where the RNA quality indicator (RQI) returns a number between 10 (intact RNA) and 1 (highly degraded RNA)<sup>[31]</sup>. The analysis showed that RNA quality was not affected after tissue fraction collection (Figure 4A), HE staining (Figure 4B), LMPC (Figure 4C) or after IHC staining (Figure 4D). However, electropherograms of RNA collected by LMPC could not be analysed systematically because the amount of RNA collected was too low. In Figure 4C, RNA quality from large microdissected patches of either epithelial or smooth muscle cells is shown, indicating that RNA was remained mostly intact, but the small ribosomal RNA peaks (18S/28S) indicate a low amount of RNA.

### Expression of the 5-HT<sub>4</sub> receptor in porcine tissue fractions

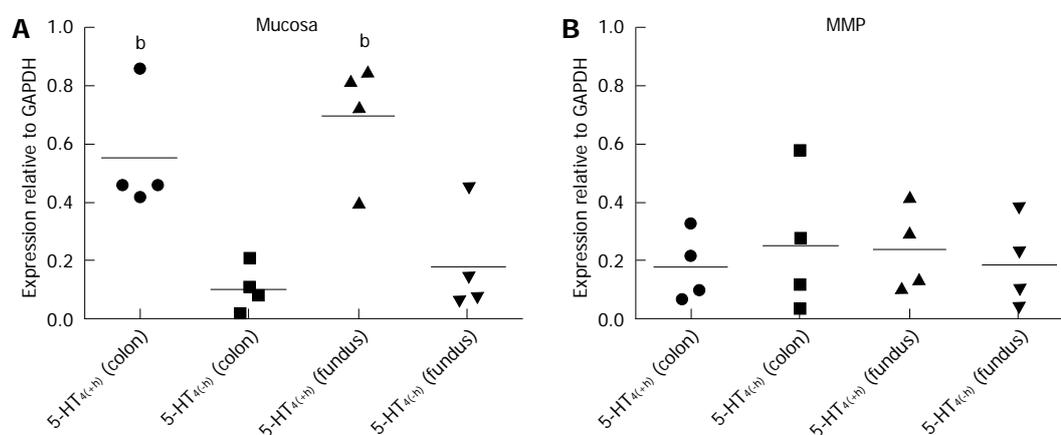
Tissue samples from pig colon descendens and gastric fundus were dissected into the mucosa fraction and the MMP fraction before freezing in liquid N<sub>2</sub>. After RNA extraction and endpoint PCR analysis of these fractions, 5-HT<sub>4</sub> receptor expression was detected in mucosa as well as MMP fractions of the colon descendens and gastric fundus (Figure 5). All tissue samples were positive for GAPDH, confirming the integrity of the samples and completed PCR reactions. In all samples, 5-HT<sub>4</sub> receptors containing the h-exon [277 bp; 5-HT<sub>4(+h)</sub> receptor] as well as 5-HT<sub>4</sub> receptors without the h-exon [235 bp; 5-HT<sub>4(-h)</sub> receptor] were present (Figure 5). However, a third band, corresponding to a fragment of more than 300 bp was also observed. Therefore, we isolated this unknown PCR band using a QIA quick Gel extraction kit (Qiagen) and determined its DNA sequence with an ABI3130XL sequencer (Life Technologies Europe). After sequence analysis, we aligned the unknown sequence with the 5-HT<sub>4(+h)</sub> and 5-HT<sub>4(-h)</sub> receptor sequences and observed that the unknown band contained the same sequence and



**Figure 4** Representative experion electropherograms of collected RNA. A: Mucosa and MMP tissue fractions of colon descendens and gastric fundus; B: The mucosal part and the MMP part of HE stained tissue sections of colon descendens; C: Large patches of epithelial cells and smooth muscle cells obtained by LMPC from HE stained tissue sections and the whole HE stained tissue section scraped off after LMPC in colon descendens; D: The mucosal part of an IHC stained tissue section of gastric fundus. Electropherograms show fluorescence (ordinate) vs time (abscissa) with RQI values. Positions of 18S and 28S ribosomal RNA and marker (M) peaks are indicated. MMP: Muscle-myenteric plexus; HE: Hematoxylin and eosin; IHC: Immunohistochemically; RQI: RNA quality indicator.



**Figure 5** Invert color image of endpoint polymerase chain reaction analysis of the 5-HT<sub>4</sub> receptor and of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene expressed in mucosa and muscle-myenteric plexus tissue fractions of pig colon descendens and gastric fundus. Dominant expression of 5-HT<sub>4</sub>(+h) receptor in the mucosa of the colon descendens and gastric fundus is observed. Part of the ladder is increased to indicate the size of the expected polymerase chain reaction (PCR) products. A third unknown upper band is shown above the 277 bp band, due to dimerization of the PCR product with other PCR fragments after the PCR reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MMP: Muscle-myenteric plexus.



**Figure 6** Expression of 5-HT<sub>4</sub>(+h) and 5-HT<sub>4</sub>(-h) receptors in the mucosa (A) and muscle-myenteric plexus (B) fractions of colon descendens and gastric fundus. Data are given as ratio relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The line indicates the mean of  $n = 4$  for each region-fraction. <sup>b</sup> $P < 0.01$  vs values for 5-HT<sub>4</sub>(-h) receptors in colon descendens or gastric fundus. MMP: Muscle-myenteric plexus.

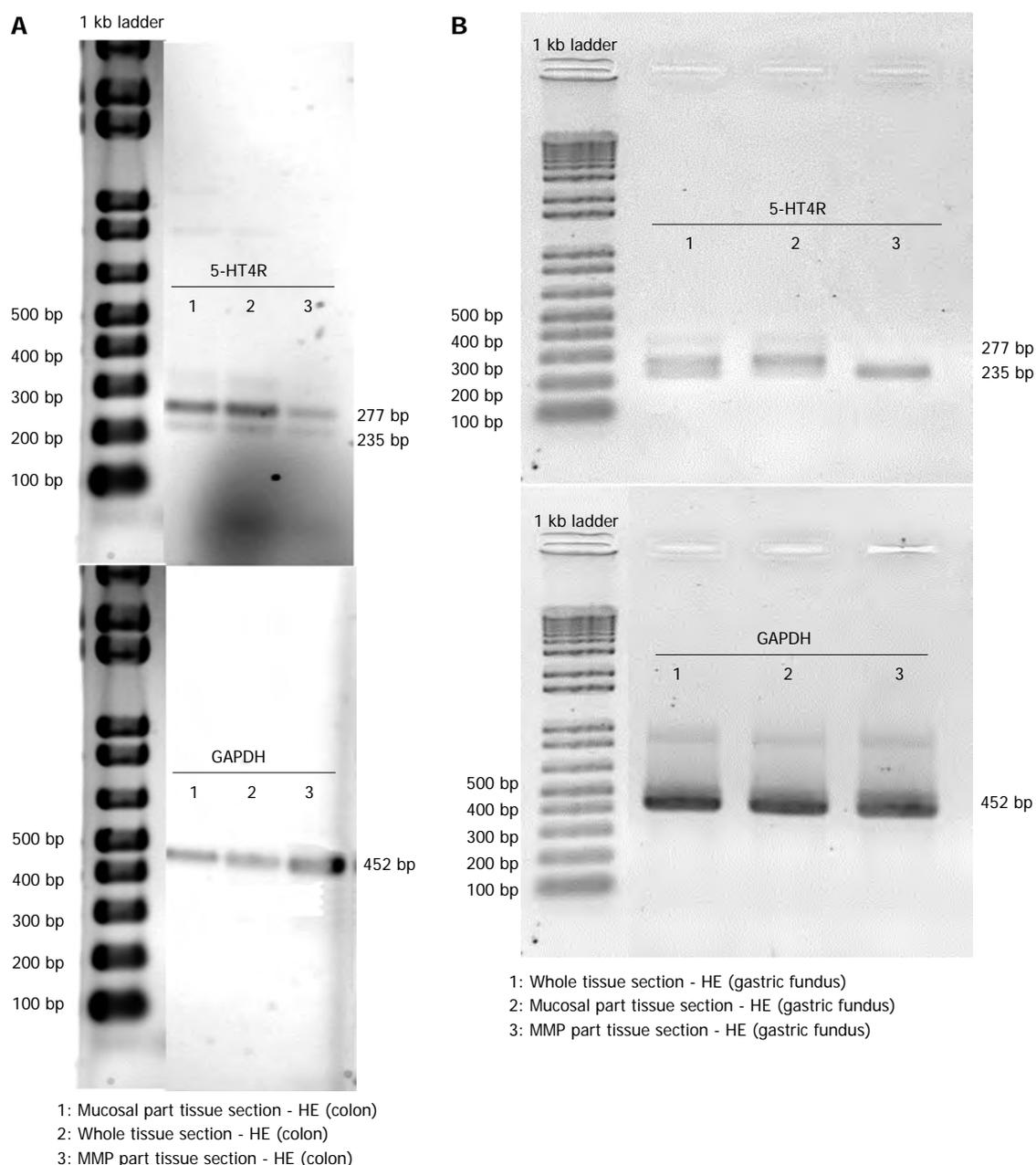
length as the 5-HT<sub>4</sub>(+h) receptor, but chromatogram details suggested the presence of additional nucleotides in the tail of the sequence, possibly caused by the formation of a heteroduplex with a 5-HT<sub>4</sub>(+h) strand and a 5-HT<sub>4</sub>(-h) strand, or formation of a triplex with other PCR fragments, resulting in a different electrophoretic separation.

Mucosa fractions of the colon descendens and gastric fundus contained relatively more h-exon containing 5-HT<sub>4</sub> receptors. Semi-quantification by expressing the intensity of the bands compared with the intensity of GAPDH and statistical analysis (Figure 6), confirmed the significantly ( $P < 0.01$ ) more pronounced expression of 5-HT<sub>4</sub>(+h) receptor within the mucosa fractions (the ratio vs GAP-

DH was  $0.55 \pm 0.10$  in the colon descendens and  $0.69 \pm 0.13$  in the gastric fundus;  $n = 4$ ) compared to the expression of the 5-HT<sub>4</sub>(-h) receptor (colon descendens:  $0.12 \pm 0.03$ ; gastric fundus:  $0.21 \pm 0.10$ ;  $n = 4$ ). Within the MMP fraction of the colon descendens and gastric fundus there was no difference in expression of the 5-HT<sub>4</sub>(+h) receptor (colon descendens:  $0.18 \pm 0.06$ ; and gastric fundus:  $0.18 \pm 0.05$ ;  $n = 4$ ) and 5-HT<sub>4</sub>(-h) receptor (colon descendens:  $0.26 \pm 0.12$ ; gastric fundus:  $0.17 \pm 0.09$ ;  $n = 4$ ).

**Expression of the 5-HT<sub>4</sub> receptor in porcine HE stained tissue sections**

Whole tissue sections, and the mucosal or MMP part of



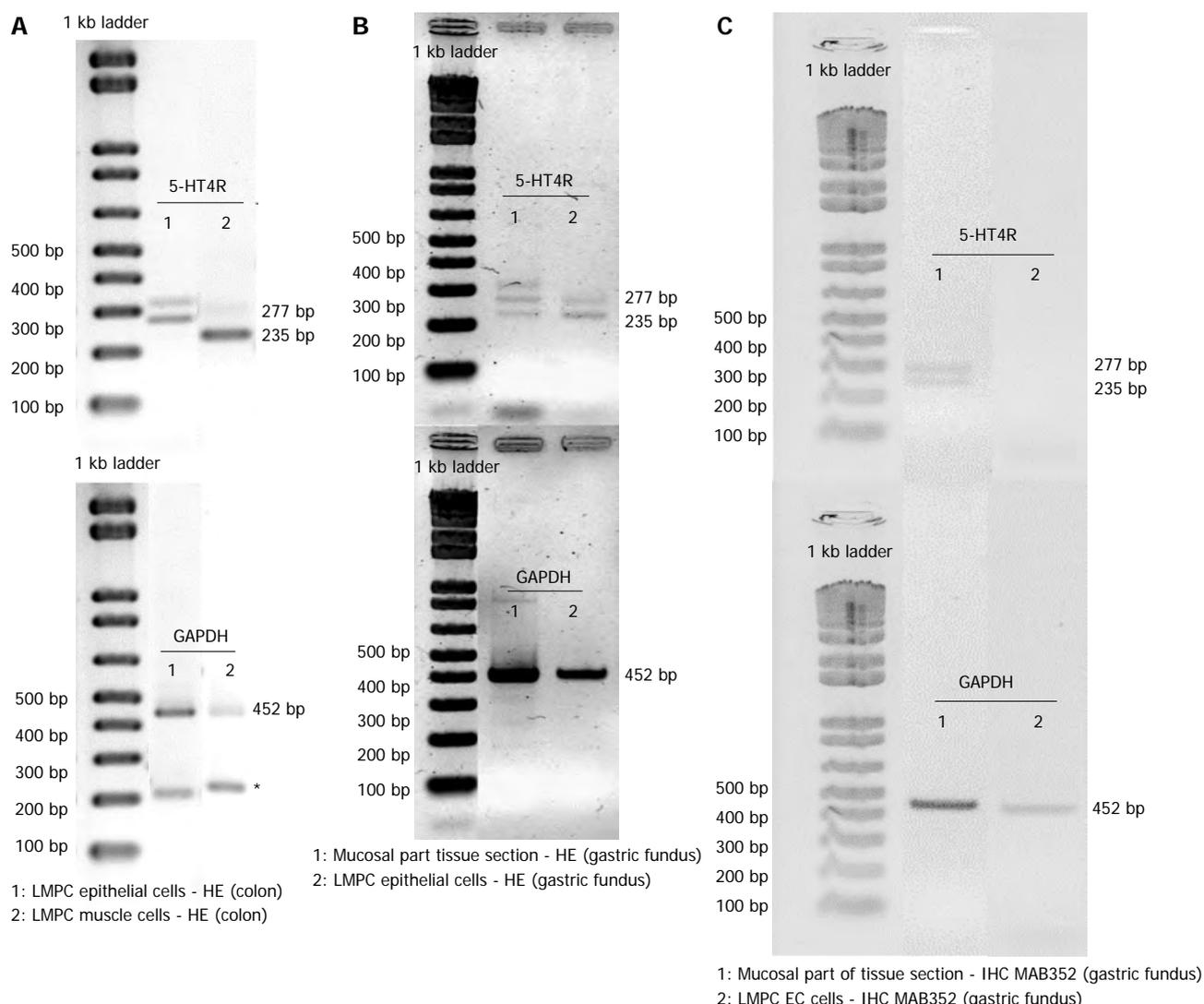
**Figure 7** Invert color image of endpoint polymerase chain reaction analysis. 5-HT<sub>4</sub> receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in a hematoxylin and eosin (HE) stained whole tissue section, and in the mucosal as well as the muscle-myenteric plexus (MMP) part of a HE stained tissue section of colon descendens (A) and gastric fundus (B). The size of the expected polymerase chain reaction products is indicated.

tissue sections of the colon descendens and gastric fundus were scraped off a membrane slide and 5-HT<sub>4</sub> receptor expression was analyzed.

**Colon descendens:** 5-HT<sub>4</sub> receptor and GAPDH expressions were detected in the whole tissue sections, and in the mucosal and MMP parts of tissue sections of the colon descendens (Figure 7A). After semi-quantification, the values for 5-HT<sub>4</sub> receptor expression were 5-HT<sub>4(+h)</sub>R  $1.21 \pm 0.66$  and 5-HT<sub>4(-h)</sub>R  $0.63 \pm 0.45$  within whole tissue sections ( $n = 3$ ); 5-HT<sub>4(+h)</sub>R  $0.86 \pm 0.39$ , 5-HT<sub>4(-h)</sub>R  $0.28 \pm 0.14$  within the mucosal part of tissue sections ( $n = 3$ ); and 5-HT<sub>4(+h)</sub>R  $0.47 \pm 0.05$ , 5-HT<sub>4(-h)</sub>R  $0.24 \pm 0.11$  within the MMP part of tissue sections ( $n = 3$ ). The

tendency for more pronounced expression of the h-exon containing splice variant did not reach significance.

**Gastric fundus:** 5-HT<sub>4</sub> receptor and GAPDH expression was also detected in the whole tissue sections, and mucosal and MMP parts of tissue sections of the gastric fundus (Figure 7B). After semi-quantification, expression values were 0.51 and 0.19 for 5-HT<sub>4(+h)</sub>R, 0.23 and 0.11 for 5-HT<sub>4(-h)</sub>R in whole tissue sections, 1.13 and 0.24 for 5-HT<sub>4(+h)</sub>R and 0.31 and 0.05 for 5-HT<sub>4(-h)</sub>R in the mucosal part of tissue sections. In the two samples with the MMP part of tissue sections, the 5-HT<sub>4(-h)</sub> receptor was found in both (0.24 and 0.45), while the 5-HT<sub>4(+h)</sub> receptor was only found in one of the samples (0.53) (Figure 7B



**Figure 8** Invert color representation of the double round of endpoint polymerase chain reaction analysis. A, B: 5-HT<sub>4</sub> receptor and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression is shown in large patches of epithelium cells and smooth muscle cells obtained by laser microdissection and pressure catapulting (LMPC) from hematoxylin and eosin (HE) stained tissue sections of colon descendens (A) and gastric fundus (B, only epithelial cells were obtained), and in enterochromaffin cells obtained by LMPC from MAB352 immunohistochemical (IHC) stained tissue sections of gastric fundus (C). For comparison, the result obtained in the mucosal part of the HE or IHC stained tissue section of gastric fundus is also shown in B and C. The size of the expected polymerase chain reaction products and presence of non-specific amplification are indicated (asterisk).

lane 3, showing the result with only the 5-HT<sub>4(h)</sub> receptor detected).

**Expression of the 5-HT<sub>4</sub> receptor in LMPC-isolated cell populations from HE stained porcine tissue sections**

After HE staining, large microdissected patches of cells were taken from the epithelium and the circular smooth muscle layer of the colon descendens and from the epithelium of the gastric fundus. Low RNA yields meant that only after a second round of PCR, with the same 5-HT<sub>4</sub> receptor specific-primers, was 5-HT<sub>4</sub> receptor expression detected in large microdissected patches of epithelial cells (Figure 8A and B) or smooth muscle cells (Figure 8A). Non-specific amplification was occasionally observed because of the high number of cycles (Figure 8A). In the colon descendens (Figure 8A), higher expression of the 5-HT<sub>4(+h)</sub> receptor was observed com-

pared with the 5-HT<sub>4(h)</sub> receptor within the LMPC-isolated epithelial cells [5-HT<sub>4(+h)</sub>R 1.03 and 0.85, 5-HT<sub>4(h)</sub>R 0.24 and 0.11], while the opposite occurred for the smooth muscle cells [5-HT<sub>4(+h)</sub>R 1.14 and 0.91, 5-HT<sub>4(h)</sub>R 5.07 and 4.03]. In the gastric fundus epithelial cells (Figure 8B), the expression appeared similar for the 5-HT<sub>4(+h)</sub>R (0.45 and 0.20) and 5-HT<sub>4(h)</sub>R (0.67 and 0.24).

**Expression of the 5-HT<sub>4</sub> receptor in LMPC-isolated EC cells from IHC stained porcine tissue sections**

After IHC staining, EC cells (MAB352; Figure 8C), were isolated by LMPC from gastric fundus tissue sections. After a second round of PCR with the same 5-HT<sub>4</sub> receptor specific-primers, no 5-HT<sub>4</sub> receptor expression was detected in LMPC-isolated EC cells, although the cells were positive for the *GAPDH* gene, confirming the integrity of the samples and completed PCR reactions at

least for GAPDH. Additionally, in the mucosal part (Figure 8C) of IHC stained tissue sections, scraped off after LMPC of EC cells, 5-HT<sub>4</sub> receptor and GAPDH expressions were detected.

## DISCUSSION

The aim of this study was to investigate the 5-HT<sub>4</sub> receptor distribution in the pig GI tract by confining gene expression analysis to site-specific regions of interest, with special attention being paid to the mucosal layer of the pig colon descendens and gastric fundus by isolating epithelial cells using the LMPC technique. A stepwise approach was used by first studying mucosal and MMP tissue fractions, then tissue sections where different cell layers were discerned morphologically by HE staining, and finally tissue sections stained for a particular cell type by IHC. The impact of the freezing method and staining method on the RNA quality was evaluated in mucosa and MMP tissue fractions (Figure 4A), and mucosal and MMP parts of HE stained tissue sections (Figure 4B) of the pig colon descendens and gastric fundus. The major advantages of LMPC are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity<sup>[32]</sup>. The main obstacles, when using LMPC to analyse 5-HT<sub>4</sub> receptor mRNA expression in different cell types, are firstly to recognize the cells of interest and secondly to obtain RNA of good quality. To recognize the cells of interest while preserving RNA integrity, an HE protocol and a cell-specific IHC protocol were developed in RNase-free conditions. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standardized IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling; however, long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation<sup>[33]</sup>. Our attempts to develop a fast IHC protocol resulted in diminished visualization because of the short antibody labeling time. Therefore, our IHC staining protocol was based on the report published by Brown *et al.*<sup>[24]</sup>, where overnight antibody incubation and RNA integrity could be maintained with the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although we can speculate that normal saline protects the integrity of cell membranes, which may prevent the release of intracellular RNases<sup>[34]</sup>. Our results confirm the preservation of RNA after HE staining (Figure 4B) as well as after overnight IHC staining (Figure 4D). EC cells in the mucosal layer of the pig gastric fundus were visualized by IHC staining with the MAB352 antibody against 5-HT<sub>4</sub> (Figure 3). Indeed, Penkova *et al.*<sup>[35]</sup> showed that the MAB352 antibody is selective for EC cells in the human gastric mucosa, as verified by electron microscopy.

In this study, 5-HT<sub>4</sub> receptor expression was detected in both mucosa and MMP tissue fractions (Figures 5 and 6), and in mucosal and MMP parts of HE stained tissue sections (Figure 7) of the colon descendens, as well as of

the gastric fundus, confirming previously reported data by De Maeyer *et al.*<sup>[16]</sup>. Expressions of both a variant with the h-exon [5HT<sub>4(+h)</sub> receptor, 277 bp] and one without the h-exon [5HT<sub>4(-h)</sub> receptor, 235 bp] were observed. This was made possible by analysis of the 5-HT<sub>4</sub> receptor mRNA expression profile using forward and reverse primers based on exon 4 and 5, which were designed to amplify part of the common receptor region encoded by exon 4 and 5, which flank exon h (Figure 1B). The study was not designed to discriminate the C-terminal tail associated with the h-exon; the 5HT<sub>4(+h)</sub> receptor detected might indeed be associated with several C-terminal splice variants, because the h-exon has already been associated with the a, m and r C-terminal in pigs<sup>[16]</sup>. Semi-quantitative analysis revealed that in the mucosa tissue fractions of colon descendens and gastric fundus, the expression level of the 5-HT<sub>4(+h)</sub> receptor was significantly higher compared with the 5-HT<sub>4(-h)</sub> receptor, and a similar trend was obtained in the mucosal part of HE stained tissue sections. While evaluation of 5-HT<sub>4</sub> receptor RNA expression in human GI full-thickness tissue samples showed similar levels in the stomach compared with more distal levels<sup>[36]</sup>, expression in human gastric mucosal specimens was much less pronounced than in mucosal specimens of more distal regions of the GI tract<sup>[21,22]</sup>. In the pig gastric fundus mucosa, however, the expression of the 5-HT<sub>4</sub> receptor was similar to that in the mucosa of the colon descendens, with a clear-cut preponderance of the h-exon-containing receptor. The predominant mucosal location of h-exon containing 5-HT<sub>4</sub> receptor splice variants might correspond to the preferential involvement of this type of 5-HT<sub>4</sub> receptor splice variant in the mucosal effects of 5-HT<sub>4</sub> receptor activation, such as goblet cell degranulation, chloride secretion and control of 5-HT release<sup>[21]</sup>.

Both the mucosal and the MMP part of the GI tract contain several cell types on which the presence of 5-HT<sub>4</sub> receptors has been suggested, at least in some regions in some species, such as EC cells, smooth muscle cells of the muscularis mucosae and submucosal intrinsic neurons in the mucosal part; and myenteric cholinergic neurons, smooth muscle cells and interstitial cells of Cajal in the MMP part<sup>[37-41]</sup>. To obtain more information on the cell-specific distribution of the 5-HT<sub>4</sub> receptors, cell layers or particular cell types were isolated by LMPC. In the colon descendens, patches of the epithelial cell layer obtained by LMPC showed expression of 5-HT<sub>4</sub> receptors, predominantly the 5HT<sub>4(+h)</sub> receptor. Possible cell types involved might be EC cells and goblet cells, which were recently shown to express 5-HT<sub>4</sub> receptors in the mouse intestine<sup>[21]</sup>. In mouse, application of 5-HT<sub>4</sub> receptor agonists led to mucosal 5-HT release and mucus secretion in a tetrodotoxin-insensitive manner, indicating direct activation of stimulatory 5-HT<sub>4</sub> receptors on the EC cells and goblet cells<sup>[21]</sup>. In the porcine and human small intestine however, analysis of 5-HT release suggested the presence of inhibitory 5-HT<sub>4</sub> receptors on the EC cells<sup>[42]</sup>. Relaxant 5-HT<sub>4</sub> receptors have been proposed on circular smooth muscle in the human colon on the basis

of functional data<sup>[13]</sup>; therefore, patches of cells were also obtained from the circular muscle layer of the pig colon descendens, which revealed 5-HT<sub>4</sub> receptor expression. However, some authors were not able to confirm the presence of relaxant 5-HT<sub>4</sub> receptors in human colonic circular muscle strips<sup>[43]</sup>; we were also unable to obtain evidence for muscular 5-HT<sub>4</sub> receptors in pig colonic circular muscle strips<sup>[12]</sup>. Thus, we can not exclude the possibility that the 5-HT<sub>4</sub> receptor expression observed in LMPC-isolated cell patches from the pig colonic circular muscle layer represents the 5-HT<sub>4</sub> receptors on intercalated interstitial cells of Cajal. In addition, in the pig gastric fundus, LMPC-isolated cell patches of the epithelial cell layer showed 5-HT<sub>4</sub> receptor expression. The human gastric mucosa was shown to contain a considerable number of EC cells scattered within the lining epithelium<sup>[35]</sup>; therefore, we stained EC cells in porcine gastric mucosa immunohistochemically and isolated them by LMPC. Although the 5-HT<sub>4</sub> receptor was still detected in the full mucosal part of these IHC stained sections, 5-HT<sub>4</sub> receptor expression was not detected in the isolated EC cells. The EC cells showed expression of GAPDH; therefore, this might be related to the small amount of 5-HT<sub>4</sub> receptor RNA obtained, even when pooling 500 LMPC-isolated cells. In the gastric fundus, LMPC was not used to obtain cell patches from the muscle layer, as there are no functional data suggesting 5-HT<sub>4</sub> receptors to be present on muscle cells in the stomach.

In conclusion, this study, using endpoint RT-PCR, confirmed the presence of 5-HT<sub>4</sub> receptors in the mucosa and in the MMP part of porcine gastric fundus and colon descendens, and showed that the mucosa predominantly expresses h-exon-containing 5-HT<sub>4</sub> receptors. The mucosal h-exon-containing 5-HT<sub>4</sub> receptors might form additional sites of action for 5-HT<sub>4</sub> receptor agonists. 5-HT<sub>4</sub> receptors were detected in LMPC-isolated epithelial cell patches in the gastric fundus and colon descendens, and in circular muscle cell patches in the colon descendens. No 5-HT<sub>4</sub> receptor expression was detected in gastric LMPC-isolated EC cells stained by immunohistochemistry; the expression of 5-HT<sub>4</sub> receptors in individual cell types might be too low to detect by LMPC and endpoint PCR.

## ACKNOWLEDGMENTS

The authors thank Mrs. An Neesen, Sandra Soetaert and Trees Lopez for their technical help.

## COMMENTS

### Background

5-HT<sub>4</sub> receptors are distributed throughout the gastrointestinal (GI) tract. They are expressed on excitatory motor neurons, promoting the stimulatory effect of these neurons on GI motility. This explains the therapeutic use of 5-HT<sub>4</sub> receptor agonists in conditions with impaired GI motility, such as constipation. Several reports indicate that 5-HT<sub>4</sub> receptors are also expressed in the GI mucosa. 5-HT<sub>4</sub> receptors are G-protein coupled receptors containing seven transmembrane domains. The intracellular tail shows splice variation, with different lengths of the intracellular amino acid sequence. Exceptionally among G-protein coupled

receptors, 5-HT<sub>4</sub> receptors can also have an extra insertion of 14 amino acids in the second extracellular loop, encoded by the h-exon (42 base pairs). Previous distribution studies of 5-HT<sub>4</sub> receptors in the GI tract did not consider the all or none presence of the h-sequence.

### Research frontiers

Using homogenates of tissues limits the potential to study cell-specific expression: important cell-specific transcript information is lost because of cell heterogeneity of tissues such as GI tissues. Techniques have been developed to enable collection of particular cells from mixed populations, which generally involve either fluorescence activated cell sorting (FACS) purification of dissociated cells or laser-assisted microdissection. In contrast to FACS, microdissection can be applied to most tissues.

### Innovations and breakthroughs

The major advantages of laser microdissection and pressure catapulting (LMPC) are the isolation of biological material without direct user contact, thereby avoiding contamination, and the preservation of cellular integrity. The main concerns when using LMPC to analyse 5-HT<sub>4</sub> receptor mRNA expression of a specific cell type is the efficient and selective isolation of the right cells, and obtaining RNA of good quality. To address these issues, and to optimize a LMPC experimental design for GI tissue that is highly heterogeneous, rich in endogenous RNase and enzymes, a systematic approach was required to evaluate the impact on RNA integrity of different critical steps. Therefore, RNA yield and quality were determined using the Experion automated electrophoresis system after tissue collection, hematoxylin and eosin (HE) staining or immunohistochemistry (IHC) staining, and LMPC by analysing RNA extracted from mucosal and muscular myenteric plexus (MMP) tissue fractions, and from scraped off tissue sections after staining and after LMPC. Developing a suitable IHC staining procedure was more complex compared with the HE staining, because a standard IHC procedure requires a long overnight antibody incubation to obtain good antibody labeling, but long incubation in aqueous buffers activates endogenous RNases, resulting in RNA degradation. The authors attempted to develop a fast IHC protocol, which resulted in diminished visualization because the short antibody labeling time. Therefore, the authors used an overnight antibody incubation for the IHC staining protocol whereby RNA integrity was maintained by the addition of 1 mol/L NaCl to all aqueous solutions, resulting in superior protection of RNA. It is not yet clear why a saline solution preserves RNA, although the authors speculate that normal saline protects the integrity of cell membranes, preventing the release of intracellular RNases.

### Applications

This study, using endpoint reverse transcription-polymerase chain reaction (PCR) and LMPC, confirms the presence of 5-HT<sub>4</sub> receptors in the mucosal and MMP parts of the porcine gastric fundus and colon descendens, and shows that the mucosa predominantly expresses h-exon containing 5-HT<sub>4</sub> receptors. The mucosal h-exon-containing 5-HT<sub>4</sub> receptors might be preferentially involved in the mucosal response to 5-HT<sub>4</sub> receptor activation and might form potential drug targets for 5-HT<sub>4(+h)</sub> receptor-selective agonists.

### Terminology

5-HT<sub>4</sub> receptors: The 5-HT<sub>4</sub> receptor is a G-protein coupled receptor that activates the adenylyl cyclase/cyclic adenosine monophosphate/protein kinase A pathway in response to serotonin (5-hydroxytryptamine; 5-HT). The 5-HT<sub>4</sub> receptor is expressed on excitatory motor neurons in the gut, facilitating acetylcholine release, which stimulates GI motility; LMPC: Under direct microscopic visualization, LMPC permits sampling of histologically or immunohistologically defined tissue and cell samples. The LMPC system uses a focused pulsed nitrogen UV-A laser beam (wavelength 355 nm) whose source is positioned below the material and the high energy generated beam is focused through a microscope ocular lens onto the biological material on the slide. The RoboMover stage is used to move the sample through the laser beam path to allow the user to control the size and shape of the area to be cut. The beam is then defocused and this energy is used to catapult the membrane and corresponding biological material from the slide. When the laser beam strikes the material, it is blasted off the glass surface and catapulted into the cap of the vial.

### Peer review

This manuscript describes the expression of the +h 5-HT<sub>4</sub> receptor variant in porcine stomach and colon. The significance of this work is the use of LMPC and end-point PCR to allow the positive identification of the specific HTR<sub>4</sub> variant (+ exon h) in discernible functional cell types and tissue regions relevant to serotonergic responses. This study also discusses several technique modifications and improvements and artifacts in detail, which might be helpful for wider application of similar interests.

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**P- Reviewers** Hori M, Tache Y **S- Editor** Huang XZ  
**L- Editor** Stewart GJ **E- Editor** Li JY



## Assessment of vascular invasion in gastric cancer: A comparative study

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Supported by Grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

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Received: January 30, 2013 Revised: April 3, 2013

Accepted: April 13, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To evaluate and compare detection of lymphatic and blood vessel invasion (LVI and BVI) by hematoxylin-eosin (HE) and immunohistochemistry (IHC) in gastric cancer specimens, and to correlate with lymph node status.

**METHODS:** IHC using D2-40 (a lymphatic endothelial marker) and CD34 (a pan-endothelial marker) was performed to study LVI and BVI in surgical specimens from

a consecutive series of 95 primary gastric cancer cases. The results of the IHC study were compared with the detection by HE using McNemar test and kappa index. The morphologic features of the tumors and the presence of LVI and BVI were related to the presence of lymph node metastasis. A  $\chi^2$  test was performed to obtain associations between LVI and BVI and other prognostic factors for gastric cancer.

**RESULTS:** The detection rate of LVI was considerably higher than that of BVI. The IHC study identified eight false-positive cases and 13 false-negative cases for LVI, and 24 false-positive cases and 10 false-negative cases for BVI. The average Kappa value determined was moderate for LVI ( $\kappa = 0.50$ ) and low for BVI ( $\kappa = 0.20$ ). Both LVI and BVI were statistically associated with the presence of lymph node metastasis (HE:  $P = 0.001$ ,  $P = 0.013$ , and IHC:  $P = 0.001$ ,  $P = 0.019$ ). The morphologic features associated with LVI were location of the tumor in the distal third of the stomach ( $P = 0.039$ ), Borrmann's macroscopic type ( $P = 0.001$ ), organ invasion ( $P = 0.03$ ) and the depth of tumor invasion ( $P = 0.001$ ). The presence of BVI was related only to the depth of tumor invasion ( $P = 0.003$ ).

**CONCLUSION:** The immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of vessel invasion and for predicting lymph node metastasis.

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**Key words:** Gastric cancer; Tumour-node-metastasis staging; Lymph node metastasis; Predictive factor; Lymphatic vessel invasion; Blood vessel invasion; Immunohistochemistry; CD34; D2-40

**Core tip:** The presence of lymphatic vessel invasion in gastric cancer is the strongest risk factor for lymph node metastasis and is known as an independent prog-

nostic factor. The subjective evaluation of vessel invasion performed with conventional hematoxylin-eosin staining can lead to inaccurate false-positive and false-negative results. This study shows that the immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of lymphatic and blood vessel invasion and for predicting lymph node metastasis in gastric cancer.

Gresta LT, Rodrigues-Júnior IA, de Castro LPF, Cassali GD, Cabral MMDA. Assessment of vascular invasion in gastric cancer: A comparative study. *World J Gastroenterol* 2013; 19(24): 3761-3769 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3761.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3761>

## INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer deaths in the world<sup>[1]</sup>. A steady decline in the incidence and mortality rates of gastric carcinoma has been observed worldwide over the past several decades, but there is a significant variation in incidence between the populations at the greatest and least risk<sup>[2]</sup>. In areas without endoscopic screening for GC, especially in developing countries, GC presents as an advanced disease and has a high frequency of nodal involvement<sup>[3]</sup>. Surgery is the only effective intervention for a cure or for long-term survival and nodal status is one of the most important independent predictors of patient survival<sup>[4]</sup>.

The depth of invasion is an independent prognostic factor for gastric carcinoma and is associated with patient survival<sup>[5,6]</sup>. Early GC is limited to the mucosa and submucosa and is associated with a better prognosis. In Japan, where asymptomatic patients are screened, there is a high incidence of early diagnosis, ranging from 30% to 50%, in contrast with the smaller fraction of 16%-24% in Western countries<sup>[2]</sup>. Minimizing the number of invasive procedures used in cancer treatment is critical for improving the patient's quality of life. Minimally invasive treatments, such as endoscopic mucosal resection, may be possible only in highly selective cases of early GC<sup>[7-9]</sup>.

Lymph node metastasis is one of the most important prognostic factors in patients with GC<sup>[2,10]</sup>. Studies have estimated that the lymph nodes will be involved in 3%-5% of cases of gastric adenocarcinoma limited to the mucosa, in 11%-25% of cases limited to the submucosa, in 50% of T2 tumors and in 83% of T3 tumors<sup>[11]</sup>. Hence the accurate assessment of potential lymph node metastasis is an important issue for the appropriate treatment of early GC.

The histologic identification of lymphatic vessel invasion (LVI) by tumor cells has long been recognized as a potential prognostic indicator and a predictor of patient outcomes in various malignancies<sup>[12-18]</sup>. One of the earliest steps in the metastatic cascade is (lympho)vascular inva-

sion, *i.e.*, the penetration of tumor cells into lymph and/or blood vessels in and around the primary tumor<sup>[19-21]</sup>. Therefore, tumor cell emboli in the lymph and blood vessels are considered to be the morphological correlates of metastases to loco-regional lymph nodes and to distant hematogenous sites, respectively. Consistent with the distribution of lymphatic vessels in the gastric wall, LVI is most frequently observed in the muscularis mucosa layer and in the superficial submucosa<sup>[22,23]</sup>.

Usually, LVI and blood vessel invasion (BVI) are identified based on conventional hematoxylin-eosin (HE) staining, and the diagnosis is made based on the presence of tumor emboli within the vascular channels lined by a single layer of endothelial cells, with or without red blood cells<sup>[14,19,20]</sup>. However, if the cancer cells completely obliterate the lumen, it is not possible to diagnose vascular invasion. Additionally, retraction artifacts that isolate tumor aggregates *via* tissue shrinkage during fixation are sometimes confused with true tumor emboli in lymphatic vessels. Besides, using that criterion, vascular invasion detected on HE sections does not always allow for a distinction between BVI and LVI<sup>[14]</sup>.

Recently, interest in vascular invasion has increased because of the development of specific markers for the lymphatic endothelium used in immunohistochemistry (IHC), such as Prox-1, which is a transcription factor; Lyve-1, which is a hyaluronan receptor; podoplanin, which is a glomerular podocyte membrane protein and D2-40<sup>[21]</sup>. It has been demonstrated that D2-40 is the best marker for the lymphatic endothelium<sup>[24]</sup>. Used in combination with panendothelial markers such as CD34 or CD31, D2-40 permits the differentiation between BVI and LVI and the study of both processes in GC metastasis<sup>[25]</sup>.

There have been numerous studies regarding LVI and BVI in GC. However, most of them have not defined the criteria used to determine the presence or absence of lymphatic and vascular invasion. Additionally, many large retrospective series of GC cases have extracted the reporting of (lympho)vascular invasion from the patients' medical records, without histological reviews by central pathologists for consistency and without immunohistochemical studies<sup>[6,9,15,26]</sup>. Uncertain criteria for the diagnosis of (lympho)vascular invasion may affect the clinical assessment of prognosis and may change the course of therapy for the patients<sup>[27-30]</sup>.

The aim of this study was to evaluate, in a consecutive series of patients with GC, a technique that uses a combined immunohistochemical expression profile to detect LVI and BVI and compare this technique to routine HE assessment. In addition, we analyzed the relationship between lymph node metastasis and clinicopathological findings, especially those of LVI and BVI re-evaluated by IHC staining.

## MATERIALS AND METHODS

This study was reviewed and approved by the university's research ethics committee (COEP-UFGM). Ninety-five consecutive cases of GC, diagnosed and treated between

**Table 1 Clinicopathological characteristics of 95 patients with gastric cancer *n* (%)**

| Clinicopathological data |           |
|--------------------------|-----------|
| Sex                      |           |
| Male                     | 62 (65.3) |
| Female                   | 33 (34.7) |
| Curvature                |           |
| Small curvature          | 54 (56.8) |
| Large curvature          | 10 (10.5) |
| Small and large          | 14 (14.7) |
| Not evaluated            | 17 (17.9) |
| Primary tumor            |           |
| pT1a                     | 9 (9.5)   |
| pT1b                     | 12 (12.6) |
| pT2a                     | 12 (12.6) |
| pT2b                     | 8 (8.4)   |
| pT3                      | 51 (53.7) |
| pT4                      | 3 (3.2)   |
| Regional lymph nodes     |           |
| pN0                      | 28 (29.5) |
| pN1                      | 36 (37.9) |
| pN2                      | 13 (13.7) |
| pN3                      | 12 (12.6) |
| pNx                      | 6 (6.3)   |
| Distant metastasis       |           |
| pMx                      | 87 (91.6) |
| pM1                      | 8 (8.4)   |
| Laurén classification    |           |
| Intestinal               | 45 (47.4) |
| Diffuse                  | 25 (26.3) |
| Mixed/indeterminate      | 25 (26.3) |
| WHO classification       |           |
| Adenocarcinoma NOS       | 48 (50.5) |
| Tubular                  | 5 (5.3)   |
| Papillary                | 5 (5.3)   |
| Mucinous                 | 4 (4.2)   |
| Signet-ring cell         | 22 (23.2) |
| Undifferentiated         | 9 (9.5)   |
| Others                   | 2 (2.2)   |
| Ming classification      |           |
| Expansive                | 40 (42.1) |
| Infiltrative             | 40 (42.1) |
| Not evaluated            | 15 (15.8) |

NOS: Not otherwise specified; WHO: World Health Organization; pT: Primary tumor; pN: Regional lymph nodes; pM: Distant metastasis.

2000 and 2006 and identified from the pathology archives, were selected for study. All patients underwent curative gastrectomy with standard lymphadenectomy at the Clinical Hospital of the Federal University of Minas Gerais. None of the patients had received preoperative radiation therapy or chemotherapy. In total, 57 patients underwent distal gastrectomy, 33 had total gastrectomy and five had partial gastrectomy.

All surgical specimens of the primary tumors and regional lymph nodes had been processed and examined histologically by routine HE staining, according to the institutional protocol<sup>[31]</sup>. The definitions of stages and the criteria for histological classification followed the World Health Organization classification<sup>[2]</sup> and the Japanese classification for GC<sup>[32]</sup>. The resected primary tumors and regional lymph nodes were reviewed histologically by two pathologists using HE staining.

IHC for CD34 and D2-40 was then performed on

the corresponding paraffin blocks. Serial 4- $\mu$ m-thick sections were deparaffinized in xylene and rehydrated. A hydrogen peroxide quench, using 3% H<sub>2</sub>O<sub>2</sub> with methyl alcohol, was performed for 20 min. A 15-min incubation with a serum-free protein blocking agent was then performed. Antigen retrieval was performed using EDTA buffer at pH 9.0 (CD34), a citrate buffer at pH 6.0 (D2-40) and a steam cooker for 20 min, with a bench cool down period of 15 min. A 30-min incubation in the primary antibodies (Biogenex CD34 monoclonal mouse antihuman, clone QBEND/10, at 1:10 dilution; Dako D2-40 monoclonal mouse antihuman, clone D2-40, at 1:30 dilution) was performed at room temperature. Secondary detection consisted of a 30-min incubation using Labeled Streptavidin-Biotin link (Dako LSAB<sup>®</sup>+ System), and the staining was visualized by a 5-min incubation with diaminobenzidine tetrahydrochloride. The slides were counterstained with Harris' hematoxylin and cover-slipped. A normal stomach was used for the control slide for each immunohistochemical stain. For the negative control, all of the reagents except for the primary antibody were used.

All slides were assessed simultaneously by two investigators (Gresta LT and Cabral MMDA) on a double-observation microscope, without knowledge of the clinicopathological data. The slides (HE, CD34 and D2-40) were screened for (lympho)vascular invasion using strict criteria<sup>[14]</sup>. With HE, invasion was considered only if the tumor cells were within an endothelium-lined, vessel-like structure. With IHC, vessels with endothelial cells stained by CD34, but not by D2-40, were recognized as blood vessels, and vessels with endothelial cells stained by both CD34 and D2-40 were recognized as lymphatic vessels. Every vessel with tumor cell invasion on one of the three consecutive sections was identified in the other slides and was classified as a blood vessel or lymphatic invasion, based on the immunohistochemical staining profile.

### Statistical analysis

The statistical calculations were performed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, United States). The McNemar test was used to determine the significance of intergroup differences.  $P \leq 0.05$  was considered to be statistically significant. The estimation of the agreement rate between the two methods was obtained using the Kappa statistic ( $\kappa$ ). A  $\chi^2$  test was applied for the analysis of associations between categorical variables.

## RESULTS

The clinical and pathological characteristics of the 95 patients with GC are summarized in Table 1. Lymphatic vessels were identified by immunostaining as D2-40-positive and CD34-positive (Figure 1A-C). Blood vessels were identified by immunostaining as D2-40-negative and CD34-positive (Figure 1D-F).

### Lymphatic and BVI (HE and IHC)

Histological HE staining revealed LVI from the primary

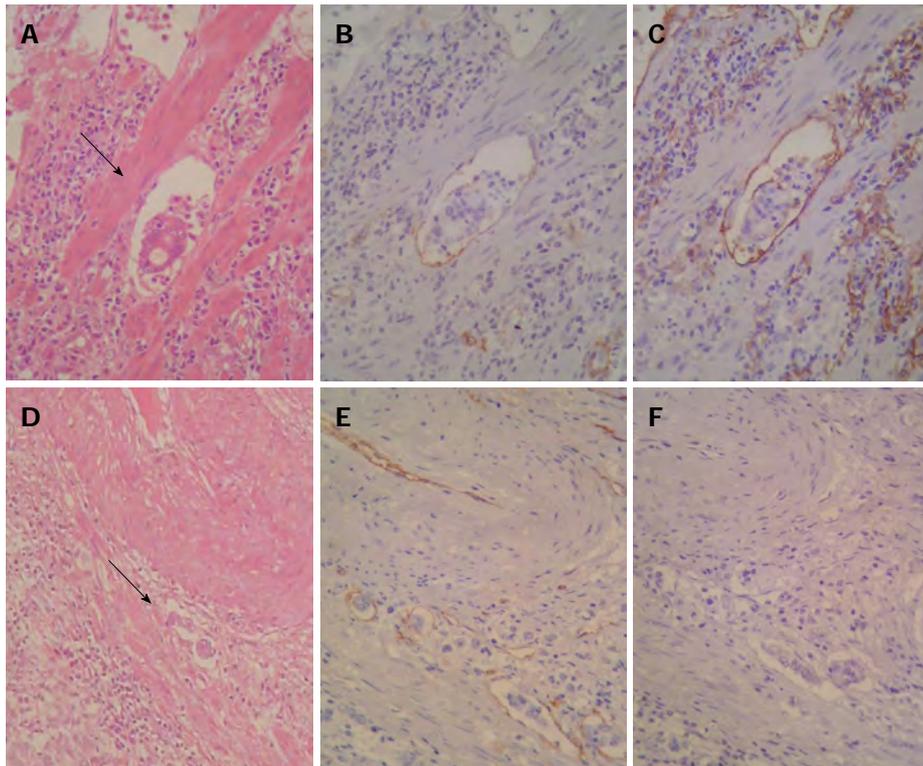


Figure 1 Sequential sections stained with hematoxylin and eosin and Immunohistochemistry showing neoplastic cell emboli within a space surrounded by the endothelial lining (arrows). A: Lymphatic vessel invasion (LVI)-hematoxylin and eosin (HE,  $\times 400$ ); B: LVI CD34 ( $\times 400$ ); C: LVI D2-40 ( $\times 400$ ); D: Blood vessel invasion (BVI) (HE,  $\times 100$ ); E: BVI CD34 ( $\times 200$ ); F: BVI D2-40 ( $\times 200$ ).

**Table 2** Diagnostic agreement between methods of detection for lymphatic and blood vessel invasion ( $n = 95$ )

| Variables |          | HE | IHC | P value | $\kappa$ |
|-----------|----------|----|-----|---------|----------|
| LVI       | Positive | 61 | 66  | 0.38    | 0.50     |
|           | Negative | 34 | 29  |         |          |
| BVI       | Positive | 38 | 26  | 0.02    | 0.20     |
|           | Negative | 57 | 69  |         |          |

BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion; HE: Hematoxylin and eosin; IHC: Immunohistochemistry.

tumor in 61 of the 95 patients (64.2%). In 53 of those cases, LVI detected by HE staining was confirmed with D2-40 staining. In contrast, LVI was newly detected in 13 of 34 patients who had been diagnosed as free of LVI by HE staining. Figure 2 shows examples of false-positive and false-negative for LVI.

The specimens examined using HE staining showed a false-negative BVI rate of 12.6% (12/95) and a false-positive rate of 25.2% (24/95). The positive rate of BVI determined by HE staining was 40% (38/95); however, BVI was confirmed by CD34 in only 27.4% of the cases (26/95).

Figure 3 shows the prevalence of LVI and BVI with conventional HE staining and IHC in 95 primary tumors. Table 2 shows the average kappa values for both methods determined separately for LVI and BVI. The agreement was fair for BVI ( $\kappa = 0.20$ ) and medium for LVI ( $\kappa = 0.50$ ).

**Table 3** Correlation between lymphatic and blood vessel invasion and lymph node status ( $n = 89$ )  $n$  (%)

| Vascular invasion |          | Lymph node metastasis |           | P value |
|-------------------|----------|-----------------------|-----------|---------|
|                   |          | Negative              | Positive  |         |
| LVI-HE            | Negative | 23 (71.8)             | 9 (28.2)  | 0.001   |
|                   | Positive | 5 (8.8)               | 52 (91.2) |         |
| LVI-IHC           | Negative | 20 (74.0)             | 7 (26.0)  | 0.001   |
|                   | Positive | 8 (13.0)              | 54 (87.0) |         |
| BVI-HE            | Negative | 22 (41.5)             | 31 (58.5) | 0.013   |
|                   | Positive | 6 (16.6)              | 30 (83.4) |         |
| BVI-IHC           | Negative | 25 (38.5)             | 40 (61.5) | 0.019   |
|                   | Positive | 3 (12.5)              | 21 (87.5) |         |

IHC: Immunohistochemistry; HE: Hematoxylin and eosin; BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion.

**Correlation of LVI and BVI with other prognostic factors**

The LVI and BVI diagnosed by both HE and IHC were significantly correlated with lymph node metastasis, as shown in Table 3.

Table 4 shows other clinical-pathologic variables that were significantly correlated with LVI and BVI when detected by IHC.

**DISCUSSION**

To improve the detection of vascular invasion in GC, which is normally performed by routine HE staining, and to distinguish LVI from BVI, we introduced an IHC method using the combination of two markers: one spe-

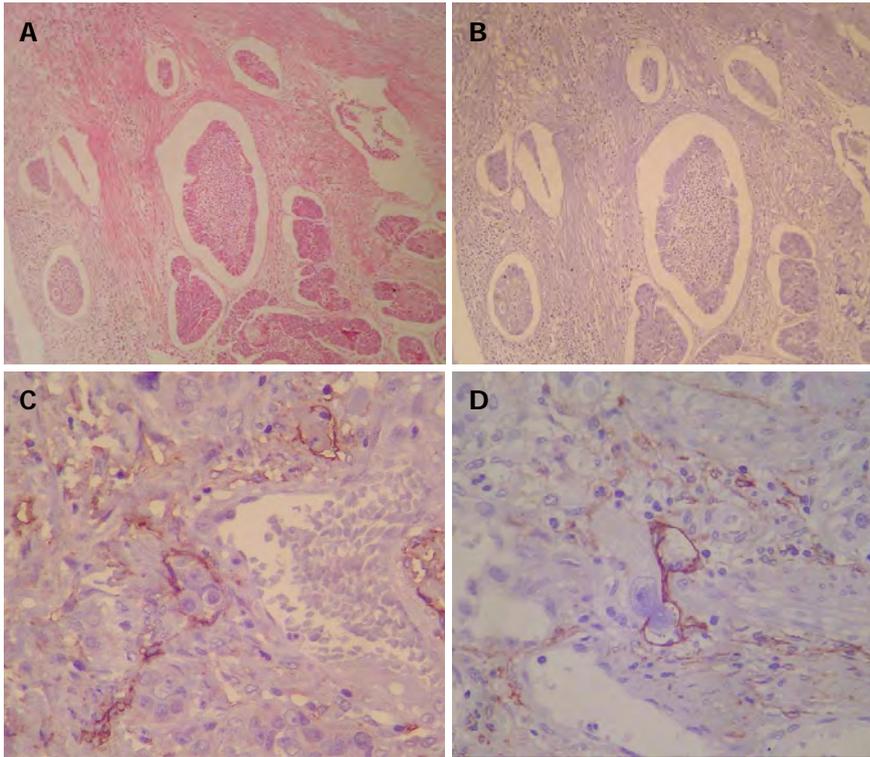
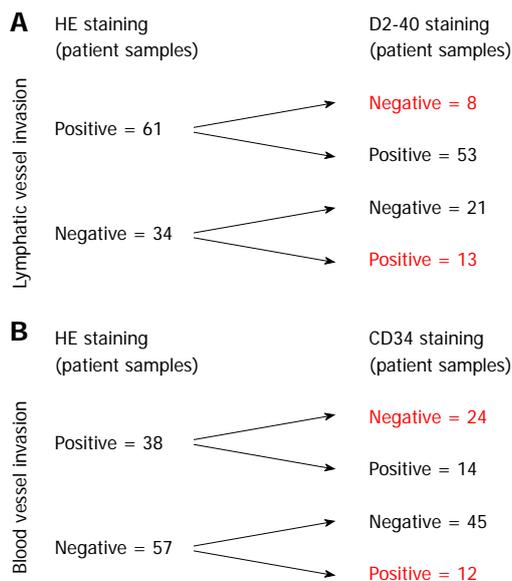


Figure 2 Example of a patient diagnosed for lymphatic vessel invasion by routine histological examination. A: Example of a patient diagnosed as positive for lymphatic vessel invasion (LVI) by routine histological examination; B: As false-positive for lymphatic vessel invasion by D2-40 ( $\times 100$ ); C, D: Examples of patients diagnosed as free of LVI by routine histological examination. False-negatives for LVI detected by D2-40 ( $\times 400$ ).

**Table 4** Correlation of lymphatic and blood vessel invasion detected by immunohistochemistry with other prognostic factors ( $n = 95$ )  
 $n$  (%)

| Data                 | LVI       |            |                | BVI        |           |                |
|----------------------|-----------|------------|----------------|------------|-----------|----------------|
|                      | Negative  | Positive   | <i>P</i> value | Negative   | Positive  | <i>P</i> value |
| Tumor location       |           |            |                |            |           |                |
| Distal third         | 15 (27.3) | 40 (72.7)  | 0.0391         | 42 (76.3)  | 13 (23.7) | 0.281          |
| Other locations      | 14 (35.0) | 26 (65.0)  |                | 29 (72.5)  | 11 (27.5) |                |
| Curvature            |           |            |                |            |           |                |
| Small curvature      | 19 (35.2) | 35 (64.8)  | 0.122          | 41 (76.0)  | 13 (24.0) | 0.131          |
| Large curvature      | 3 (30.0)  | 7 (70.0)   |                | 8 (80.0)   | 2 (20.0)  |                |
| Small and large      | 1 (7.2)   | 13 (92.8)  |                | 7 (50.0)   | 7 (50.0)  |                |
| Macroscopy           |           |            |                |            |           |                |
| Borrmann I           | 5 (71.4)  | 2 (28.6)   | 0.0011         | 7 (100.0)  | 0 (0.0)   | 0.24           |
| Borrmann II          | 4 (13.3)  | 26 (86.7)  |                | 21 (70.0)  | 9 (30.0)  |                |
| Borrmann III         | 5 (18.5)  | 22 (81.5)  |                | 17 (62.9)  | 10 (37.1) |                |
| Borrmann IV          | 0 (0.0)   | 12 (100.0) |                | 7 (58.3)   | 5 (41.7)  |                |
| Organ invasion       |           |            |                |            |           |                |
| Negative             | 26 (45.6) | 31 (54.4)  | 0.0031         | 46 (80.7)  | 11 (19.3) | 0.297          |
| Duodenum             | 1 (4.3)   | 22 (95.7)  |                | 15 (65.2)  | 8 (34.8)  |                |
| Esophagus            | 1 (12.5)  | 7 (87.5)   |                | 6 (75.0)   | 2 (25.0)  |                |
| Both E + D           | 0 (0.0)   | 3 (100.0)  |                | 1 (33.3)   | 2 (66.7)  |                |
| Other                | 1 (25.0)  | 3 (75.0)   |                | 3 (75.0)   | 1 (25.0)  |                |
| Tumor depth          |           |            |                |            |           |                |
| Early                | 17 (80.9) | 4 (19.1)   | 0.0011         | 21 (100.0) | 0 (0.0)   | 0.0031         |
| Advanced             | 12 (16.2) | 62 (83.8)  |                | 50 (67.5)  | 24 (32.5) |                |
| Laurén histology     |           |            |                |            |           |                |
| Intestinal           | 12 (26.6) | 33 (73.3)  | 0.228          | 36 (80.0)  | 9 (20.0)  | 0.332          |
| Diffuse              | 11 (44.0) | 14 (56.0)  |                | 19 (76.0)  | 6 (24.0)  |                |
| Mixed/not classified | 6 (24.0)  | 19 (76.0)  |                | 16 (64.0)  | 9 (36.0)  |                |

BVI: Blood vessel invasion; LVI: Lymphatic vessel invasion; E + D: Esophagus + duodenum.



**Figure 3** Diagnostic comparison in 95 patients with gastric cancer. A: Diagnostic comparison of lymphatic vessel invasion; B: Diagnostic comparison of blood vessel invasion. HE: Hematoxylin and eosin.

cific to the lymphatic vessel endothelium (D2-40) and the other pan-endothelial (CD34). In addition to the effective detection of LVI and BVI, this method also enabled the correct evaluation of the predictive value of vascular invasion in GC for the occurrence of lymph node metastasis. As we expected, the identification of LVI and BVI was also correlated with other prognostic factors important for GC, such as tumor depth and organ invasion, which have been detected in other studies.

In this study, the group of 95 patients generally reflected the profile of GC described in the literature with regard to age, gender, location of tumor and Laurén histological type. The patients with diffuse-type carcinoma were significantly younger than those with the intestinal-type ( $P = 0.04$ ). This peculiar feature of diffuse-type neoplasms is well established and reflects differences in pathogenesis that are generally linked to genetic factors, whereas intestinal-type neoplasms are more influenced by environmental factors, such as diet and infection with *Helicobacter pylori*.

Data from the literature indicate that most diagnoses in developing countries occur late, when the disease is already in the advanced stage<sup>[31]</sup>. In our study, 53.7% of the GCs were in-depth stage pT3 tumors. Additionally, more than half of our sample (64.2%) had lymph node metastasis at the time of diagnosis.

The prevalence of LVI and BVI in GC has been determined in various studies to vary from 7.2% to 86%<sup>[9,15,28,33-35]</sup>. This wide variation in results could be explained by the different methods used to evaluate vascular invasion, *i.e.*, HE only or usage of IHC staining with endothelium-specific markers. Three consistent instances of this variation include the studies of Arigami *et al.*<sup>[36]</sup>, of Sako *et al.*<sup>[22]</sup> and of Yonemura *et al.*<sup>[23]</sup>, who reported higher rates of detection of LVI with the IHC method when compared

to routine staining with HE. All three studies strengthened the role of IHC in the analysis of vascular invasion in GC<sup>[36]</sup>.

We observed that the difference between HE and IHC when detecting LVI was not statistically significant ( $P = 0.38$ ). However, there were 8 cases of false-positive and 13 cases of false-negative that were isolated only after IHC. Thus, the Kappa coefficient was considered to be only moderate for LVI ( $\kappa = 0.50$ ). The evaluation of LVI by only HE is subject to these misconceptions because of the inability to distinguish retraction artifacts around glands or cell groups from true vascular invasion. Occasionally, neoplastic cells occupy the vascular lumen completely, which makes their identification impossible without specific marking of the lymphatic endothelium. Additionally, false-positive results can occur when BVI is misinterpreted as LVI with HE staining only<sup>[36]</sup>.

The correlation between LVI and the presence of metastasis was statistically significant when assessed by both methods ( $P = 0.001$ ). This finding agrees with published data, in which LVI of the primary tumor was found to be crucial for the occurrence of lymph node metastasis<sup>[37]</sup>. Therefore, it is possible to infer that LVI is more widely found in patients with lymph node metastases than in those in which the examined lymph nodes are negative.

We found 24 cases of false-positives for BVI with HE and 10 false-negatives identified by IHC. Therefore, the detection of BVI was more accurate and significantly less frequent with IHC than with HE ( $P = 0.02$ ). This result produced a very low Kappa coefficient ( $\kappa = 0.20$ ) because of the identification of a large number of cases as false-positives. The false-positive results obtained could be explained as cases of LVI that were inadvertently interpreted as BVI, as it is not possible to distinguish between blood vessels and lymphatic vessels in all cases using only HE<sup>[14]</sup>.

Our results show that BVI evaluated by both methods is positively correlated with the presence of lymph node metastasis, in contrast to what has been demonstrated by some previous studies<sup>[7]</sup>. It is interesting to note that although the previous studies have examined large numbers of cases, they performed retrospective review studies that included only cases of early GC, which explains the low occurrence of lymph node metastasis and BVI. Our study, in contrast, analyzed BVI and lymph node metastasis not only in early GC but also in advanced cases of GC, which resulted in the statistical significance described in Table 4.

The presence of lymph node metastasis is considered to be the most important prognostic factor in GC, and it is related to the presence of vascular invasion<sup>[37,38]</sup>. Retrospective studies have shown that the presence of LVI and BVI detected by the IHC method is related to tumor recurrence in patients with and without lymph node metastasis and is also related to a low survival rate<sup>[16,36,39]</sup>. In this regard, our study revealed the importance of LVI and BVI as predictive factors, even in the absence of lymph node involvement.

The early GC concept applies to those tumors with more superficial infiltration of the gastric wall. It is thought that cases of early GC are less likely to show invasion of blood and lymph vessels. Our data show that, compared with advanced GC, early GC exhibits less lymph node involvement ( $P = 0.001$ ), less LVI ( $P = 0.001$ ) and less BVI ( $P = 0.007$ ) detected by IHC. However, studies of lymphatic network density in the normal gastric wall have found that the concentration of lymphatic vessels is considerably greater in the muscularis mucosa, which can be infiltrated in early GC<sup>[22]</sup>.

The risk of lymph node metastasis in early GC is only 3.2% for the intramucosal and is approximately 19.2% when invasion reaches the submucosa<sup>[40]</sup>. Our results agree with these findings. We found two cases of early GC (11.2%) with invasion of the submucosa and lymph node metastasis. Conversely, in 59 cases of advanced GC (83.0%), the lymph nodes were positive for metastasis.

At present, non invasive imaging methods to properly evaluate the likelihood of lymph node metastasis in GC do not exist. Thus, lymph node staging in early GC still relies on the assessment of specific tumor characteristics that are related to increased lymph node metastasis, *i.e.*, depth of tumor infiltration in the gastric wall, tumor size greater than 2.0 cm, Laurén histological classification and LVI. It is noteworthy that, among these factors, the presence of lymphatic vessel involvement is the most significant isolated predictive factor for the occurrence of lymph node metastasis<sup>[7]</sup>. Thus, it is essential to include LVI and BVI evaluation by IHC in routine pathologic protocols of GC surgical specimens.

Gastrectomy with lymphadenectomy is indicated in poorly differentiated intramucosal carcinomas, with dimensions larger than 20 mm or in submucosal carcinomas. However, these criteria are quite strict and may result in unnecessary surgery. Gotoda *et al.*<sup>[41]</sup> proposed more expanded criteria for the endoscopic treatment of early GC that combined histological type, LVI and BVI, ulceration and tumor size, thereby enabling the expansion of the universe of patients with early GC who could potentially be eligible for endoscopic resection, even with submucosal invasion.

The meta-analysis published by Kwee *et al.*<sup>[40]</sup> revealed several variables significantly associated with the presence of lymph node metastasis in early GC. Most of these predictive factors may be perfectly evaluated through pre-operative exams, endoscopy with biopsy and non-invasive imaging methods, such as computed tomography and endoscopic ultrasound. However, the presence or absence of LVI and BVI can only be judged by a histopathological study after tumor resection.

LVI and BVI must be systematically analyzed as the histological parameters with the greatest prognostic significance and as decisive factors in the choice of complementary adjuvant therapy. Therefore, we suggest that more sensitive and more specific methods be incorporated into the routine protocols for histopathological examination of GC.

Our results show that the application of IHC using

two combined markers (CD34 and D2-40) provides a more accurate detection of LVI and BVI when compared to routine staining with HE. These findings may be of great value in clinical practice, especially in cases in which it is not possible to determine the precise lymph node status because of an insufficient number of lymph nodes or because lymphadenectomy was not performed.

## ACKNOWLEDGMENTS

The authors would like to pay tribute to professor Ana Margarida MF Nogueira in recognition of her great contribution to our work and to gastrointestinal pathology.

## COMMENTS

### Background

Gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer deaths in the world. Lymph node metastasis is one of the most important prognostic factors in patients with GC. The presence of lymphatic vessel invasion in GC is the strongest risk factor for lymph node metastasis and is known as an independent prognostic factor.

### Research frontiers

Usually, lymphatic and blood vessel invasion (BVI) are identified based on conventional hematoxylin-eosin staining, and the diagnosis is made based on the presence of tumor emboli within the vascular channels lined by a single layer of endothelial cells, with or without red blood cells. However, this subjective evaluation can lead to inaccurate false-positive and false-negative results.

### Innovations and breakthroughs

Recently, interest in vascular invasion has increased because of the development of specific markers for the lymphatic endothelium used in immunohistochemistry (IHC), such as Prox-1, which is a transcription factor; Lyve-1, which is a hyaluronan receptor; podoplanin, which is a glomerular podocyte membrane protein and D2-40. It has been demonstrated that D2-40 is the best marker for the lymphatic endothelium. Used in combination with panendothelial markers such as CD34 or CD31, D2-40 permits the differentiation between lymphatic and BVI and the study of both processes in GC metastasis.

### Applications

Uncertain criteria for the diagnosis of (lympho)vascular invasion may affect the clinical assessment of prognosis and may change the course of therapy for the patients. This study shows that the immunohistochemical identification of lymphatic and blood vessels is useful for increasing the accuracy of the diagnosis of lymphatic and BVI and for predicting lymph node metastasis in GC.

### Terminology

Blood and lymphatic vessels show different functions and phenotypic expression. IHC is a procedure which identifies specific antigens expressed by the cells or tissues, making it possible to determine their origin. Vascular invasion is the presence of tumor cells within the lumen of blood or lymphatic vascular spaces, which can be transported into the bloodstream, producing metastasis.

### Peer review

Authors demonstrated that the IHC of two different endothelial markers D2-40 and CD34 is useful for accurate diagnosis of vessel invasion and for predicting lymph node metastasis.

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**P- Reviewers** Langley RR, Pan WS, Shen LZ, Shibata MA  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Xiong L



## Interaction of 14-3-3 $\sigma$ with KCMF1 suppresses the proliferation and colony formation of human colon cancer stem cells

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Author contributions: Zou J and Yu XF contributed equally to this work; Zou J designed the research; Zou J, Mi L, Yu XF and Dong J performed the majority of the experiments; Zou J, Mi L and Dong J analysed the data; Zou J and Yu XF wrote the paper. Supported by The Medical Guidance Projects of Shanghai Science Committee, No. 10411961800; National Natural Science Foundation of China, No. 81101617

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Received: December 17, 2012 Revised: February 14, 2013

Accepted: March 21, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the biological function of 14-3-3 $\sigma$  protein and to look for proteins that interact with 14-3-3 $\sigma$  protein in colon cancer stem cells.

**METHODS:** Reverse transcription polymerase chain reaction was performed to amplify the 14-3-3 $\sigma$  gene from the mRNA of colon cancer stem cells. The gene was then cloned into the pGEM-T vector. After being sequenced, the target gene 14-3-3 $\sigma$  was cut from the pGEM-T vector and cloned into the pGBKT7 yeast expression plasmid. Then, the bait plasmid pGBKT7-14-3-3 $\sigma$  was transformed into the yeast strain AH109. After the expression of the pGBKT7-14-3-3 $\sigma$  fusion protein in the AH109 yeast strain was accomplished, a yeast two-hybrid screening assay was performed by mating AH109 with Y187 that contained a HeLa cDNA library plasmid. The interaction between the 14-3-3 $\sigma$  protein and the proteins obtained from positive colonies was further confirmed by repeating the yeast two-hybrid

screen. After extracting and sequencing the plasmids from the positive colonies, we performed a bioinformatics analysis. A coimmunoprecipitation assay was performed to confirm the interaction between 14-3-3 $\sigma$  and the proteins obtained from the positive colonies. Finally, we constructed 14-3-3 $\sigma$  and potassium channel modulatory factor 1 (KCMF1) siRNA expression plasmids and transfected them into colon cancer stem cells.

**RESULTS:** The bait plasmid pGBKT7-14-3-3 $\sigma$  was constructed successfully, and the 14-3-3 $\sigma$  protein had no toxic or autonomous activation effect on the yeast. Nineteen true-positive colonies were selected and sequenced, and their full-length sequences were obtained. We searched for homologous DNA sequences for these sequences from GenBank. Among the positive colonies, four coding genes with known functions were obtained, including *KCMF1*, quinone oxidoreductase (*NQO2*), hydroxyisobutyrate dehydrogenase (*HIBADH*) and 14-3-3 $\sigma$ . For the subsequent coimmunoprecipitation assay, the plasmids PCDEF-Flag-14-3-3 $\sigma$ , PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH were successfully constructed, and the sequences were further confirmed by DNA sequencing. The Fugene 6 reagent was used to transfect the plasmids, and fluorescence-activated cell sorting analysis showed the transfection efficiency was 97.8% after 48 h. The HEK 293FT cells showed the stable expression of the PCDEF-Flag-14-3-3 $\sigma$ , PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids. After anti-Myc antibody immunoprecipitation with Myc-KCMF1, Myc-NQO2 and Myc-HIBADH from cell lysates, the presence of Flag-14-3-3 $\sigma$  protein in the immunoprecipitated complex was determined by western blot analysis. The knock-down expression of the 14-3-3 $\sigma$  and KCMF1 proteins significantly inhibited cell proliferation and colony formation of SW1116csc.

**CONCLUSION:** Genes of the proteins that interacted

with 14-3-3 $\sigma$  were successfully screened from a HeLa cDNA library. KCMF1 and 14-3-3 $\sigma$  protein may affect the proliferation and colony formation of human colon cancer stem cells.

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**Key words:** 14-3-3 $\sigma$  protein; Interacting proteins; Yeast two-hybrid system; Colon cancer stem cells

**Core tip:** Of the 14-3-3 proteins, tumor-suppressor activity has most clearly been defined for 14-3-3 $\sigma$ . In the study, we constructed 14-3-3 $\sigma$  bait gene and expressed as a fusion to the GAL4 DNA-binding domain successfully. Using the yeast two-hybrid system, we found novel binding proteins from the HeLa cDNA library which closely interact with 14-3-3 $\sigma$ . Our results also suggest that 14-3-3 $\sigma$  may interact with potassium channel modulatory factor 1 (KCMF1) protein. The knock-down expression of 14-3-3 $\sigma$  and KCMF1 proteins significantly inhibited proliferation and colony formation of SW1116csc cells. So, 14-3-3 $\sigma$  and other proteins may be involved in proliferation and colony formation of human colon cancer stem cells.

Zou J, Mi L, Yu XF, Dong J. Interaction of 14-3-3 $\sigma$  with KCMF1 suppresses the proliferation and colony formation of human colon cancer stem cells. *World J Gastroenterol* 2013; 19(24): 3770-3780 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3770.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3770>

## INTRODUCTION

The 14-3-3 proteins are among the most abundant proteins within the cell, having been initially identified in 1967 as a family of acidic proteins within the mammalian brain. This family of highly conserved proteins consisting of seven isotypes in human cells ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\eta$ ,  $\sigma$ ,  $\tau$ ,  $\xi$ ) plays crucial roles in regulating multiple cellular processes, including the maintenance of cell cycle checkpoints and DNA repair, the prevention of apoptosis, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility. All 14-3-3 proteins bind to phosphoserine/phosphothreonine-containing peptide motifs corresponding to the sequences RSXp-SXP or RXXXpSXP<sup>[1]</sup>. Many 14-3-3-binding proteins contain sequences that closely match these motifs, although a number of ligands bind to 14-3-3 in a phospho-independent manner using alternative sequences that do not closely resemble these motifs. Pozuelo Rubio *et al*<sup>[2]</sup> recently used 14-3-3-affinity chromatography and mass spectrometry to identify more than 200 14-3-3-binding ligands. All of these ligands lost their ability to bind to 14-3-3 upon dephosphorylation by the serine/threonine phosphatase PP2A *in vitro*. Some of the proteins bound to the 14-3-3-affinity column contained sequences that

closely matched the optimal binding motifs, while others diverged significantly from the consensus sequences, despite their apparent phospho-specific binding.

Among the 14-3-3 proteins, 14-3-3 $\sigma$  is the isoform most directly linked to cancer<sup>[3]</sup>. There are several lines of evidence indicating that 14-3-3 $\sigma$  acts as a tumour suppressor gene and that its inactivation is crucial in tumorigenesis. The downregulation of 14-3-3 $\sigma$  by CpG methylation is detected in adenocarcinoma of the breast (96%), squamous cell carcinoma of the vulva (60%), lung cancer (83%), hepatocellular carcinoma (89%), ovarian carcinoma (60%), endometrioid endometrial adenocarcinoma (74%), gastric adenocarcinoma (43%), basal cell carcinoma (68.3%), squamous cell carcinoma of the bladder, neuroendocrine tumours (85%), and prostate cancer (45%)<sup>[4-10]</sup>. In our former proteomic study on human colon cancer stem cells, we found the expression of 14-3-3 $\sigma$  was obviously increased in colon cancer stem cells compared with differentiated cancer cells<sup>[11]</sup>. 14-3-3 $\sigma$  may be involved in the course of self-renewal, proliferation and differentiation of colon cancer stem cells.

To better understand the role of 14-3-3 $\sigma$  in the tumorigenesis, self-renewal, and differentiation of stem cells, we used the yeast two-hybrid system to find novel binding proteins that interact with 14-3-3 $\sigma$ . The bait gene, 14-3-3 $\sigma$ , was expressed as a fusion to the GAL4 DNA-binding domain, while the HeLa cDNA library was expressed as a fusion to the GAL4 activation domain. When the bait and library fusion proteins interact, the DNA-binding domain and activation domain are brought into proximity, thus activating the transcription of the reporter genes. Using this system, we found novel binding proteins from the HeLa cDNA library that closely interact with 14-3-3 $\sigma$ . This investigation provides functional clues for further exploration into novel cancer-related proteins for the treatment of colon cancer.

## MATERIALS AND METHODS

### *Bacteria, yeast strains and plasmids*

All yeast strains and plasmids for the yeast two-hybrid experiments were obtained from Clontech (Palo Alto, CA, United States) as components of the MATCHMAKER two-hybrid system 3. The *Escherichia coli* (*E. coli*) strain DH5 $\alpha$  was used to clone every shuttle plasmid. The pGBKT7 DNA binding domain (DNA-BD) cloning plasmid, pGADT7 AD cloning plasmid, pGBKT7-53 control plasmid, pGADT7, pGBKT7-Lam control plasmid and pCL1 plasmid were obtained from Clontech Ltd. (K1612-1). The HeLa MATCHMAKER cDNA library was also obtained from Clontech Ltd.

### *Chemical agents and culture media*

Taq DNA polymerase, T4 DNA ligase, EcoRI and BglII restriction endonuclease were purchased from Takara Company, Japan. Lithium acetate, semi-sulphate adenine, acrylamide and N,N'-bis-acrylamide were purchased from Sigma Company United States. Tryptone and yeast ex-

tracts were purchased from the Oxoid Company, United States. X- $\alpha$ -gal and the culture media YPDA, SD/-Trp, SD/-Leu, SD/-Trp/-Leu, SD/-Trp/-Leu/-His, SD/-Trp/-Leu/-His/-Ade were purchased from Clontech, United States.

#### Total RNA isolation human colon cancer stem cells

Human colon cancer stem cells (SW1116csc) were isolated and maintained in serum-free DMEM/F12 medium supplemented with human recombinant epidermal growth factor (20  $\mu$ g/L; Invitrogen), human recombinant basic fibroblast growth factor (20  $\mu$ g/L; Invitrogen), L-glutamine (2 mmol/L), insulin (4 U/L), penicillin G ( $1 \times 10^5$  U/L), and streptomycin (100 mg/L). Total RNA was isolated with TRIzol reagent (Invitrogen, United States) according to the manufacturer's instructions. The total RNA recovered from the DNase I digestion was measured at 260 and 280 nm with a spectrophotometer (Ultraspec 2000, Pharmacia Biotech), with the 260 nm reading used to estimate the concentration of total RNA. The 260/280-nm ratios and a 1% agarose-formaldehyde gel stained with ethidium bromide were used to confirm the RNA quality of the samples.

#### Construction of bait plasmid and expression of the 14-3-3 $\sigma$ protein

To make the bait plasmid, reverse transcription polymerase chain reaction (RT-PCR) was performed to amplify the 14-3-3 $\sigma$  gene from SW1116csc cells. The sequences of the primers contained *Eco*RI and *Bgl*II restriction enzyme sites. The PCR conditions were as follows: 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min, for 35 cycles. Ten nanograms of the 747 bp PCR product were cloned into the pGEM-T vector. The primary structure of the insert was confirmed by direct sequencing. The fragment encoding 14-3-3 $\sigma$  was released from the pGEM-T-14-3-3 $\sigma$  by digestion with *Eco*RI and *Bgl*II and was then ligated into pGBKT7. Vector pGBKT7-expressing proteins were fused with amino acids 1-147 of the GAL4 DNA-BD, and pGADT7-expressing proteins were fused with amino acids 768-881 of the GAL4 activation domain. The plasmid pGBKT7-14-3-3 $\sigma$ , containing the full-length 14-3-3 $\sigma$  gene, could directly express the DNA binding domain, c-Myc and 14-3-3 $\sigma$  fusion protein. The plasmid was transformed into the yeast strain AH109 with the lithium acetate method.

#### Toxicity and autonomous activation assays

The purified bait plasmid was transformed into the AH109 strain and was then cultured on SD/-Trp/agar plates for detection. Approximately 2 mm of AH109 colonies, transformed by pGBKT7-14-3-3 $\sigma$  and pGBKT7, were incubated in 3 mL YPDA liquid medium at 30 °C for 16 h with shaking. The absorbance values at 600 nm ( $A_{600}$ ) in different groups were compared. Additionally, transformants containing the pGBKT7-14-3-3 $\sigma$  and pGBKT7 plasmids were transferred to SD/-Trp/X-a-gal,

SD/-Trp/-his/X-a-gal and SD/-Ade/-Trp/X-a-gal plates at 30 °C for 5 d. In parallel, AH109 cells transformed by pCL1 and pGBKT7-Lam served as the positive and negative controls, respectively.

#### Screening of the HeLa cDNA library by the yeast two-hybrid system

The screening of the HeLa cDNA library was performed as follows. One large (2-3 mm), fresh (< 2 mo old) colony of AH109 (bait) was inoculated into 50 mL SD/-Trp and incubated and shaken at 250-270 r/min at 30 °C overnight (16-24 h). Then, the cells were pelleted by centrifuging the entire 50-mL culture at 1000 r/min for 5 min. After the supernatant was decanted, the cell pellet was resuspended in the residual liquid by vortexing. A HeLa cDNA library was cloned into pACT2 and the yeast reporter strain Y187. The entire AH109 (bait) culture and 1 mL of the HeLa cDNA library were combined and cultured in a 2-L sterile flask; 45 mL of 2  $\times$  YPDA/Kan was added and swirled gently. After a 20-h mating period, the cells were pelleted, re-suspended and spread on 50 large (150 mm) plates containing 100 mL SD/-Ade/-His/-Leu/-Trp. After 6-15 d, the yeast colonies were transferred onto plates containing X- $\alpha$ -gal to evaluate the expression of the MEL1 reporter gene (blue colonies).

#### Isolation and transformation of yeast plasmids

Approximately  $1 \times 10^6$  colonies were screened, and positive clones were identified. The yeast plasmids were isolated from the positive colonies with the lyticase method and transformed into super-competent *E. coli* DH5 $\alpha$  by electroporation. The transformants were plated on ampicillin SOB selection media and grown under selection conditions. Subsequently, the pACT2-cDNA constructs were re-isolated following the standard protocol, analysed by restriction digestion and sequenced.

#### Bioinformatics analysis

After the positive colonies were sequenced, the sequences were blasted against sequences in GenBank to analyse the function of the genes (<http://www.ncbi.nlm.nih.gov/blast>). Other bioinformatics analyses, including their molecular weight, theoretical PI, estimated half-life, secondary structure prediction, and so on, were respectively performed with different software tools ([www.expasy.ch/tools/protparam.html](http://www.expasy.ch/tools/protparam.html); [www.cmpharm.ucsf.edu/nom/npredict.html](http://www.cmpharm.ucsf.edu/nom/npredict.html); [http://www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html); [www.expasy.org/tools/protscale.html](http://www.expasy.org/tools/protscale.html); [http://www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html); <http://www.cbs.dtu.dk/services/SignalP/>).

#### Plasmid constructs and transfection

The plasmids in positive yeast clones were isolated from the colonies by the lyticase method. The 14-3-3 $\sigma$ , potassium channel modulatory factor 1 (*KCMF1*), quinone oxidoreductase (*NQO2*) and hydroxyisobutyrate dehydrogenase (*HIBADH*) genes were PCR amplified with

**Table 1** Different plasmids transfected into HEK 293FT cells

| Plasmid     | 1        | 2         | 3        | 4         | 5        | 6         | 7         | 8        | Transfection control |          |
|-------------|----------|-----------|----------|-----------|----------|-----------|-----------|----------|----------------------|----------|
|             | Sample 1 | Negative  | Sample 2 | Negative  | Sample 3 | Negative  | Negative  | Positive | Positive             | Negative |
|             | KCMF1    | Control-1 | NQO2     | Control-2 | HIBADH   | Control-3 | Control-4 |          |                      |          |
| Flag-mfrP53 | -        | -         | -        | -         | -        | -         | -         | +        |                      |          |
| Myc-Large T | -        | -         | -        | -         | -        | -         | -         | +        |                      |          |
| Flag-14-3-3 | +        | -         | +        | -         | +        | -         | +         | -        |                      |          |
| Myc-HIBADH  | -        | -         | -        | -         | +        | +         | -         | -        | pEGFP                | 293FT    |
| Myc-NQO2    | -        | -         | +        | +         | -        | -         | -         | -        |                      |          |
| Myc-KCMF1   | +        | +         | -        | -         | -        | -         | -         | -        |                      |          |
| VECTOR-Myc  | -        | -         | -        | -         | -        | -         | -         | -        |                      |          |
| VECTOR-Flag | -        | +         | -        | +         | -        | +         | -         | -        |                      |          |

KCMF1: Potassium channel modulatory factor 1; NQO2: Quinone oxidoreductase; HIBADH: Hydroxyisobutyrate dehydrogenase.

specific primers, and the products were characterised by restriction digest using SfiI. After PCDEF-Flag and PCDEF-Myc plasmids were digested with the SfiI restriction enzyme, 14-3-3 $\sigma$ , KCMF1, NQO2 and HIBADH cDNA were cloned into them. The correct plasmids were named PCDEF-Flag-14-3-3 $\sigma$ , PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH. The plasmid sequences were analysed by DNA sequencing. HEK 293FT cells ( $5 \times 10^5$  cells) were cultured in a 6-cm dish until 60% confluence was reached. Various types of plasmids were transfected using the Fugene 6 Transfection Kit (Roche) according to the manufacturer's instructions (Table 1). Briefly, 1.5  $\mu$ L Fugene 6 was diluted with 100  $\mu$ L Opti-MEM (Invitrogen), mixed, and incubated at room temperature for 5 min. A 0.5  $\mu$ g quantity of plasmid was added to the Fugene 6/Opti-MEM combination at a 3 (DNA):1 (Fugene) ratio, then mixed and incubated at room temperature for 45 min. The mixture of Fugene 6 and plasmid DNA was then added to cells cultured in 0.5 mL fresh Opti-MEM and incubated at 37 °C in 5% CO<sub>2</sub>. After 14-16 h, the transfection reagent was replaced with fresh culture medium; 48 h later, the transfected cells were harvested and fluorescence-activated cell sorting (FACS) was performed.

### Fluorescence-activated cell sorting

FACS of enhanced green fluorescent protein (EGFP) positive cells was performed on a FACS Aria (Becton Dickinson). The cells were washed twice with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS and then incubated with 50 U papain (Worthington) and 100 U DNase I (Sigma) in PIPES at 37 °C for 10 min with gentle shaking. The samples were spun, resuspended in 2 mL DMEM/F12/N2 and dissociated by sequentially titrating with three serially narrowed glass Pasteur pipettes. The papain was inactivated with DMEM/F12/N2 plus 20% FBS, and the cells were pelleted. The pelleted cells were resuspended in HBSS and then passed over a 40  $\mu$ m cell strainer. The cells were resuspended at a final concentration of (3-6)  $\times 10^6$  cells/mL with 1.0  $\mu$ g 7-amino-actinomycin D (7-AAD). The cells were analysed by forward and side scatter for EGFP fluorescence through a 530  $\pm$  30 nm bandpass filter and for 7-AAD fluorescence through a 695  $\pm$  40 nm

bandpass filter. The EGFP-positive cells were sorted at 2000-5000 events/s with a purification mode algorithm. Untransfected cells were used as a control to set the background fluorescence; a false-positive rate of 0.1% was accepted to ensure an adequate yield.

### Coimmunoprecipitation and Western blotting

The HEK 293FT cell line was grown in accordance with the ATCC recommendations. Forty-eight hours after plasmid transfection, the cells were lysed in ice-cold 1% Triton X-100 buffer containing a cocktail of protease inhibitors. The lysates were cleared by centrifugation at 12000 *g* for 15 min at 4 °C. Coimmunoprecipitation assays using cleared cell lysates were performed at 4 °C for 2 h with the appropriate antibody. Immune complexes were precipitated with protein G Sepharose beads for an additional 1 h, washed three times with cold lysis buffer, resuspended in 16 Laemmli sample buffer, boiled for 5 min, subjected to SDS-PAGE and transferred to NC filters. The NC filters were blocked for 1 h at 4 °C in 5% nonfat milk in TBS (50 mmol/L Tris, 150 mmol/L NaCl) containing 0.1% Tween-20 (Sigma). They were then incubated for 2 h with primary antibodies (1:1000 dilution) in the blocking solution. After extensive washes in TBS 0.1% Tween-20, the filters were incubated for 1 h with HRP-conjugated anti-mouse antibody (Serotech) diluted 1:5000 in TBS 5% nonfat milk solution. After final washes in TBS 0.1% Tween, Western blottings were developed with the ECL kit from Amersham Biosciences.

### siRNA plasmid constructs and transfection

Selection of the siRNA sequence was based on the siRNA Target Finder and Design Tool available at the Ambion Inc. web site and related reference. The siRNAs targeting human 14-3-3 $\sigma$  and KCMF1 mRNA common sequence 5'-CCCAGAAGAUGGACUUCUA-3' and 5'-CGCGU-GUCGAAGACUAUUU-3' were synthesised and purified by Shanghai Sangon Corporation. The sense strand of the pU-siRNA inserts was 5'-GATCCACCTCACCAAGGC-CAGCACTTCAAGAGAGCTGGCCTTGGTGAG-GTTTTTTTTTGGAAAGTCGACA-3'; it was inserted into *Bam*HI-*Hind*III linearised pRNAT-U6.1/neo vector (Ambion Inc., Austin, TX, United States). The inserted

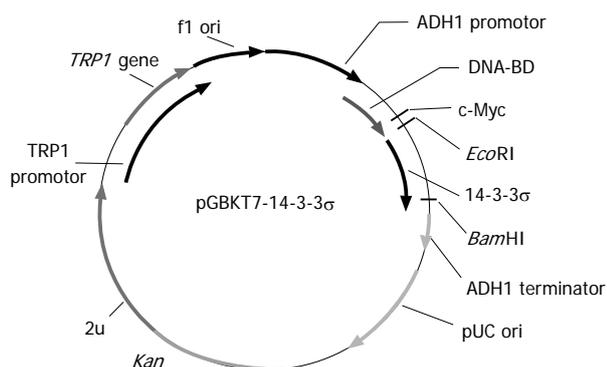


Figure 1 Map of bait plasmid pGBKT7-14-3-3 $\sigma$ .

sequences were verified by DNA sequencing. The control siRNA vector (pU-siCONT; Ambion Inc.) was constructed by the insertion of a sequence that expressed a hairpin siRNA with no significant homology to any known sequences in the human, mouse or rat genomes. The control insert sequence was: 5'-GATCCACTACCGTTGTTATAGGTGTTCAAGAGACACCTATAACAACGGTAGTTTTTTGGAAA-3'. One microgram per well of pU-14-3-3 $\sigma$ -siRNA, pU-KCMF1-siRNA expression plasmid or control plasmid (pU-siCONT) were transfected into SW1116csc. Forty-eight hours later, transfection-positive cells were observed under a fluorescence microscope.

#### Cell proliferation assay and colony formation in soft agar

SW1116csc and siRNA-transfected SW1116csc cells were seeded at a density of  $1 \times 10^4$  in 35-mm Petri dishes. The cultured cells stained with trypan blue were observed and counted in triplicate over 6 wk. The cells were disassociated, suspended in medium containing 0.3% agar, and plated onto a bottom layer containing 0.6% agar. The cells were plated at a density of  $3 \times 10^4$  cells/6-cm dish, and the number of colonies that were  $> 0.5$  mm in diameter was counted 14 d later.

## RESULTS

### RNA quality characterisation

Total RNA was extracted from  $1 \times 10^8$  SW1116csc cells and yielded approximately 10  $\mu$ g of high purity total RNA. The absorbance ratios of the RNA at 260/280 and 230/260 nm were 2.03 and 2.00, respectively, indicating that the RNA was of the highest quality and was therefore useful for the following experiments.

### Construction of the pGBKT7-14-3-3 $\sigma$ plasmid

The bait plasmid pGBKT7-14-3-3 $\sigma$  was constructed with complete E6 cDNA successfully, and the results of sequencing analysis suggested that the cDNA was in-frame, no artefacts were added to the E6 sequence and the restriction sites were correct (Figure 1).

### Toxicity and autonomous activation assays

Two to three millimetre AH109 colonies, transformed

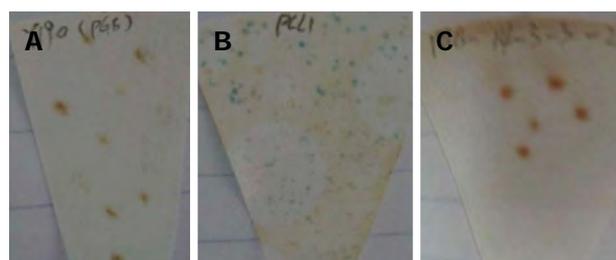


Figure 2 X-Gal filter assay analysis result. A: Negative control plasmid pGBKT7; B: Positive control plasmid pCL1; C: pGBKT7-14-3-3 $\sigma$  plasmid.

by pGBKT7-14-3-3 $\sigma$  and pGBKT7, were incubated in 3 mL YPDA liquid medium. The  $A_{600}$  nm values in the AH109-pGBKT7-14-3-3 $\sigma$  and AH109-pGBKT7 groups were 0.98 and 0.99, respectively, which suggested that the pGBKT7-14-3-3 $\sigma$  plasmid was not toxic to yeast and had no effect on the growth of the yeast. Furthermore, the AH109-pGBKT7-14-3-3 $\sigma$  clones were white and were detected on the SD/-Trp/-His/X- $\alpha$ -gal and SD/-Ade/-Trp/X- $\alpha$ -gal plates (Figure 2). Therefore, the 14-3-3 $\sigma$  protein was believed to have no autonomous activation effect.

### Screening for positive clones

Diploids were detected under an inverted microscope 20 h after co-incubation, which indicated that yeast mating was successful. Nineteen positive colonies grew on the SD/-Ade/-His/-Leu/-Trp/X- $\alpha$ -gal agar medium and restreaked three times. Finally, 19 putative positive yeast colonies were obtained.

### Gene sequencing and analysis

Nineteen positive yeast colonies were selected, and each purified plasmid DNA was directly transferred to competent DH5 $\alpha$  cells by electroporation. The transformants containing only the pGADT7-HeLa cDNA library plasmids were obtained by plating on LB/Amp agar medium. Sequencing analysis was performed, and 4 genes that interacted with 14-3-3 $\sigma$ , including *KCMF1*, *NQO2*, *HIBADH* and 14-3-3 $\sigma$ , were identified (Table 2).

### Verification of protein interactions between X and Y in yeast

The diploids were detected under an inverted microscope, and blue colonies were grown on the SD/-Ade/-His/-Leu/-Trp/X- $\alpha$ -gal agar medium.

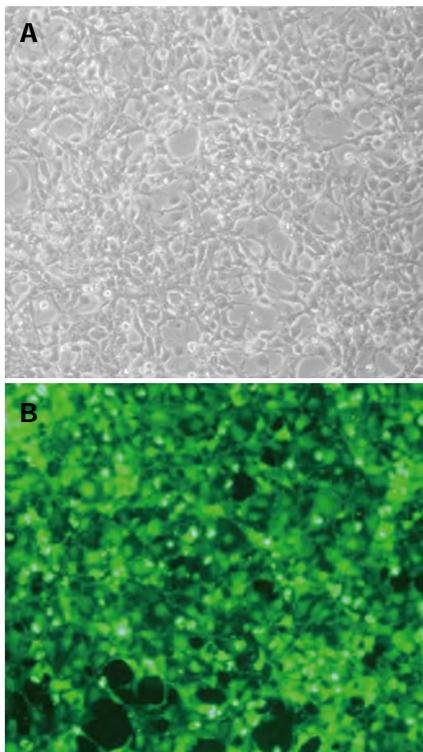
### Plasmid constructs and transfection efficiency

The PCDEF-Flag-14-3-3 $\sigma$ , PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids were successfully constructed. The sequences of the plasmids were further confirmed by DNA sequencing. The plasmids were transfected into HEK 293FT cells with the Fugene 6 reagent (Figure 3). After 48 h, FACS assay was performed and showed that the transfection efficiency was 97.8% (Figure 4).

**Table 2** Positive hit of the screening HeLa cDNA library

| Bait       | Prey library                       | Identical colonies | Positive clone name | Positive gene identified | NCBI accession number | Gene code match |             |             |
|------------|------------------------------------|--------------------|---------------------|--------------------------|-----------------------|-----------------|-------------|-------------|
| pGB-14-3-3 | Human HeLa MATCHMAKER cDNA library | 19                 | Sigma 12            | SFN                      | NM_006142.3           | Yes             | Full Length |             |
|            |                                    |                    | Sigma 13            |                          |                       |                 |             | Length      |
|            |                                    |                    | Sigma 30            |                          |                       |                 |             |             |
|            |                                    |                    | Sigma 4             | HIBADH                   | NM_152740.3           | Yes             | Full Length |             |
|            |                                    |                    | Sigma 15            |                          |                       |                 |             | Length      |
|            |                                    |                    | Sigma 21            | NQO2                     | NM_000904.3           | Yes             | Full Length |             |
|            |                                    |                    | Sigma 32            |                          |                       |                 |             | Length      |
|            |                                    |                    | Sigma 23            |                          |                       |                 |             |             |
|            |                                    |                    | Sigma 33            |                          |                       |                 |             | Full Length |
|            |                                    |                    | Sigma 28            | KCMF1                    | NM_020122.4           | Yes             | Length      |             |

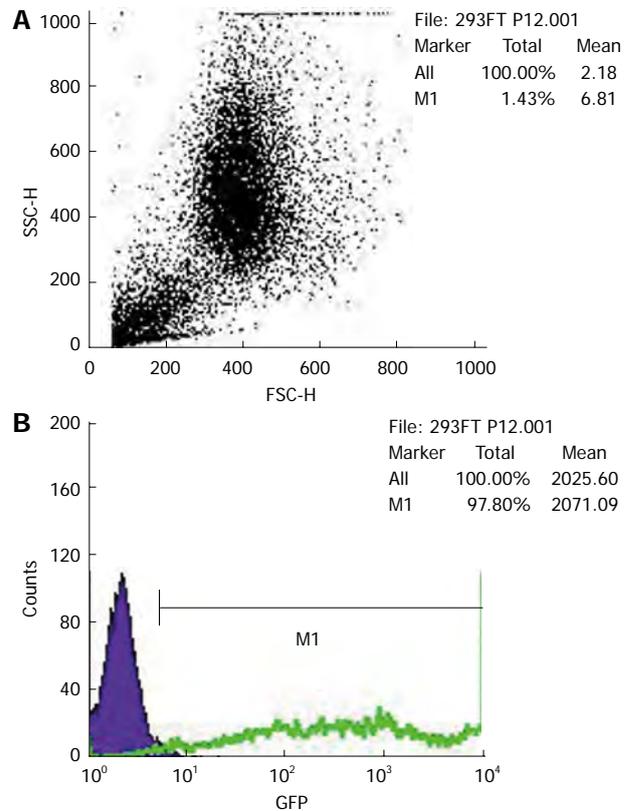
KCMF1: Potassium channel modulatory factor 1; NQO2: Quinone oxidoreductase; HIBADH: Hydroxyisobutyrate dehydrogenase.



**Figure 3** Plasmids transfected with the Fugene 6 transfection reagent. A: HEK 293FT cells in light phase 48 h after transfection with plasmids; B: HEK 293FT cells in fluorescent phase 48 h after transfection with plasmids.

### Confirmation of interaction by coimmunoprecipitation

Three interactions detected in this two-hybrid screen were further confirmed in HEK 293FT cells by specific coimmunoprecipitation of Flag-tagged bait proteins with the three Myc-tagged prey proteins. HEK 293FT cells stably expressed the PCDEF-Flag-14-3-3 $\sigma$ , PCDEF-Myc-KCMF1, PCDEF-Myc-NQO2 and PCDEF-Myc-HIBADH plasmids (Figure 5A). An anti-Myc antibody was used to immunoprecipitate Myc-KCMF1, Myc-NQO2 and Myc-HIBADH from cell lysates. The presence of Flag-14-3-3 $\sigma$  protein in the immunoprecipitated complex was determined by western blot analysis (Figure 5B). These coimmunoprecipitations confirm that several of the novel interactions identified in the present two-hybrid screen are reproducible in the context of mammalian

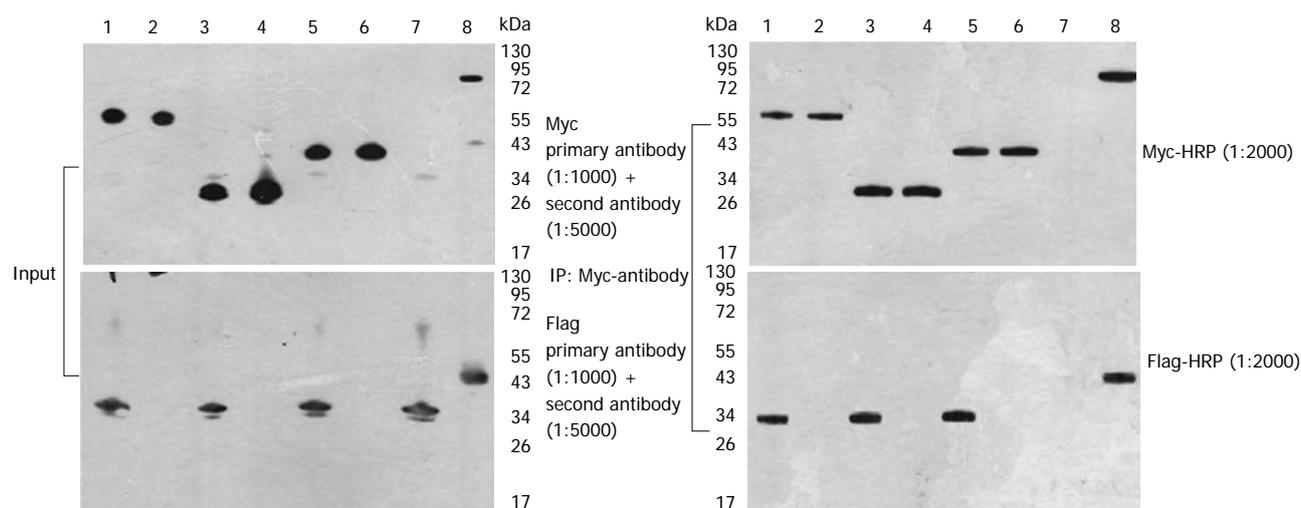


**Figure 4** Fluorescence-activated cell sorting assay. It demonstrates the method for calculating green fluorescent protein (GFP)-positive cells with fluorescence-activated cell sorting. The debris is gated out, and the target populations are identified and positively or negatively selected. The dot plots represent 10000 events, with the side scatter and forward scatter plots (A) and the fluorescence intensity of GFP (B). Figure B shows a histogram demonstrating the GFP-positive cell populations. The histogram plots the fluorescence of GFP-positive cells on region M1 against counts on the Y-axis.

cells and therefore validate the results obtained by the two-hybrid assay.

### Inhibition of cell proliferation and colony formation of SW1116csc by siRNA plasmid transfection

After pU-14-3-3 $\sigma$ -siRNA or pU-KCMF1-siRNA expression plasmid transfection, the knock-down of the 14-3-3 $\sigma$  and KCMF1 proteins in SW1116csc cells was observed (Figure 6A). We tested for differences in the



**Figure 5** Expression and interaction of 14-3-3 $\sigma$ , potassium channel modulatory factor 1, quinone oxidoreductase, hydroxyisobutyrate dehydrogenase and 14-3-3 $\sigma$  plasmids in HEK 293FT cells. A: Expression of the 14-3-3 $\sigma$ , potassium channel modulatory factor 1 (KCMF1), quinone oxidoreductase (NQO2), hydroxyisobutyrate dehydrogenase (HIBADH) plasmids. HEK 293FT cells were transfected with different plasmids. Forty-eight hours post-transfection, the cells were lysed, and Western blotting analysis was performed with an anti-Myc or anti-Flag antibody (1:1000); B: Interaction of 14-3-3 $\sigma$  and KCMF1, NQO2 or HIBADH. HEK 293FT cells were transfected with different plasmids. Forty-eight hours post-transfection, the cells were lysed and immunoprecipitations were performed with an anti-Myc antibody (1:2000). After the proteins were resolved by SDS-PAGE, immunoblot analysis was performed with antibodies against Flag protein (1:2000). 1: Sample 1 KCMF1; 2: Negative Control-1; 3: Sample 2 NQO2; 4: Negative Control-2; 5: Sample 3 HIBADH; 6: Negative Control-3; 7: Negative Control-4; 8: Positive.

proliferation rate between SW1116csc and siRNA-transfected SW1116csc. The cells were examined from week 1 to week 7 after seeding. As shown in Figure 6B, there was a difference in the growth rate between the transfected and control cells. The transfected cells grew slowly and showed growth inhibition after week 4. The self-renewing capacity of the transfected cells was also examined with the colony formation assay. When plated at a density of 100 cells/well, 14-3-3 $\sigma$ -siRNA, KCMF1-siRNA and 14-3-3 $\sigma$  + KCMF1-siRNA transfected cells generated a lower mean number of tumour spheres ( $57.4 \pm 3.6$ ,  $55.3 \pm 3.3$  and  $23.7 \pm 2.8$ , respectively) compared to the SW-1116csc cells ( $71.4 \pm 4.1$ ) (Figure 6C).

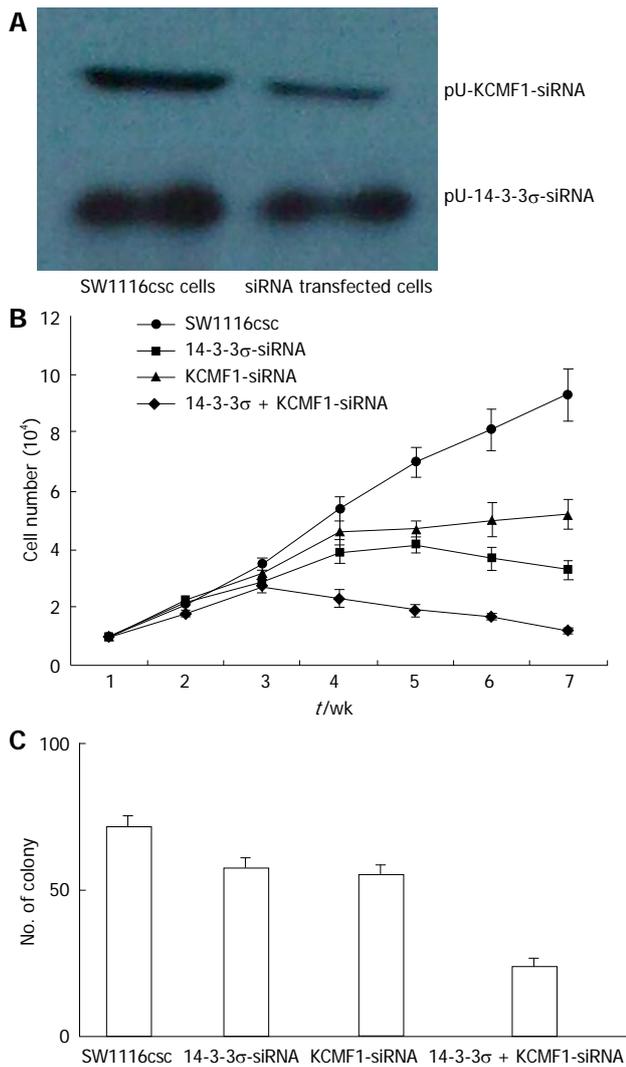
## DISCUSSION

Protein-protein interactions occur in a wide variety of biological processes and essentially control cell fate from division to death. Yeast two-hybrid assays represent a versatile tool to study protein interactions *in vivo*. The yeast two-hybrid system 3, based on the system originally designed by Fields and Song, takes advantage of the properties of the GAL4 protein of the yeast *Saccharomyces cerevisiae*. The GAL4-based assay uses the yeast transcription factor GAL4 for the detection of protein interactions by transcriptional activation. GAL4 possesses a characteristic phenomenon in which the transactivation function can be restored when the factor's DNA-binding domain (DBD) and its transcription-AD are brought together by two interacting heterologous proteins. The GAL4-yeast two-hybrid assay uses two expression vectors, one with DBD and the other with AD. The GAL4-DBD fuses to protein "X" and GAL4-AD fuses to protein "Y" to form the bait and the target of the interaction

trap, respectively. A selection of host cells with different reporter genes and different growth selection markers provides a means to detect and confirm protein-protein interactions and has significantly fewer false positives.

To investigate the role of 14-3-3 $\sigma$  in tumorigenesis, the yeast two-hybrid system 3 was used to screen the proteins interacting with 14-3-3 $\sigma$ . In this study, the bait plasmid pGBKT7-14-3-3 $\sigma$  was transformed into the yeast strain AH109. To further confirm the expression of 14-3-3 $\sigma$  protein in the AH109 yeast strain, we performed Western blotting analysis and observed strong 14-3-3 $\sigma$  expression. After the bait plasmid pGBKT7-14-3-3 $\sigma$  yeast strain AH109 was mated with the HeLa cDNA library yeast strain Y187, resulting diploid yeast cells were plated on QDO media containing X-a-gal. Nineteen true positives were confirmed and obtained. Through sequencing analysis of isolated library plasmids, we obtained the sequences of four types of genes with known functions.

In addition to their well-known pro-proliferative and anti-apoptotic effects, 14-3-3 proteins have also been found to suppress cell growth and cell-cycle progression, especially after DNA damage, indicating functions in tumour suppression<sup>[12-15]</sup>. Of the 14-3-3 proteins, the tumour-suppressor activity has most clearly been defined for 14-3-3 $\sigma$ <sup>[16-19]</sup>. 14-3-3 $\sigma$  is unique among the 14-3-3 proteins in that it is expressed primarily in epithelial cells and forms homodimers almost exclusively<sup>[20]</sup>. Further insight as to why the loss of 14-3-3 $\sigma$  might facilitate tumor formation comes from the discoveries by Wilker *et al*<sup>[21]</sup> reported that 14-3-3 $\sigma$  is a crucial regulator of translation during mitosis and that 14-3-3 $\sigma$  function is required for proper mitotic exit and cytokinesis. In eukaryotic cells, most mRNA translation occurs *via* a cap-dependent



**Figure 6** Effects of 14-3-3 $\sigma$  and potassium channel modulatory factor 1 protein expression on cell proliferation and colony formation of colon cancer stem cells. A: Expression of 14-3-3 $\sigma$  and potassium channel modulatory factor 1 (KCMF1) proteins in SW1116csc and siRNA transfected cells; B: Growth curves of SW1116csc, 14-3-3 $\sigma$  siRNA transfected, KCMF1 siRNA transfected and 14-3-3 $\sigma$  + KCMF1 siRNA transfected cells. The mean  $\pm$  SD are shown; C: Colony formation after the incubation of 100 separate cells for 14 d. The mean  $\pm$  SD are shown.

mechanism in which ribosome recruitment begins with the binding of eukaryotic initiation factors, such as eIF4B, to a modified guanosine residue (known as a “cap”) at the 5’ end of the mRNA. However, some mRNAs contain internal ribosome entry sites and are translated in a cap-independent manner. During mitosis, cap-dependent translation is suppressed and cap-independent translation is stimulated, allowing for the translation of key cell-cycle regulators such as cell division cycle 2-like 1. Experiments by Wilker *et al*<sup>[21]</sup> showed that 14-3-3 $\sigma$  is needed for the mitotic switch from cap-dependent to cap-independent translation and that 14-3-3 $\sigma$  appears to mediate this switch by binding to eIF4B and perhaps other factors involved in cap-dependent translation. When cells are depleted of 14-3-3 $\sigma$ , cap-dependent translation is not suppressed and cytokinesis is impaired, resulting in

the generation of binucleated cells, a phenotype observed in the early stages of tumour formation.

14-3-3 acts as an adaptor or “chaperone molecule”, which is able to move freely from the cytoplasm to the nucleus and vice-versa<sup>[22]</sup>. 14-3-3 proteins are mainly cytoplasmic molecules; they can form homodimers or heterodimers, and interact with various cellular proteins. 14-3-3 proteins are phosphoserine-binding proteins that bind the consensus motifs RSXpSXP and RXY/FXp-SXP. These consensus motifs are present in almost all of the 14-3-3 binding proteins<sup>[1]</sup>. More than a hundred small molecules interact with 14-3-3 in a phosphorylation-dependent manner. These proteins include protein kinases (murine leukaemia viral oncogene homologue-RAF1, MEK kinase, PI3 kinase and Grb10), receptor proteins (insulin-like growth factor 1 and glucocorticoid receptors), enzymes (serotonin N-acetyltransferase, tyrosine and tryptophan hydroxylase), structural and cytoskeletal proteins (vimentins and keratins), scaffolding molecules (calmodulin), proteins involved in cell cycle control (cdc25, p53, p27 and wee1) proteins involved in transcriptional control (histone acetyltransferase, and TATA box binding proteins), and proteins involved in apoptosis (BAD)<sup>[1,23]</sup>. However, a few proteins interact with 14-3-3 in a phosphorylation-independent manner such as *Bax*. Recently, using direct proteomic analysis, researchers have identified a large number of polypeptides (> 200) that can associate with 14-3-3 proteins. These polypeptides are involved in numerous cell functions, including fatty acid synthesis, reductive metabolism, iron and other metabolisms, DNA/chromatin interactions including transcription factors, RNA binding, protein synthesis, protein folding and processing, proteolysis, protease inhibitors, ubiquitin metabolism, cellular signaling and apoptosis, actin dynamics, cellular trafficking and transporters, signaling kinases, cell division, nuclear proteins, oncogenic signaling, and cytoskeletal proteins<sup>[2,24]</sup>. A study demonstrated that some of the 14-3-3 binding proteins are involved in the regulation of the cytoskeleton, GTPase functions, membrane signaling, and cell fate determination<sup>[25]</sup>. In this study, we found that 14-3-3 $\sigma$  could interact with the proteins KCMF1, NQO2, HIBADH and 14-3-3 $\sigma$ .

The function of NQO2 is not clearly understood. NQO2 is expressed selectively in the kidneys, skeletal muscles, liver, heart, and lungs, suggesting a tissue-specific action of the enzyme. Some studies have suggested that greater NQO2 expression may activate certain types of chemicals in the brain, leading to oxidative stress and neuronal damage<sup>[26]</sup>. Other studies have implied that NQO2 can protect against quinone-induced skin carcinogenesis<sup>[27]</sup>. Recently, new evidence has shown for the first time that NQO2 catalyses the reduction of electrophilic oestrogen quinones and thereby acts as a detoxification enzyme. Gaikwad *et al*<sup>[28]</sup> successfully demonstrated that oestrogen-3,4-quinone binds to NQO2 and established that oestrogen quinines are endogenous biological substrates of NQO2. Moreover, they demonstrated that NQO2 is faster at reducing oestrogen quinones than its

homologue NQO1. Such findings reveal a possible relationship between breast cancer and NQO2, although no studies to date have addressed this issue. In addition, NQO2 can stabilise the p53 protein<sup>[29]</sup>, a known breast tumor suppressor gene product. *p53* is recognised as a highly penetrant breast cancer susceptibility gene, and loss of both p53 and breast cancer type 1 susceptibility protein (BRCA1) results in the rapid and efficient formation of mammary carcinomas<sup>[30]</sup>. Interestingly, the expression of 14-3-3 $\sigma$  is coordinately upregulated by the cellular tumour antigen p53 and BRCA1 and contributes to the DNA-damage-induced cell-cycle checkpoint mediated by these tumour suppressors<sup>[31]</sup>. It is logical to assume that 14-3-3 $\sigma$  binds to and sequesters NQO2 in the cytoplasm, thus enabling DNA damage to be repaired before the cell cycle progresses. In this study, we found that 14-3-3 $\sigma$  could interact with NQO2 directly and further confirmed the important function of 14-3-3 $\sigma$  protein in DNA repair and cell cycle progress.

*KCMF1* encodes a zinc-finger protein with hitherto barely characterised function. *KCMF1* was mentioned in 2001 at NCBI as an expressed sequence tag clone potentially involved in the regulation of potassium channels. A partial expressed sequence tag sequence of *KCMF1* was identified as a differentially regulated gene during kidney tubulogenesis *in vitro* and designated as developing branching tubulogenesis 91 (Debt91)<sup>[32]</sup>. In addition, *KCMF1* was shown to be downregulated in Ewing's sarcoma cell lines after the overexpression of CD99 and upregulated through fibroblast growth factor (FGF) receptor signalling pathways in gastric cancer cells and was consequently named basic FGF induced in gastric cancer<sup>[33]</sup>. Kreppel *et al.*<sup>[34]</sup> showed that the nuclear zinc-finger protein *KCMF1* was overexpressed in epithelial cancers and especially in human and mouse pancreatic cancer. *KCMF1* enhanced proliferation, migration and invasion. The downregulation of *KCMF1* *in vivo* reduced preneoplastic changes in the transforming growth factor- $\alpha$  transgenic pancreatic cancer model. One study showed that 14-3-3 $\sigma$  was highly expressed in pancreatic adenocarcinoma<sup>[35]</sup>. Our results suggest that 14-3-3 $\sigma$  may also interact with *KCMF1*. The knock-down of 14-3-3 $\sigma$  and *KCMF1* proteins expression significantly inhibited cell proliferation and colony formation of human colon cancer stem cells. Further study is required to understand how the interaction between 14-3-3 $\sigma$  and *KCMF1* proteins affects cell proliferation and colony formation of SW1116csc.

In summary, we constructed a 14-3-3 $\sigma$  bait gene and successfully expressed it as a fusion with the GAL4 DNA-binding domain. Using the yeast two-hybrid system, we found novel binding proteins (*KCMF1*, NQO2, HIBADH and 14-3-3 $\sigma$ ) from the HeLa cDNA library that closely interact with 14-3-3 $\sigma$ . The knock-down of 14-3-3 $\sigma$  and *KCMF1* protein expression significantly inhibited cell proliferation and colony formation of colon cancer stem cells.

## COMMENTS

### Background

The cancer stem cell (CSC) hypothesis is currently at the center of a rapidly evolving field, involving a change of perspective on the development and treatment of cancers. However, research has been hampered by the lack of distinct molecular markers of CSCs. Among all 14-3-3 proteins, 14-3-3 $\sigma$  is the isoform most directly linked to cancer. There are several lines of evidence indicating that 14-3-3 $\sigma$  acts as a tumor suppressor gene and that its inactivation is crucial in tumorigenesis.

### Research frontiers

All 14-3-3 proteins bind to phosphoserine/phosphothreonine-containing peptide motifs corresponding to the sequences RSXpSXP or RXXpSXP. Many 14-3-3-binding proteins contain sequences that closely match these motifs, although a number of ligands bind to 14-3-3 in a phospho-independent manner using alternative sequences that do not closely resemble these motifs. The interaction of 14-3-3 $\sigma$  with other proteins may be involved in proliferation and colony formation of human colon cancer stem cells.

### Innovations and breakthroughs

The authors constructed 14-3-3 $\sigma$  bait gene and expressed as a fusion to the GAL4 DNA-binding domain successfully. Using the yeast two-hybrid system, we found novel binding proteins [potassium channel modulatory factor 1 (*KCMF1*), quinone oxidoreductase, hydroxyisobutyrate dehydrogenase and 14-3-3 $\sigma$ ] from the HeLa cDNA library which closely interact with 14-3-3 $\sigma$ . The knock-down expression of 14-3-3 $\sigma$  and *KCMF1* proteins significantly inhibited cell proliferation and colony formation of colon cancer stem cells.

### Applications

The study of CSCs has important implications for future cancer treatment and therapies. The CSC hypothesis states that if the CSCs were eliminated, the tumor would simply regress due to differentiation and cell death. By selectively targeting CSCs relative proteins, it may be possible to treat patients with aggressive, non-resectable tumors and prevent the tumor from metastasizing.

### Terminology

14-3-3 proteins are among the most abundant proteins within the cell, having been initially identified in 1967 as a family of acidic proteins within the mammalian brain. This family of highly conserved proteins consisting of seven isotypes in human cells ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\eta$ ,  $\sigma$ ,  $\tau$ ,  $\xi$ ) plays crucial roles in regulating multiple cellular processes including the maintenance of cell cycle checkpoints and DNA repair, the prevention of apoptosis, the onset of cell differentiation and senescence, and the coordination of cell adhesion and motility.

### Peer review

The paper presents an original work about colon CSCs, and shows an original pathway for colon cancer management.

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**P-Reviewer** Torigoe T **S-Editor** Zhai HH **L-Editor** A  
**E-Editor** Li JY



## Glycyrrhizic acid attenuates CCl<sub>4</sub>-induced hepatocyte apoptosis in rats *via* a p53-mediated pathway

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Supported by Leading Academic Discipline Project of State Administration of Traditional Chinese Medicine of China

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Received: February 24, 2013 Revised: May 14, 2013

Accepted: May 18, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the effect of glycyrrhizic acid (GA) on carbon tetrachloride (CCl<sub>4</sub>)-induced hepatocyte apoptosis in rats *via* a p53-dependent mitochondrial pathway.

**METHODS:** Forty-five male Sprague-Dawley rats were randomly and equally divided into three groups, the control group, the CCl<sub>4</sub> group, and the GA treatment group. To induce liver fibrosis in this model, rats were given a subcutaneous injection of a 40% solution of CCl<sub>4</sub> in olive oil at a dose of 0.3 mL/100 g body weight biweekly for 8 wk, while controls received the same isovolumetric dose of olive oil by hypodermic injection, with an initial double-dose injection. In the GA group,

rats were also treated with a 40% solution of CCl<sub>4</sub> plus 0.2% GA solution in double distilled water by the intraperitoneal injection of 3 mL per rat three times a week from the first week following previously published methods, with modifications. Controls were given the same isovolumetric dose of double distilled water. Liver function parameters, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. Pathologic changes in the liver were detected by hematoxylin and eosin staining. Collagen fibers were evaluated by Sirius red staining. Hepatocyte apoptosis was investigated using the terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay and the cleaved caspase-3 immunohistochemistry assay. The expression levels of p53 and apoptosis-related proteins were evaluated by immunohistochemistry or Western blotting analysis.

**RESULTS:** After 8 wk of treatment, GA significantly reduced serum activity of ALT (from 526.7 ± 57.2 to 342 ± 44.8, *P* < 0.05) and AST (from 640 ± 33.7 to 462.8 ± 30.6, *P* < 0.05), attenuated the changes in liver histopathology and reduced the staging score (from 3.53 ± 0.74 to 3.00 ± 0.76, *P* < 0.05) in CCl<sub>4</sub>-treated rats. GA markedly reduced the positive area of Sirius red and the ratio of the hepatic fibrotic region (from 7.87% ± 0.66% to 3.68% ± 0.32%, *P* < 0.05) compared with the CCl<sub>4</sub> group. GA also decreased the expression level of cleaved caspase-3 compared to the CCl<sub>4</sub> group. TUNEL assay indicated that GA significantly diminished the number of TUNEL-positive cells compared with the CCl<sub>4</sub> group (*P* < 0.05). GA treatment clearly decreased the level of p53 (*P* < 0.05) detected by immunohistochemistry and Western blotting analysis. Compared with the CCl<sub>4</sub> group, we also found that GA reduced the Bax/Bcl-2 ratio (*P* < 0.05), the expression of cleaved caspase-3 (*P* < 0.05), cleaved caspase-9 (*P* < 0.05), and inhibited cytochrome C and second mitochondria-derived activator of caspases (Smac) release from mitochondria to cytoplasm, *i.e.*, GA reduced the expression

level of Smac, which inhibited c-IAP1 activity ( $P < 0.05$ ), ultimately inhibiting the activity of caspase-3, according to Western blotting analysis. As a result, GA suppressed activation of the caspase cascades and prevented hepatocyte apoptosis.

**CONCLUSION:** GA can inhibit CCl<sub>4</sub>-induced hepatocyte apoptosis *via* a p53-dependent mitochondrial pathway to retard the progress of liver fibrosis in rats.

**Key words:** P53; Apoptosis; Liver fibrosis; Glycyrrhizic acid; Mitochondria

**Core tip:** This study is the first to investigate the effects of glycyrrhizic acid (GA) on p53-dependent apoptosis in carbon tetrachloride (CCl<sub>4</sub>)-induced hepatic injury. The results indicated that GA can attenuate hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and retard the progression of liver fibrosis induced by CCl<sub>4</sub> in rats.

Guo XL, Liang B, Wang XW, Fan FG, Jin J, Lan R, Yang JH, Wang XC, Jin L, Cao Q. Glycyrrhizic acid attenuates CCl<sub>4</sub>-induced hepatocyte apoptosis in rats *via* a p53-mediated pathway. *World J Gastroenterol* 2013; 19(24): 3781-3791. Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3781.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3781>

## INTRODUCTION

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and is a serious threat to human health. It is known that the foundation of liver fibrosis is the imbalance between synthesis and degradation of extracellular matrix (including collagen, glycoproteins, polysaccharides, amines, *etc.*).

It has been shown that hepatocyte apoptosis can induce liver fibrosis<sup>[1-3]</sup>. Hepatocyte apoptosis is a major form of cell death which is primarily triggered by activation of the caspase family of cysteine proteases during the progression of chronic liver disease<sup>[4]</sup>. Many reports have shown that p53 is accumulated in hepatocytes in several fibrotic liver diseases<sup>[5-7]</sup>. The protein p53 can lead to apoptosis predominantly through p53-regulated genes such as *P21*, *PUMA*, *NOXA* and *Bax*<sup>[8]</sup>. The intensity of inflammation induces pro-apoptotic protein p53 with inhibition of anti-apoptotic Bcl-2 in non-alcoholic fatty liver disease<sup>[5]</sup>. Thioacetamide activates p53, increases caspase-3, Bax and Bad protein contents, and possibly causes the release of cytochrome C from mitochondria and the disintegration of membranes, eventually leading to apoptosis of cells in thioacetamide (TAA)-induced liver fibrosis and cirrhosis<sup>[9]</sup>. The pro-apoptotic protein, Bax, is a positive regulator and the anti-apoptotic protein, Bcl-xL, is a negative regulator that regulates the release of cytochrome C from mitochondria to the cytoplasm<sup>[10,11]</sup>. The presence of Bax protein is a direct result of the release of cytochrome C from mitochondria and activation of caspase-9<sup>[12]</sup>. Inhibi-

tors of apoptosis proteins (IAPs), which regulate apoptosis through various factors, play a vital role in inhibition of the apoptotic process<sup>[13]</sup>. c-IAP1, c-IAP2 and Survivin, as key members of IAPs, can inhibit the activity of caspase-3 and -7, thus blocking cell apoptosis<sup>[14,15]</sup>. During the apoptotic process, second mitochondria-derived activator of caspases (Smac), released from mitochondria into the cytoplasm, bind and antagonize IAPs, subsequently reducing the inhibition of caspases by IAPs resulting in apoptosis<sup>[16-18]</sup>. p53 activation enhances X-IAP inhibition-induced cell death by promoting mitochondrial release of Smac<sup>[19]</sup>. Therefore, inhibiting p53-dependent hepatocyte apoptosis may be an effective therapeutic strategy for the treatment and prevention of hepatic fibrosis.

Chinese herbal medicine has been widely used to cure diseases for thousands of years in China, especially chronic liver diseases. In recent years, the efficacy of Chinese herbal medicine has been appraised by modern biological technology<sup>[20,21]</sup>. Glycyrrhizic acid (GA), also known as Glycyrrhizin<sup>[22]</sup>, is the major bioactive component of licorice root extract. GA, a glycosylated saponin, which has one molecule of glycyrrhetic acid and two molecules of glucuronic acid, has adrenal cortex hormone-like effects<sup>[23,24]</sup>. GA has numerous pharmacologic effects, such as anti-inflammatory, anti-viral, anti-tumor and hepatoprotective activities<sup>[25]</sup>. GA also exerts an anti-apoptotic effect through the inhibition of hepatocyte apoptosis<sup>[26,27]</sup>. Recent findings indicate that GA significantly inhibits hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of cytochrome C from mitochondria into the cytoplasm<sup>[28]</sup>.

It has been reported that carbon tetrachloride (CCl<sub>4</sub>) can induce hepatocyte apoptosis and liver fibrosis in animal models<sup>[29-33]</sup>. The damage responses, induced by CCl<sub>4</sub> injection in rat and mouse models, are similar to liver cirrhosis in humans<sup>[34]</sup>. Thus, we presumed here that GA treatment started from the early stage of chronic liver disease could effectively attenuate hepatocyte apoptosis, consequently inhibit liver fibrosis and retard disease progression in rats. This study sought to investigate the effects of GA on p53-dependent apoptosis in CCl<sub>4</sub>-induced hepatic injury.

## MATERIALS AND METHODS

### Materials

GA was purchased from Sigma (St Louis, MO, United States). Anti-caspase-3, anti-caspase-9, anti-c-IAP1, anti-cytochrome C, anti-Smac, anti-Bcl-2, anti-Bax and anti-COX-IV antibodies were purchased from Cell Signaling Technology (Beverly, MA, United States). Anti-GADPH and anti-p53 antibodies were bought from Abcam (Cambridge, United Kingdom), horseradish peroxidase-conjugated anti-mouse and anti-rabbit immunoglobulin G antibodies were purchased from Cell Signaling Technology. The chemiluminescence reaction kit (ECL Plus) was purchased from Millipore (Billerica, MA, United States). Anti-cleaved-caspase-3 antibody and the mitochondria/cytoplasm fractionation kit were purchased from Beyotime Biotechnology (Haimen, Jiangsu Province, China).

**Animal model of liver fibrosis and treatment**

Male SD rats weighing 150-200 g were purchased from the Experimental Animal Center of Zhongshan Hospital, Fudan University. Rats were kept in a temperature-controlled room with an alternating 12-h dark and light cycle. Forty-five rats were randomly and equally divided into three groups, the control group, the CCl<sub>4</sub> group, and the GA treatment group. To induce liver fibrosis in this model, rats were given a subcutaneous injection of a 40% solution of CCl<sub>4</sub> (Wako Pure Chemical, Osaka, Japan) in olive oil at a dose of 0.3 mL/100 g body weight biweekly for 8 wk, while controls received the same isovolumetric dose of olive oil by hypodermic injection, with an initial double-dose injection. In the GA group, rats were also treated with a 40% solution of CCl<sub>4</sub> plus 0.2% GA solution in double distilled water by the intraperitoneal injection of 3 mL per rat three times a week from the first week following previously published methods<sup>[35,36]</sup>, with modifications. Controls were given the same isovolumetric dose of double distilled water. Animals were sacrificed 24 h after the last injection. Blood was obtained from the left ventricular apex for measurements of aminotransferases and the samples were stored at -20 °C. The liver was removed and rinsed with 0.9% saline, some liver sections were fixed in 10% buffered formaldehyde and embedded in paraffin for, and the remaining liver was stored at -70 °C for protein experiments.

**Liver function**

Blood was centrifuged at 3500 *g* at 4 °C for 10 min to separate the plasma. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were detected using a Siemens Advia 1650 automatic analyzer.

**Sirius-red and hematoxylin and eosin staining**

The thick sections (5 μm) were stained with hematoxylin and eosin (HE) and Sirius-red. HE staining was performed to assess pathologic changes in the liver. The standard of pathological grade was according to consensus on evaluation of the diagnosis and severity of hepatic fibrosis<sup>[37]</sup>. Sirius-red staining was performed to detect hepatic fibrosis. The Sirius red-positive areas were assessed in four different fields for each section by Image J Software (National Institutes of Health, Bethesda, MD, United States) and were in accordance with the following expression (collagen area/total area-vascular lumen area) × 100<sup>[38]</sup>.

**Immunohistochemical staining**

Liver tissue sections were subjected to dewaxing, hydration and thermal induction antigen retrieval. Slices were blocked and incubated with anti-p53 antibody (1:50) and anti-cleaved-caspase-3 antibody (1:100) which were diluted in TBS-5% bovine serum albumin (BSA) at 4 °C overnight. Negative-control antibody was species-matched. The following day, the slices were washed and incubated with secondary antibodies. The slices were then incubated with 3, 3'-diaminobenzidine tetrachloride for 5-10 min to develop the color, and staining was observed under light microscopy (Olympus, Japan).

**Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling assay**

The terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay (Roche, Germany) was performed in accordance with the manufacturer's protocol. Nuclei were redyed with 4,6-diamidino-2-phenylindole (DAPI) staining. Cells marked by TUNEL were evaluated using fluorescence microscopy (Olympus, Japan).

**Protein preparation**

Mitochondria were isolated with a tissue mitochondria isolation kit according to the manufacturer's instructions. During mitochondria preparation, all samples were placed on ice. Eighty mg liver tissue was cut into pieces, tissue mitochondria isolation reagent A with phenylmethylsulfonyl fluoride (PMSF) was added, and then homogenized in an ice bath approximately 10 times. The homogenate was centrifuged at 600 rpm at 4 °C for 5 min. The supernatant was then collected and centrifuged at 11000 *g* at 4 °C for 10 min. The supernatant contained the cytoplasmic protein, and the precipitate contained the mitochondria. The cytoplasmic and mitochondrial fractions of the lysate were estimated by Western blotting. Liver tissues were homogenized in RIPA Lysis Buffer with PMSF and then centrifuged at 12000 *g* for 15 min at 4 °C, and the supernatant was the total protein.

**Western blotting analysis**

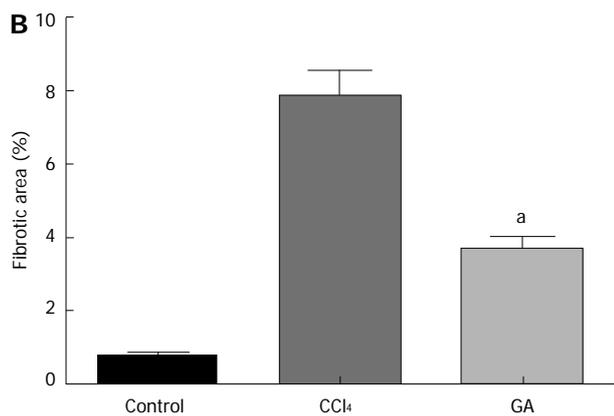
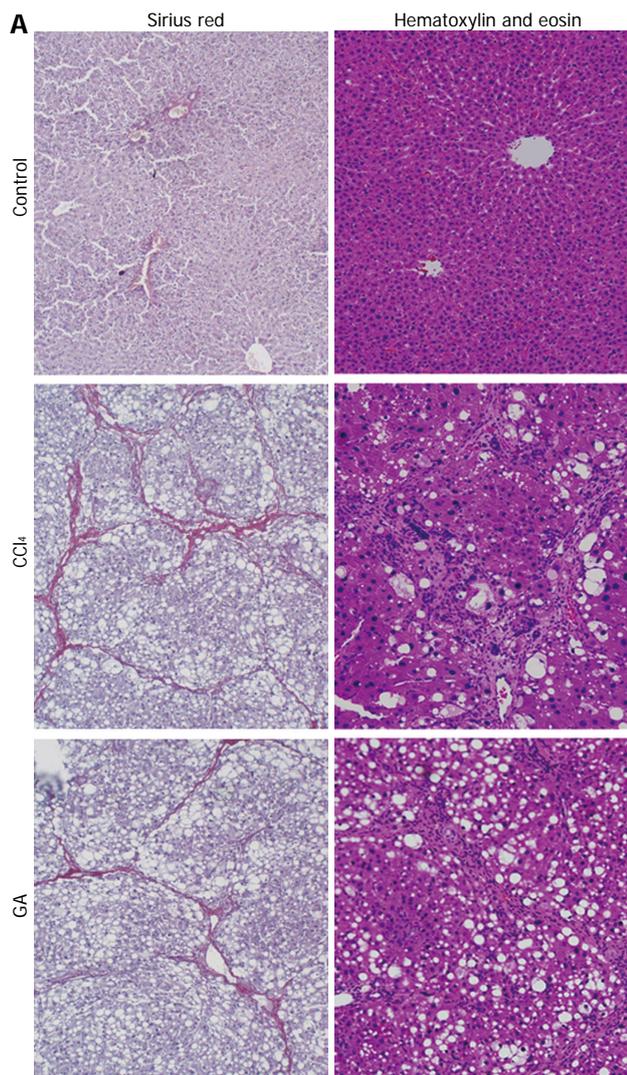
Proteins were separated by 10% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked with 5% BSA for 2 h, and then incubated overnight at 4 °C with rabbit anti-caspase-9, anti-caspase-3, anti-Smac, anti-cytochrome C, anti-c-IAP1, anti-Bcl-2, anti-Bax antibodies and mouse anti-p53, anti-GAPDH and anti-COXIV antibodies. The membranes were then incubated with HRP-conjugated goat anti-rabbit IgG and goat anti-mouse IgG (1:5000, diluted) at room temperature for 2 h, and then washed again and detected by the enhanced chemiluminescence (ECL) reaction. The intensities of the bands were analyzed by Image J software.

**Statistical analysis**

Each experiment was repeated at least 3 times. Data were estimated using analysis of variance and all values are expressed as mean ± SD. A *P* value < 0.05 was considered significant. All analyses in the study were implemented by SPSS 11.5 software for Windows (Chicago, IL, United States).

**RESULTS****Function of GA on serum parameters of hepatic fibrosis induced by CCl<sub>4</sub>**

The activities of ALT and AST were significantly increased in the CCl<sub>4</sub> treated group compared with those in the control group (*P* < 0.05). In the GA group, the activities of ALT and AST were markedly decreased compared



**Figure 1** Histological examination of liver by hematoxylin and eosin and Sirius red staining. **A:** Histological examination. Rats were treated with carbon tetrachloride (CCl<sub>4</sub>) and/or glycyrrhizic acid (GA). Liver tissue sections were stained with hematoxylin and eosin or Sirius red (original magnification, × 100); **B:** Quantitative analysis of liver fibrosis by Sirius red staining. Results are represented as fibrotic area (%), which signifies the proportion of area stained red/area of total area-vascular lumen. Values are mean ± SD. <sup>a</sup>*P* < 0.05 vs CCl<sub>4</sub>.

with those in rats with liver fibrosis not treated with GA (*P* < 0.05) (Table 1).

**Table 1** Effect of glycyrrhizic acid on plasma alanine aminotransferase and aspartate aminotransferase activity in CCl<sub>4</sub>-induced rats

| Group            | ALT (U/L)               | AST (U/L)                 |
|------------------|-------------------------|---------------------------|
| Control          | 42.4 ± 6.0              | 70.2 ± 2.3                |
| CCl <sub>4</sub> | 526.7 ± 57.2            | 640 ± 33.7                |
| GA               | 342 ± 44.8 <sup>a</sup> | 462.8 ± 30.6 <sup>a</sup> |

<sup>a</sup>*P* < 0.05 vs the carbon tetrachloride (CCl<sub>4</sub>) group. GA: Glycyrrhizic acid; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

**Table 2** Histopathological semiquantitative scores in the liver

| Group            | <i>n</i> | 0  | + 1 | + 2 | + 3 | + 4 | Staging scores           |
|------------------|----------|----|-----|-----|-----|-----|--------------------------|
| Control          | 15       | 15 | 0   | 0   | 0   | 0   | 0                        |
| CCl <sub>4</sub> | 15       | 0  | 0   | 2   | 3   | 10  | 3.53 ± 0.74              |
| GA               | 15       | 0  | 10  | 1   | 10  | 3   | 3.00 ± 0.76 <sup>a</sup> |

<sup>a</sup>*P* < 0.05 vs the carbon tetrachloride (CCl<sub>4</sub>) group. GA: Glycyrrhizic acid.

**Role of GA in the improvement of liver fibrosis induced by CCl<sub>4</sub>**

After 8 wk of CCl<sub>4</sub> administration, liver histopathology was significantly changed in the CCl<sub>4</sub> group. The livers in the control group, showed an integrated lobular structure with central venous and hepatic cord radiation (Figure 1). The staging score was 0 (Table 2). The positive area of Sirius red staining in the control group was around the central vein rather than in the hepatic parenchyma. There were numerous Steatosis and ballooning of hepatocytes in the GA and CCl<sub>4</sub> groups. In the CCl<sub>4</sub> group, the liver showed fibrous connective tissue proliferation, fiber interval formation which was associated with disorder of lobular structure in the portal area, and most rat livers appeared to have pseudo lobules (Figure 1). The score of hepatic fibrosis in the CCl<sub>4</sub> group increased to 3.53 ± 0.74 (Table 2). The positive areas of Sirius red staining in the CCl<sub>4</sub> group were in the boundaries of the hepatic lobules and the ratio of the hepatic fibrotic region was 7.87% ± 0.66%. In the GA group, livers appeared to have fibrous connective tissue proliferation, the formation of a few fiber intervals in the portal area, and the occasional pseudo lobule (Figure 1). The score was 3.00 ± 0.76 (*P* < 0.05) in the GA group (Table 2). The positive area of Sirius red staining in the GA group was decreased, and the ratio of the hepatic fibrotic region (3.68% ± 0.32%, *P* < 0.05) was reduced compared with the CCl<sub>4</sub> group (Figure 1).

**Impact of GA on hepatic apoptosis induced by CCl<sub>4</sub>**

The expression level of cleaved caspase-3 was high in the livers of rats in the CCl<sub>4</sub> group. Interestingly, this level was reduced in the GA-treated group as detected by immunohistochemistry (Figure 2A). Under fluorescence microscopy, the TUNEL assays showed no stain and non-apoptotic nuclei in the normal liver tissue. High quantities of TUNEL cells were observed in the livers of the CCl<sub>4</sub> group and numerous condensed and fragmented nuclei.

In the GA-treated group, there were few TUNEL cells, and less DAPI staining was observed in the same slice. The merged images indicated that TUNEL-positive cells were different, as numerous fused cells were observed in the CCl<sub>4</sub> group, while a significant reduction in these cells was detected in the GA-treated group (Figure 2B). Overall, these findings indicated that GA reduced apoptosis in liver lesion progression.

#### **Effect of GA on the level of proteins induced by CCl<sub>4</sub>**

The level of p53 was significantly higher in the livers of rats in the CCl<sub>4</sub> group than in the other two groups as detected by immunohistochemical staining, while in the GA group, the expression level of p53 was reduced (Figure 3A). This is consistent with the Western blotting analysis (Figure 3B) which showed that p53 was activated in the CCl<sub>4</sub> group and clearly reduced in the GA group.

We examined the impact of GA on Bcl-2 and Bax protein expression in CCl<sub>4</sub>-induced liver injury by Western blot analysis. As shown in Figure 4A, the expression level of the anti-apoptotic protein, Bcl-2, was decreased, while the expression of the pro-apoptotic protein, Bax, was increased in mitochondrial fraction of CCl<sub>4</sub>-induced hepatic injury, and the Bax/Bcl-2 ratio was elevated in the CCl<sub>4</sub> group. In contrast, GA reversed the expression levels of Bcl-2 and Bax, and improved the Bax/Bcl-2 ratio. Both pro- and anti-apoptotic Bcl-2 proteins regulate cytochrome C from mitochondria to cytoplasm. The cytoplasmic fraction in the control group contained a negligible amount of cytochrome C. However, cytochrome C accumulated in the cytoplasm of liver tissue in the CCl<sub>4</sub> group, and GA inhibited the release of cytochrome C (Figure 4B). Caspase activation plays an important role in apoptosis, and caspase-3 cleavage is a typical feature of apoptosis<sup>[39]</sup>. In the current study, we found that there was increased cleavage of caspase-9 (37 kDa) and caspase-3 (17 kDa) in the CCl<sub>4</sub> group, suggesting severe apoptosis. Intriguingly, the levels of caspase-9 and caspase-3 cleavage diminished in the GA group (Figure 4C and D).

We also found that the cytoplasmic fraction in the control group contained a negligible amount of Smac. However, Smac accumulated in the cytoplasm of livers in rats exposed to CCl<sub>4</sub>. GA treatment significantly inhibited the release of Smac induced by CCl<sub>4</sub> (Figure 4E). The expression level of c-IAP1 corresponded to the decreased expression of Smac in the GA-treated group compared with the CCl<sub>4</sub> group (Figure 4F), and the consequence was in accordance with the view that Smac has an antergic effect on c-IAP1 activity which can inhibit the activity of caspases<sup>[16-18]</sup>. These results indicated that GA could prevent CCl<sub>4</sub>-induced apoptosis by suppressing the activation of upstream caspase-3. GA treatment ameliorated CCl<sub>4</sub>-induced hepatic injury, and indicated the involvement of the p53 pathway in CCl<sub>4</sub>-induced hepatocyte apoptosis.

## **DISCUSSION**

Liver fibrosis is a common outcome in many chronic liver diseases. Liver fibrosis and cirrhosis, as shown in recent

studies, are reversible processes<sup>[40,41]</sup>. However, there have been few effective therapies for the treatment of hepatic fibrosis in recent years<sup>[42]</sup>. There is an urgent need to investigate the effect of innocuous anti-fibrotic agents<sup>[43]</sup>. CCl<sub>4</sub>-induced liver injury is one of the best-characterized models of hepatotoxicity, and can be used in the clinic to examine anti-hepatotoxic and/or hepatoprotective drugs<sup>[44]</sup>.

GA, used in the treatment and control of chronic viral hepatitis, is now routinely used in Japan, due to its well-recognized transaminase-lowering effect in clinical applications<sup>[25,45,46]</sup>. Neominophagen C is a Japanese preparation containing 0.2% glycyrrhizin, 0.1% cysteine, and 2% glycine, and mainly acts as an anti-inflammatory or cytoprotective drug rather than an antiviral. It can improve mortality in patients with subacute liver failure and ameliorate liver function in patients with subacute hepatic failure, chronic hepatitis, and cirrhosis<sup>[47]</sup>.

Apoptosis is one of the events involved in the process of liver fibrosis. Thus, factors that affect apoptosis may be used to modulate liver fibrosis<sup>[33]</sup>. A line of evidence has shown that loss of p53 function is a common and considerable occurrence in the development of many human malignancies. In unstressed cells, expression of p53 is regulated and maintained at a low level through the ubiquitin/proteasome pathway<sup>[48]</sup>. Endogenous p53 activation in hepatocytes causes spontaneous liver fibrosis in double minute 2-knockout mice<sup>[3]</sup>. It also appears to modulate ethanol-induced hepatocyte apoptosis, since it was completely abrogated in mice with a p53 null background<sup>[49]</sup>. Mitochondria react to different cytotoxic stimuli, are central death regulators and play a vital role in p53-dependent death, in other words, the p53-dependent signal induces cell death through the mitochondrial pathway<sup>[50,51]</sup>. When the death signal is conducted to the mitochondria, the cell membrane permeability is increased and apoptosis-related proteins are released<sup>[52]</sup>.

Many reports have demonstrated that drugs can ameliorate CCl<sub>4</sub>-mediated hepatic apoptosis in rats, such as branched-chain amino acids<sup>[32]</sup> and the water-soluble extract of *Salvia miltiorrhiza*<sup>[33]</sup>. GA has an anti-apoptotic effect through the inhibition of hepatic apoptosis<sup>[26,27]</sup>. It significantly inhibited hepatocyte apoptosis by down-regulating the expression of caspase-3 and inhibiting the release of cytochrome C from mitochondria into the cytoplasm<sup>[28]</sup>. GA can alter Kaposi sarcoma-associated herpesvirus latency by triggering p53-mediated apoptosis<sup>[53]</sup>. Here we demonstrated that intervention with GA from the early stage of chronic liver disease effectively attenuated p53-dependent hepatocyte apoptosis and liver fibrosis, thus retarding disease progression in rats.

Apoptosis and necrosis contribute to the process of liver fibrosis<sup>[29,33]</sup>. Whether necrotic liver injury or apoptosis is dominant in CCl<sub>4</sub>-induced liver injury models remains controversial. A previous study showed that CCl<sub>4</sub> can induce acute hepatocellular damage which is characterized by necrotic cell death<sup>[54]</sup>, while another study indicated that a substantial number of hepatocytes undergo apoptosis in the acute stage after CCl<sub>4</sub> adminis-

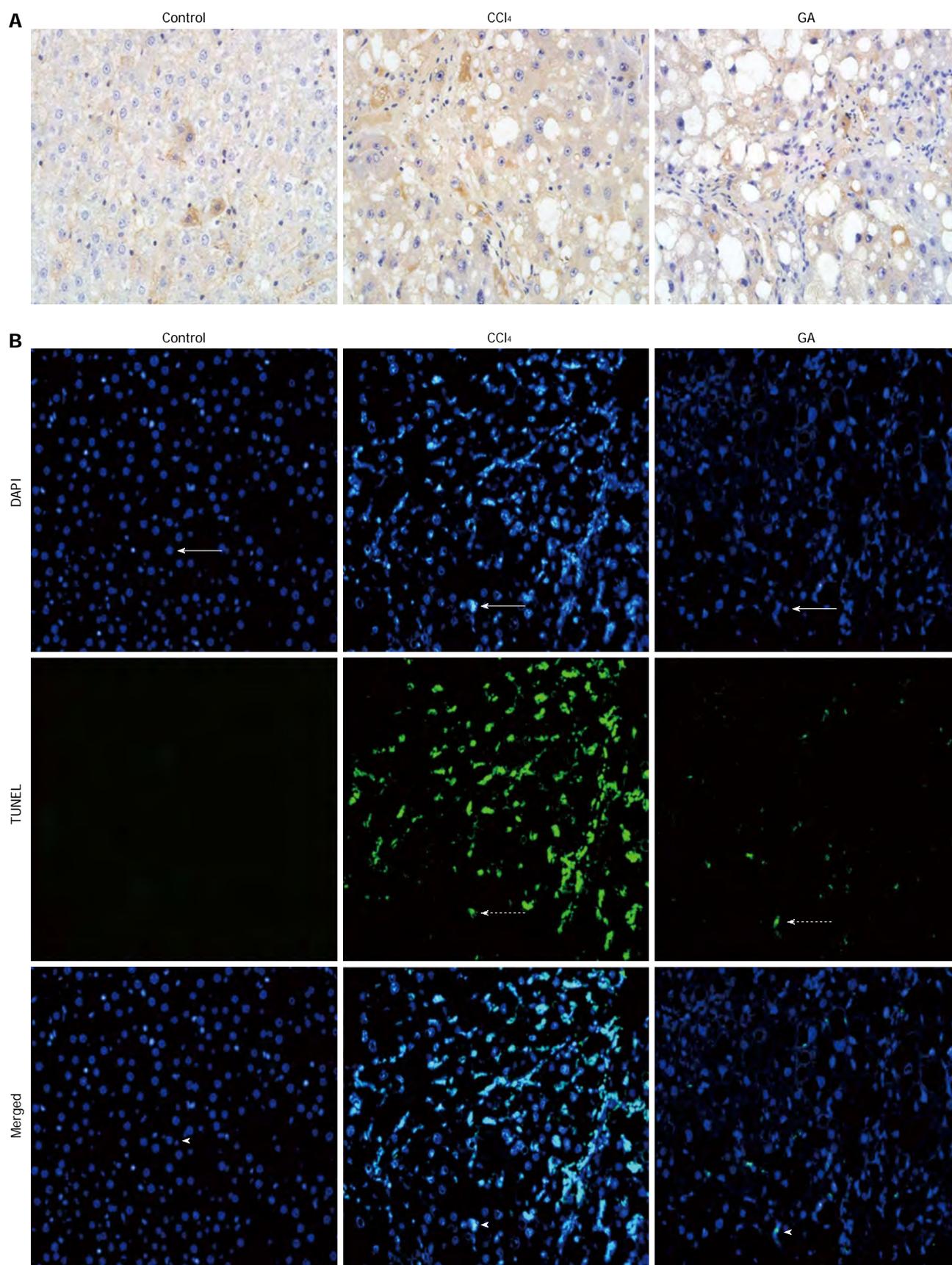
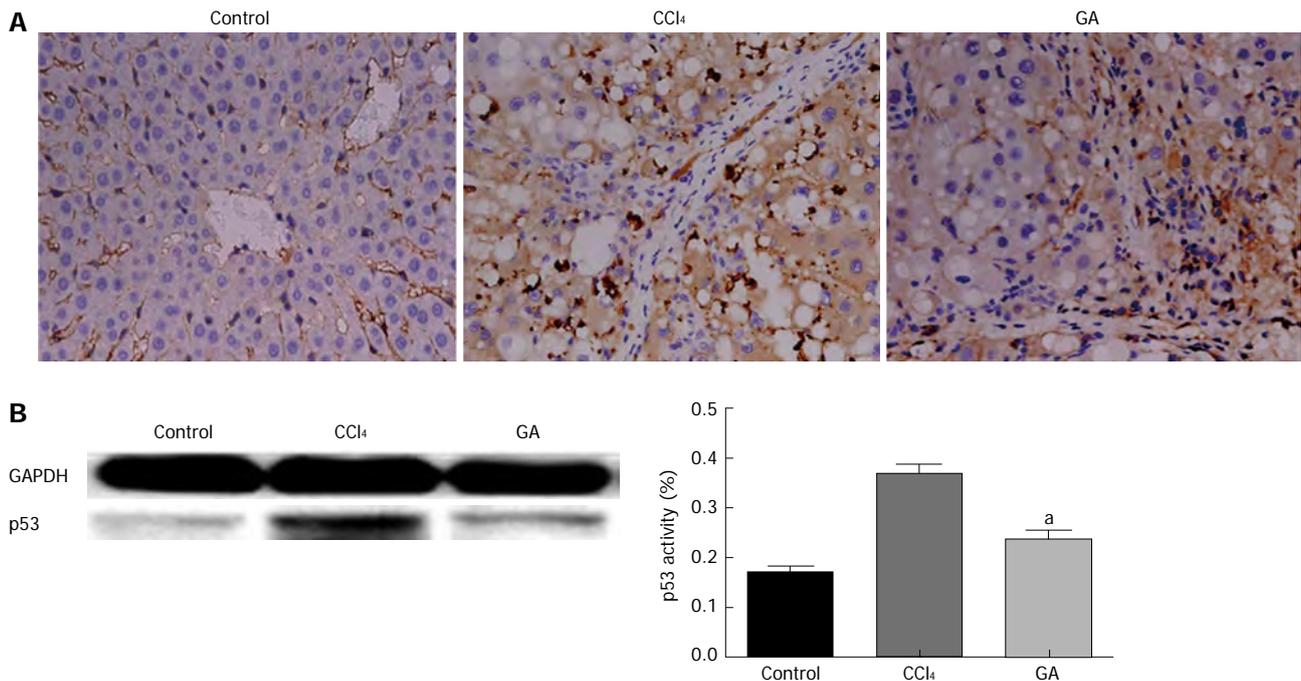


Figure 2 Impact of glycyrrhizic acid treatment on hepatic apoptosis induced by carbon tetrachloride in rats. A: Liver tissue sections from the different groups were subjected to immunohistochemistry to determine the expression level of cleaved caspase-3 (original magnification,  $\times 400$ ); B: Fluorescence microscopy image showing terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) stain (dashed arrows), and the same tissue slices were respectively counterstained with 4'6-diamidino-2-phenylindole (DAPI) to localize the nuclei (arrows). Images of combined with DAPI, indicated TUNEL-positive cells (arrow heads) (original magnification,  $\times 200$ ). GA: Glycyrrhizic acid; CCl<sub>4</sub>: Carbon tetrachloride.



**Figure 3** Effect of glycyrrhizic acid treatment on the expression level of p53 in the livers of rats injured by carbon tetrachloride. A: Liver tissue slices from the different groups were subjected to immunohistochemistry (original magnification,  $\times 400$ ). B: Total protein fractions prepared from livers were analyzed by Western blotting to assess the expression level of p53 and GAPDH to confirm the same sample loading. The results of Western blotting analysis were similar in at least three replicate independent experiments. All values are presented as mean  $\pm$  SD. Statistical significance was defined as follows: <sup>a</sup> $P < 0.05$  vs the carbon tetrachloride (CCl<sub>4</sub>) group. GA: Glycyrrhizic acid.

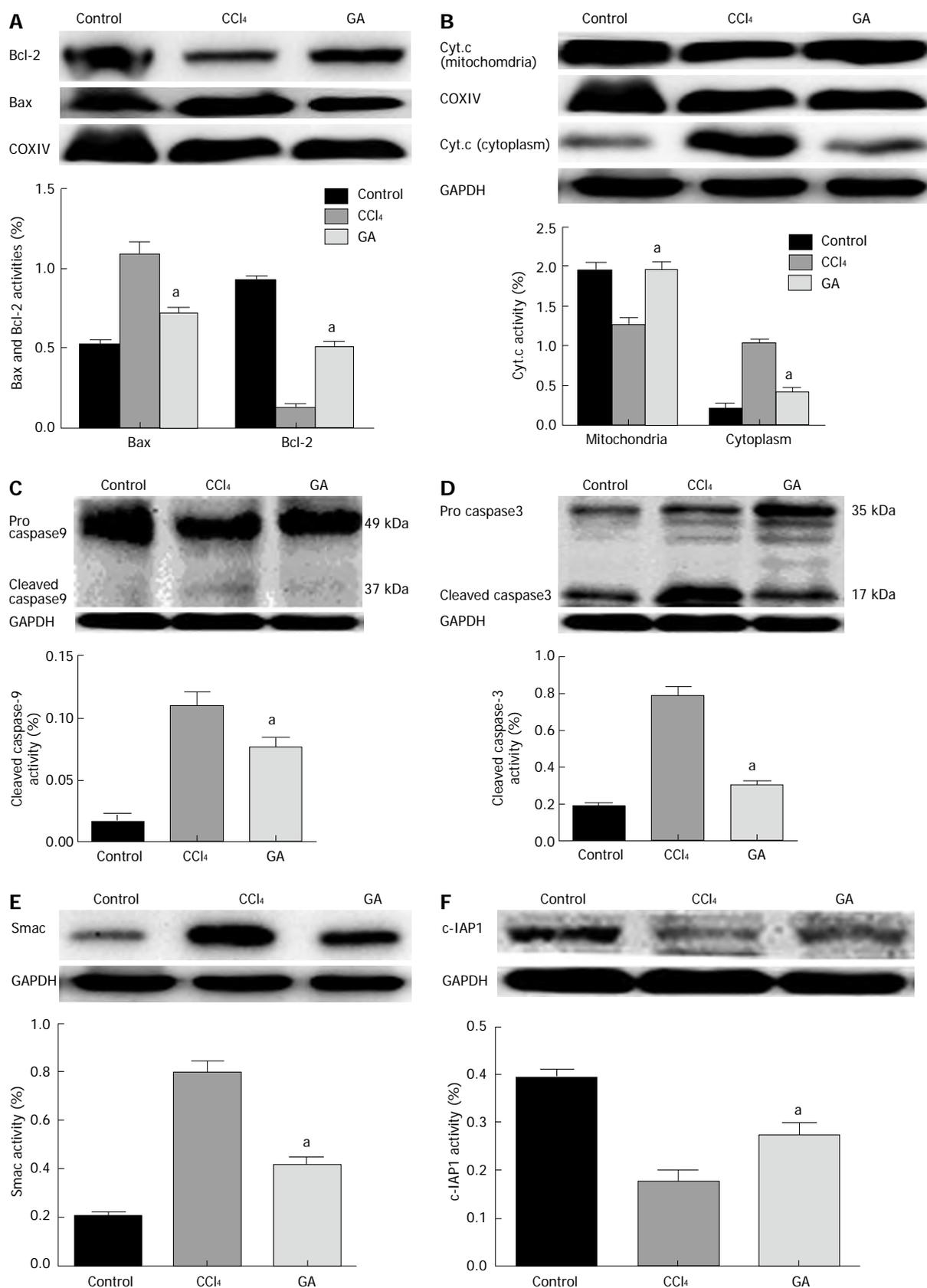
tration<sup>[29]</sup>. In the present study, we found both apoptosis and necrosis occurred in the CCl<sub>4</sub>-induced chronic liver injury model. These results were consistent with other reports<sup>[32,33]</sup>. Discrepancies may be attributed to the time points of observation.

Steatosis and ballooning of hepatocytes are the earliest, most frequent, and most striking pathological changes observed in CCl<sub>4</sub>-induced liver injury<sup>[29,55,56]</sup>, and we found this pathological change using H and E staining. According to immunohistochemical staining, p53 expression level was significantly increased in the CCl<sub>4</sub> group compared with the GA group. Western blot analysis showed that p53 was sharply up-regulated in the CCl<sub>4</sub> group compared to the GA group. This indicated that p53 was activated after CCl<sub>4</sub> administration, however, GA reduced the expression level of p53.

To date, TUNEL assay<sup>[27]</sup>, cleaved caspase-3 immunohistochemical staining<sup>[57]</sup> and serum CK18 fragment<sup>[58]</sup> have been identified as the markers of apoptosis. In the study we first detected DNA fragmentation of hepatocytes using the TUNEL assay. TUNEL-positive cells in the CCl<sub>4</sub> group were significantly increased compared with the GA group. GA reduced the number of TUNEL-labeled cells<sup>[27]</sup>. However, the TUNEL assay is not a specific marker of apoptosis, thus we performed cleaved caspase-3 immunohistochemical staining. The results coincided with those from the TUNEL assay. Apoptosis, a form of cell death, is principally caused by activation of the caspase family of cysteine proteases<sup>[4]</sup>. In accordance with Western blotting analysis, accompanied by the reduction in p53, the expression level of Bcl-2 was

sharply decreased and the expression level of Bax was obviously increased in the mitochondrial fraction of the CCl<sub>4</sub> group, and the Bax/Bcl-2 ratio was elevated, while this tendency was reversed in the GA-treated group. Our results demonstrated that GA suppressed p53 activity, resulting in an increase in Bcl-2 and a decrease in Bax. In addition, GA inhibited the release of cytochrome C into the cytoplasm from mitochondria, and then inactivated caspase-9 and caspase-3. GA also reduced the expression of Smac, which was released from mitochondria, and bound to and antagonized c-IAP1, subsequently increased the inhibitory effect of c-IAP1 on caspase-3 and finally suppressed hepatocyte apoptosis. The degree of hepatic injury was associated with a substantial number of hepatocytes undergoing apoptosis<sup>[27]</sup>. The results also demonstrated that hepatic injury in the CCl<sub>4</sub> group was more serious than that in the GA group on the basis of histological observation, Sirius red staining assay, serum transaminase and TUNEL analyses. To our knowledge, these findings were to report that the effects of GA on p53-mediated activity in hepatocyte apoptosis in the liver of CCl<sub>4</sub>-treated rats. Whether other mechanisms or pathways are involved in liver fibrosis requires further exploration.

In summary, our findings showed that GA exerted anti-apoptotic effects *via* a p53-dependent mitochondrial pathway (Figure 5). GA protected against CCl<sub>4</sub>-induced hepatocyte apoptosis by regulating the Bcl-2 family of proteins, expression of Smac and caspase cleavage. These anti-apoptotic effects were related to decreases in the expression of pro-apoptotic proteins in the cytoplasm



**Figure 4** Impact of glycyrrhizic acid on CCl<sub>4</sub>-treated hepatocyte apoptosis signal cascades. Protein extracts from livers in the different groups were subjected to Western blotting. A: Expression levels of Bax and Bcl-2 in the mitochondria; B: Expression levels of cytochrome C (Cyt.c) in the cytoplasm and mitochondria; C: Expression level of caspase-9 in the total protein; D: Expression level of caspase-3 in the total protein; E: Expression level of Smac in the cytoplasm; F: Expression level of c-IAP1 in the total protein. In all these experiments glyceraldehyde-3-phosphate dehydrogenase (GAPDH), COXIV were used to ensure equal sample loading. The Western blotting results represent three independent tests. The bar graph represents the value of in the different proteins *via* the density of bands from at least three independent tests. All values are presented as mean ± SD. Statistical significant was defined as follows: <sup>a</sup>*P* < 0.05 vs the CCl<sub>4</sub> group. GA: Glycyrrhizic acid; CCl<sub>4</sub>: Carbon tetrachloride.

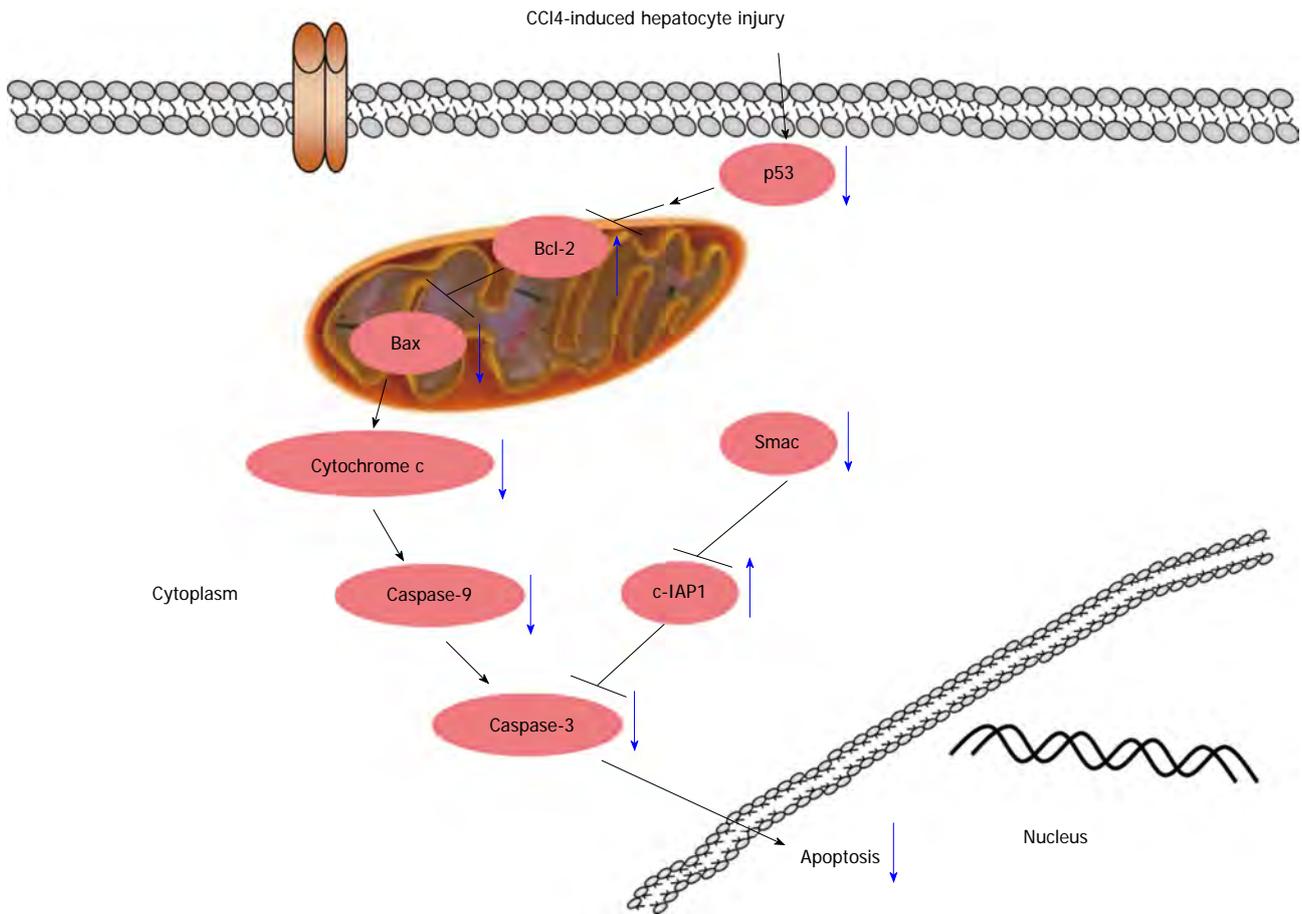


Figure 5 Schematic diagram of the effect of glycyrrhizic acid on the interruption of p53 signaling in carbon tetrachloride-induced hepatocyte apoptosis (blue arrows). Glycyrrhizic acid (GA) suppressed the activation of p53, decreased the expression level of Bax and increased the expression level of Bcl-2, which resulted in reduced cytochrome C release from the mitochondria into the cytoplasm, and inactivated caspase-9 and -3; GA also significantly inhibited Smac release from mitochondria into the cytoplasm and elevated the expression level of c-IAP1, resulting in inhibition of caspase-3 activity. Ultimately, GA suppressed the apoptosis of hepatocytes.

and the inhibition of proteins associated with apoptosis in the mitochondria. These findings suggest that GA can attenuate CCl<sub>4</sub>-induced hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and can retard the progression of liver fibrosis induced by CCl<sub>4</sub> in rats.

## COMMENTS

### Background

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and is a serious threat to human health. However, there have been few effective therapies for the treatment of hepatic fibrosis in recent years. The authors investigated whether glycyrrhizic acid (GA) could attenuate hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and retard the progression of liver fibrosis induced by CCl<sub>4</sub> in rats.

### Research frontiers

In this study, the authors found that GA attenuated hepatocyte apoptosis *via* a p53-mediated mitochondrial pathway and retarded the progression of liver fibrosis induced by carbon tetrachloride (CCl<sub>4</sub>) in rats, which may be a potential alternative treatment approach in patients with liver injury.

### Innovations and breakthroughs

This study sought to investigate the effects of GA on p53-dependent apoptosis in CCl<sub>4</sub>-induced hepatic injury. The study data showed that GA protected against CCl<sub>4</sub>-induced hepatocyte apoptosis by regulating the Bcl-2 family of proteins, expression of Smac and caspase cleavage.

### Applications

This study provides valuable experimental evidence for future anti-liver fibrosis

drug studies, and may provide an effective therapy for retarding the process of liver fibrosis.

### Terminology

Liver fibrosis, induced by various pathological factors, is a common outcome in many chronic liver diseases, and eventually leads to liver cirrhosis. Apoptosis is gene-controlled and auto-programmed cell death in order to maintain homeostasis. Apoptosis is different from necrosis, as it is an initiative process rather than a passive process and involves gene activation, expression and regulation.

### Peer review

This is a good study in which the authors presented experimental evidence that GA exerts anti-apoptotic effects *via* a p53-dependent mitochondrial pathway in CCl<sub>4</sub>-induced hepatocyte apoptosis in rats. The results are interesting and suggest that GA could protect against CCl<sub>4</sub>-induced hepatocyte apoptosis by regulating Bcl-2 family of proteins, expression of Smac and caspases cleavage.

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**P- Reviewers** Di Costanzo GG, Germanidis G, Liedtke AC, Yagi K  
**S- Editor** Wen LL **L- Editor** A **E- Editor** Ma S



## Annexin A2 silencing inhibits invasion, migration, and tumorigenic potential of hepatoma cells

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Supported by The Society Development of Nantong, No. HS2012034; the Jiangsu Health Projects, No. BL2012053 and No. K201102; the Priority Academic Program Development of Jiangsu, and the International S and T Cooperation Program of China, No. 2013DFA32150

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Received: March 13, 2013 Revised: April 30, 2013

Accepted: May 18, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the effects of Annexin A2 (ANXA2) silencing on invasion, migration, and tumorigenic potential of hepatoma cells.

**METHODS:** Human hepatoma cell lines [HepG2, SMMC-7721, SMMC-7402, and MHCC97-H, a novel human hepatocellular carcinoma (HCC) cell line with high metastasis potential] and a normal hepatocyte cell line

(LO2) were used in this study. The protein and mRNA expression levels of ANXA2 were analysed by western blotting and real-time polymerase chain reaction, respectively. The intracellular distribution profile of ANXA2 expression was determined by immunofluorescence and immunohistochemistry. Short hairpin RNA targeting ANXA2 was designed and stably transfected into MHCC97-H cells. Cells were cultured for *in vitro* analyses or subcutaneously injected as xenografts in mice for *in vivo* analyses. Effects of ANXA2 silencing on cell growth were assessed by cell counting kit-8 (CCK-8) assay (*in vitro*) and tumour-growth assay (*in vivo*), on cell cycling was assessed by flow cytometry and propidium iodide staining (*in vitro*), and on invasion and migration potential were assessed by transwell assay and wound-healing assay, respectively (both *in vitro*).

**RESULTS:** The MHCC97-H cells, which are known to have high metastasis potential, showed the highest level of ANXA2 expression among the four HCC cell types examined; compared to the LO2 cells, the MHCC97-H expression level was 8-times higher. The ANXA2 expression was effectively inhibited (about 80%) by ANXA2-specific small hairpin RNA (shRNA). ANXA2 expression in the MHCC97-H cells was mainly localized to the cellular membrane and cytoplasm, and some localization was detected in the nucleus. Moreover, the proliferation of MHCC97-H cells was obviously suppressed by shRNA-mediated ANXA2 silencing *in vitro*, and the tumour growth inhibition rate was 38.24% *in vivo*. The percentage of MHCC97-H cells in S phase dramatically decreased (to 27.76%) under ANXA2-silenced conditions. Furthermore, ANXA2-silenced MHCC97-H cells showed lower invasiveness (percentage of invading cells decreased to 52.16%) and suppressed migratory capacity (migration distance decreased to 63.49%). It is also worth noting that shRNA-mediated silencing of ANXA2 in the MHCC97-H cells led to abnormal apoptosis.

**CONCLUSION:** shRNA-mediated silencing of ANXA2

suppresses the invasion, migration, and tumorigenic potential of hepatoma cells, and may represent a useful target of future molecular therapies.

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**Key words:** Annexin A2; Small hairpin RNA; Hepatocellular carcinoma; Invasion; Migration; Tumorigenic potential

**Core tip:** The overexpression of annexin A2 (ANXA2) is closely related to the high metastasis potential and invasion ability of HCC cells, and ANXA2 deficiency inhibits the invasion, migration, and tumorigenic potential of hepatocellular carcinoma (HCC) cells, which not only provides further insight into the pathogenesis of HCC but also provides a potential predictive biomarker for HCC prognosis and a potential therapeutic target.

Zhang HJ, Yao DF, Yao M, Huang H, Wang L, Yan MJ, Yan XD, Gu X, Wu W, Lu SL. Annexin A2 silencing inhibits invasion, migration, and tumorigenic potential of hepatoma cells. *World J Gastroenterol* 2013; 19(24): 3792-3801 Available from: URL: <http://www.wjnet.com/1007-9327/full/v19/i24/3792.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3792>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the 3<sup>rd</sup> leading cause of cancer-related death, and its worldwide incidence is increasing; epidemiologic studies have revealed a strong association between HCC and chronic liver disease and cirrhosis<sup>[1,2]</sup>. Surgical therapy with liver transplantation or resection remains the mainstay of curative therapy for patients in the early stage of HCC<sup>[3,4]</sup>. However, even with radical resection, 60%-70% of patients develop metastasis and experience recurrence within 5 years of surgery<sup>[5-7]</sup>. Although several clinicopathologic features have been identified as contributing factors to this disease's poor prognosis (*e.g.*, poorly-differentiated phenotype, large-sized tumour, and portal venous invasion), the underlying mechanisms of HCC development remain unclear<sup>[8,9]</sup>. Gaining a detailed understanding of the molecular processes of HCC will likely identify specific diagnostic and prognostic markers and facilitate development of novel targeted therapeutic strategies. To this end, recent studies have identified annexin A2 (ANXA2) as an important mediator of malignant transformation and development of HCC<sup>[10,11]</sup>.

As one of the best characterized components of the annexin family, ANXA2 is a calcium-dependent phospholipid-binding protein<sup>[12,13]</sup>. Up-regulation of ANXA2 expression and its phosphorylation at tyrosine 23 (mediated by c-Src) has been observed in clinical samples of human HCC<sup>[14]</sup>; in addition, the tyrosine 23 phosphorylation-dependent cell-surface localization of ANXA2 was found to be required for the invasive and metastatic

properties of pancreatic cancer<sup>[15]</sup>. Further studies of this ability to promote tumour metastasis have elucidated the molecular process in which ANXA2 induces plasminogen conversion to plasmin, thereby leading to activation of metalloproteinases, degradation of extracellular matrix components, and promotion of neoangiogenesis<sup>[10,16-18]</sup>. Thus, ANXA2-targeted interference by small hairpin RNA (shRNA) may represent an effective therapeutic strategy to mediate the biological behaviours of hepatoma cells.

In the present study, ANXA2 overexpression was found in MHCC97-H cells, a novel human HCC cell line with high metastasis potential, which was then used to investigate the effects of ANXA2 silencing on cell invasion, migration, and tumorigenic potential of HCC cells.

## MATERIALS AND METHODS

### Plasmid construction

ANXA2-specific shRNA targeting nucleotides 94-113 downstream of the transcription start site of ANXA2 was synthesized as previously described<sup>[16]</sup> and used to construct an experimental plasmid (pRNAT-U6.1-shRNA) by inserting into a *Bam*H I -*Hind*III linearized pRNAT-U6.1/Neo shRNA expression vector (Biomics Biotechnologies Co., Ltd., Nantong, China). A negative control vector (pRNAT-U6.1-negative) was constructed similarly with a shRNA sequence that does not suppress the expression of genes expressed in humans, rats, or mice (Biomics Biotechnologies Co., Ltd.). All inserted sequences were verified by DNA sequencing.

### Cell culture and transfection

Human hepatoma cell lines (HepG2, SMMC-7721, and SMMC-7402) and a normal hepatocyte cell line (LO2) were obtained from Biomics Biotechnologies Co., Ltd. The MHCC97-H cell line was obtained from the Liver Cancer Institute of the Affiliated Zhongshan Hospital of Fudan University (Shanghai, China). For all cell lines, maintenance growth was carried out in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc., Frederick, MD, United States) supplemented with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

For experimentation, the MHCC97-H cells were seeded into 6-well plates, allowed to grow to 90%-95% confluence, and transfected with the plasmids (pRNAT-U6.1-negative or pRNAT-U6.1-shRNA) using PolyJet™ *In Vitro* DNA Transfection Reagent (SignaGen Laboratories, Gaithersburg, MD, United States) according to the manufacturer's instructions. At 48 h after transfection, cells were selected by culturing in the presence of 400 µg/mL of G418 (Life Technologies, Inc.) for 2 wk followed by 200 µg/mL of G418 for an additional 2 wk. Individual G418-resistant monoclonals were obtained by performing a limiting dilution with subsequent proliferation in medium supplemented with 200 µg/mL of G418 to generate the stably transfected experimental (MHCC97-H/

ANXA2-shRNA) and control (MHCC97-H/control-shRNA) cell lines.

### RNA isolation and cDNA synthesis

Total RNA was isolated from mouse liver tissue specimens (50 mg) using the TRIzol Reagent (Life Technologies, Inc.) according to the manufacturer's instructions. Integrity of the isolated RNA was qualitatively assessed by 1% agarose gel electrophoresis and quantitatively assessed by ultraviolet spectrophotometry (absorbance at 260 nm,  $A_{260}$ ; SmartSpec™ Plus; Bio-Rad Life Science Research and Development Co., Ltd., Shanghai, China); purity of the isolated RNA was indicated by an absorbance ratio at  $A_{260}/_{280}$  of < 1.8. The total RNA (1  $\mu$ g) was applied as template for cDNA synthesis using the First-Strand cDNA Synthesis Kit (Fermentas Inc., Burlington, Canada) and following the manufacturer's instructions.

### Quantitative real-time polymerase chain reaction

The cDNA samples (4  $\mu$ g in 4  $\mu$ L) were subjected to quantitative real-time polymerase chain reaction (qPCR) using an Applied Biosystems StepOne™ Real-Time PCR System (Life Technologies, Inc.) with the manufacturer's recommended protocol. The reaction solution (50  $\mu$ L) contained 25  $\mu$ L of 2  $\times$  SYBR Premix ExTaq (Takara Biotechnology Co., Ltd., Dalian, China), 2  $\mu$ L of gene-specific primers, 1  $\mu$ L of 50  $\times$  ROX Reference Dye I, and 18  $\mu$ L deionized water. The following primers were used: ANXA2: forward, 5'-TGAGCGGGATGCTTTGAAC-3' and reverse, 5'-ATCCTGTCTCTGTGCATTGCTG-3';  $\beta$ -actin (internal control): forward, 5'-ATTGCCGACAGGATGCAGA-3' and reverse, 5'-GAGTACTTGCCTCAGGAGGA-3'<sup>[16]</sup>. Negative control reactions with no template (deionized water) were also included in each run. The optimized PCR conditions were: one cycle at 95 °C for 2 min; 40 cycles at 95 °C for 10 s, 62 °C for 1 min; 1 cycle at 72 °C for 2 min. The relative quantitative analysis was performed by comparison of the  $2^{-\Delta\Delta Ct}$  values.

### Western blotting

Total cell protein was extracted by sonication with 2  $\times$  sample buffer (0.25 mol/L Tris-HCl, 10% 2-mercaptoethanol, 4% sodium dodecyl sulphate (SDS), and 10% sucrose). Protein concentration was determined using the Enhanced Bicinchoninic Acid Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Samples (20  $\mu$ g) were resolved by 15% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, United States) in Tris-buffered saline (pH 7.5; 100 mmol/L NaCl, 50 mmol/L Tris, and 0.1% Tween-20), the membranes were immunoblotted overnight at 4 °C with monoclonal primary antibody rabbit anti-human ANXA2 (1:500 dilution) and monoclonal primary antibody mouse anti-human  $\beta$ -actin (1:500) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, United States), washed three times, followed by incubation with the cor-

responding horseradish peroxidase-conjugated secondary antibodies (1:1000). Immunoreactive bands were visualized by chemiluminescence detection (Milipore Corp., Billerica, MA, United States) and analysed by densitometric analysis using the ImageJ software (version 1.30v; National Institutes of Health Research Services Branch, Bethesda, MD, United States). The ANXA2 protein level is expressed as relative ratio, which was calculated as signal intensity (SI) of ANXA2 divided by SI of  $\beta$ -actin<sup>[19]</sup>.

### Immunofluorescence assay

Cells ( $1 \times 10^6$ ) were plated on cover slips and cultured for 24 h in 24-well plates. After three washes with phosphate-buffered saline (PBS), the cells were fixed with 4% paraformaldehyde for 10 min and blocked with 3% BSA in PBS for 30 min at 37 °C. Then, the samples were incubated overnight at 4 °C with the anti-ANXA2 (1:100). After three washes with PBS, the samples were incubated for 1 h at 37 °C in the dark with the corresponding Cy3-labeled goat anti-rabbit immunoglobulin G (IgG) secondary antibody (1:500; Beyotime Institute of Biotechnology), washed three times with PBS, stained with 4,6-diamidino-2-phenylindole for 5 min, washed three times with PBS, and sealed with 50% glycerine. The immunostaining analysis was conducted by a multifunction microscope (IX71; Olympus Corp., Tokyo, Japan).

### Cell proliferation assay

Cell proliferation was evaluated using the cell counting kit-8 (CCK-8; Beyotime Institute of Biotechnology) and following the manufacturer's instructions. MHCC97-H (untransfected), MHCC97-H/control-shRNA and MHCC97-H/ANXA2-shRNA cells were seeded in 96-well plates ( $2 \times 10^3$  cells/well in 100  $\mu$ L medium) and cultured for 24 h. Wells with medium alone (no cells) served as blank controls. CCK-8 solution (10  $\mu$ L/well) was added to the culture medium and incubated for 2 h, after which the  $A_{450}$  was measured by a microplate reader (Synergy HT; BioTek Corp., Winooski, VT, United States) at various time points. Experiments were performed in triplicate.

### Cell cycle assay

Cell cycle assay was performed using the cell cycle and apoptosis analysis kit (Beyotime Institute of Biotechnology) and following the manufacturer's instructions. MHCC97-H (untransfected), MHCC97-H/control-shRNA and MHCC97-H/ANXA2-shRNA cells were seeded in 6-well plates ( $1.0 \times 10^6$  cells/well in 2 mL medium). After 24 h of culturing, the cells were digested with trypsin enzyme, washed with pre-cooled PBS, and fixed in pre-cooled 70% ethanol for 24 h at 4 °C. The cells were then stained with propidium iodide and analysed by flow cytometry (FACSCalibur; Becton Dickinson Medical Devices Co Ltd., Shanghai, China) to determine the cell cycle distribution. Experiments were performed in triplicate<sup>[20]</sup>.

### Transwell assay

MHCC97-H (untransfected), MHCC97-H/control-shRNA

and MHCC97-H/ANXA2-shRNA cells were plated at  $1.0 \times 10^5$  cells/well in 0.5 mL of serum-free medium in 24-well matrigel-coated transwell units with polycarbonate filters (8  $\mu\text{m}$  pore size; Costar Inc., Milpitas, CA, United States). The outer chambers were filled with 0.5 mL of medium supplemented with 10% FBS. After 24 h, the cells were fixed in methanol and stained with crystal violet. The top surface of the membrane was gently scrubbed with a cotton bud, and the cells that had invaded through the membrane filters were counted. The invasion inhibition rate (%) was calculated as  $[(A - B)/A] \times 100$ , where A was the invading cells' percentage for the MHCC97-H group and B was the invading cells' percentage for the MHCC97-H/ANXA2-shRNA group.

### *In vitro* wound-healing assay

The various HCC cell types were cultured in 12-well plates; at subconfluency, the cell monolayer was scratched (wound) with a plastic pipette tip, washed with serum-free medium, and cultured in DMEM medium supplemented with 0.1% FBS. The relative migration distance (%) travelled at various time points (0 and 8 h) was calculated as  $[(A_x - B_x)/(A_{\text{mock}} - B_{\text{mock}}) \times 100\%]$ , where A was the wound width prior to incubation and B was the wound width after incubation<sup>[21]</sup>.

### Xenograft tumour-growth assay

The animal protocol was approved by the Ethical Review Committee for Animal Experimentation of Nantong University (China). Specific-pathogen free BALB/C nude mice (6-wk-old and  $20 \pm 3$  g; Super-B and K Laboratory Animal Co., Ltd., Shanghai, China) were randomly assigned to three groups for xenografting of MHCC97-H (untransfected;  $n = 4$ ), MHCC97-H/control-shRNA ( $n = 4$ ), and MHCC97-H/ANXA2-shRNA ( $n = 4$ ) cells. For each group, right flank subcutaneous injection was made with  $2 \times 10^7$  of the respective HCC cells suspended in 0.2 mL of DMEM medium. Four mice injected with normal saline alone represented the control (non-xenografted) group. Over 21 d of growth, tumour size was routinely measured using callipers and used to calculate the tumour volume by the formula:  $[(\text{length} \times \text{width}^2)/2]$ . On post-injection day 21, the animals were sacrificed for liver excision and complete tumour resection. The tumorigenicity inhibition rate (%) was calculated for each HCC-xenografted group as  $[(\text{tumour weight}_{\text{control}} - \text{tumour weight}_{\text{shRNA}})/\text{tumour weight}_{\text{control}}] \times 100$ <sup>[22]</sup>.

### Histopathological examination of resected xenografted tumours

Resected tissues were processed for haematoxylin and eosin staining by dehydrating, sectioning (3  $\mu\text{m}$  thick), and mounting on glass slides. For analysis, the sections were rehydrated in distilled water for 2 min, stained with haematoxylin for 5 min, washed with tap water three times for 5 min each, dehydrated with 95% ethanol for 5 s stained with eosin for 2 min, washed with 70% ethanol two times for 5 min each, and air dried.

### Immunohistochemical examination of resected xenografted tumours

Resected tissues were processed for immunohistochemical analysis by formalin fixing, embedding in paraffin, sectioning (3  $\mu\text{m}$  thick), and mounting on glass slides. For analysis, the sections were deparaffinised by soaking in xylene for two times at 10 min each, dehydrated by soaking in an ethanol to distilled water gradient for 5 min at each serial dilution, washed with PBS (pH 7.4) three times, and incubated in endogenous peroxidase blocking solution for 5 min (Immunostain EliVision Kit; Maixin Biotech Inc., Fuzhou, China). The treated sections were subjected to antigen-retrieval by boiling in 0.01 mol/L citrate buffer (pH 6.0) for 10 min (650 W microwave) and blocking of non-specific antibody binding by pretreatment with 0.5% BSA in PBS. After rinsing with PBS, the processed sections were incubated overnight at 4 °C with ANXA2 antibody (1:500), washed three times with 0.05% Tween-20 in PBS, and stained with the chromogen 3, 3'-diaminobenzidine tetrahydrochloride. The slide was then rinsed with distilled water, counterstained with haematoxylin, dehydrated, and air-dried. Negative control sections were generated by the same procedure except that the non-specific mouse IgG antibody was used. All samples were evaluated by light microscope by an expert who was blinded to the group and outcome. ANXA2 staining intensity is expressed as an immunoreactive score<sup>[23]</sup>.

### Statistical analysis

Results are expressed as mean  $\pm$  SD. Significance of differences detected between groups was assessed by one-way analysis of variance followed by the least significant difference test or Newman-Keuls test. A *P* value of  $< 0.05$  was set as the threshold of significance.

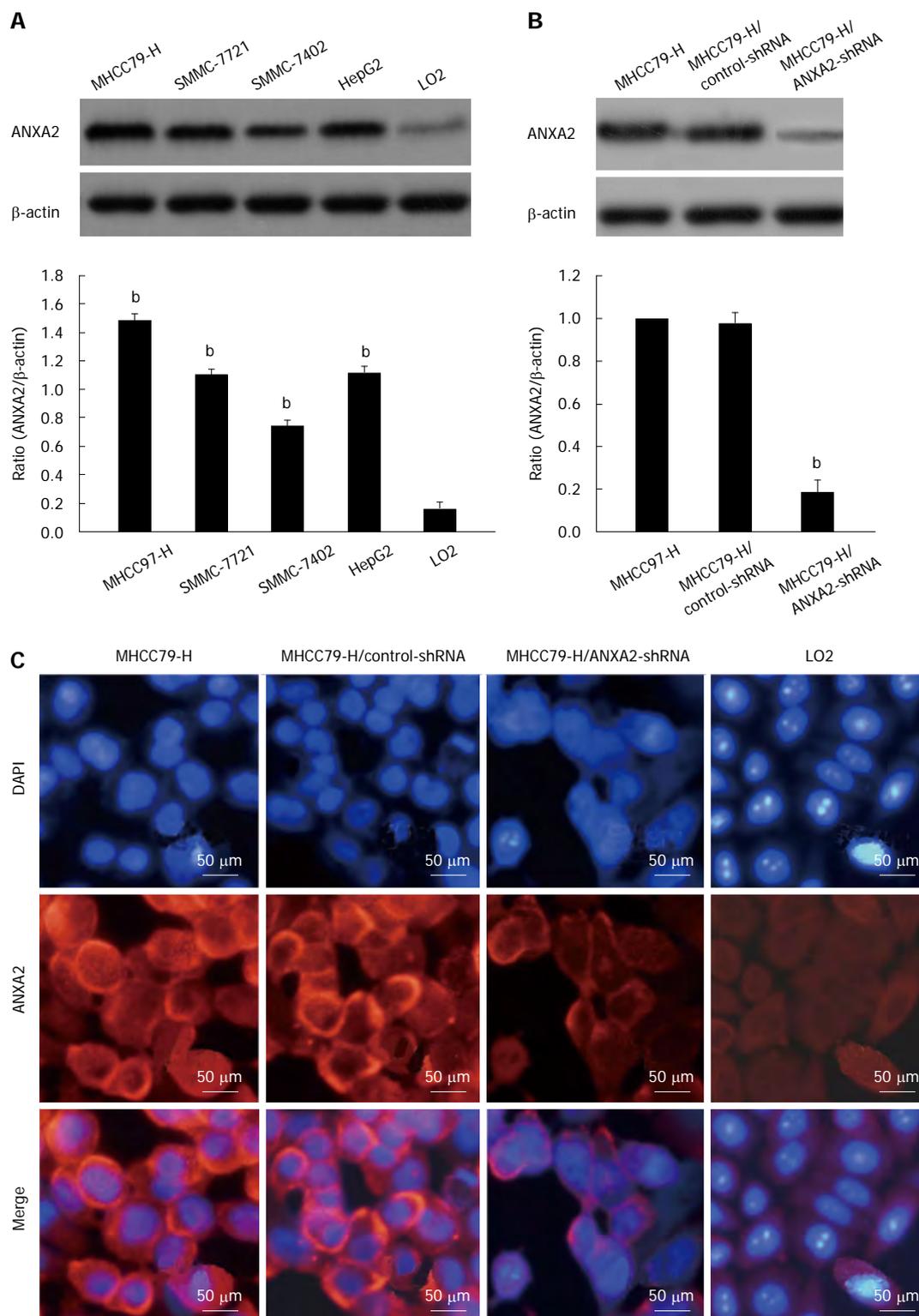
## RESULTS

### ANXA2 is over-expressed in HCC cells

As shown in Figure 1A, the ANXA2 protein level detected in MHCC97-H cells was the highest among the four HCC cell lines and was 8-times higher ( $P < 0.05$ ) than that detected in the normal cell line LO2. Additionally, the level of ANXA2 mRNA expression was significantly higher in the MHCC97-H cells than that in the other HCC cells and the LO2 cells (Table 1;  $P < 0.001$ ). Thus, the MHCC97-H cell line was selected for subsequent study of the effects of shRNA targeting ANXA2 on cell invasion, migration, and tumorigenic potential of hepatoma cells.

### ANXA2 expression in MHCC97-H cells is inhibited by shRNA *in vitro*

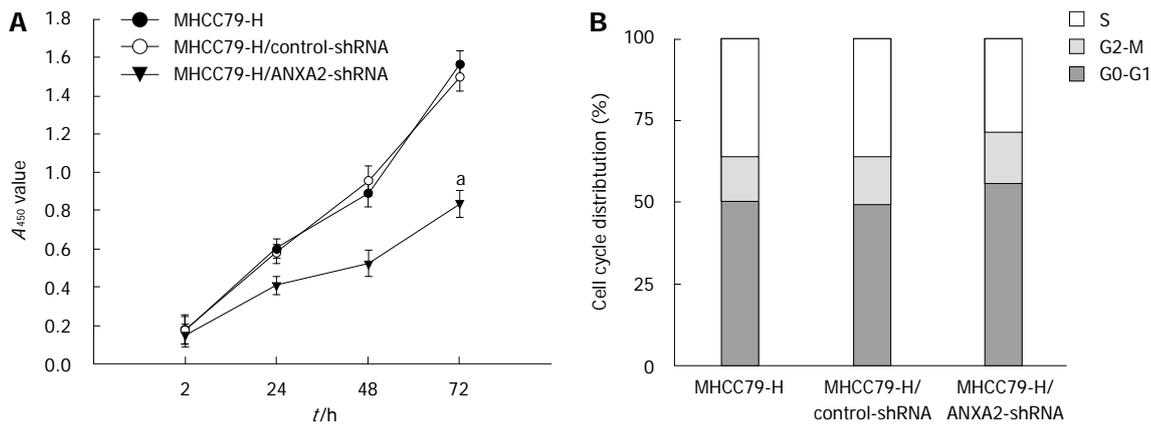
The silencing efficiency of ANXA2-specific shRNA in MHCC97-H cells reached approximately 80%. As shown in Table 2 and Figure 1B, the expression levels of ANXA2 mRNA and protein were significantly lower in the MHCC97-H/ANXA2-shRNA cells than in the MHCC97-H/control-shRNA cells and in the MHCC97-H



**Figure 1** Annexin A2 expression level in hepatoma cells and silencing efficiency of small hairpin RNA in MHCC97-H cells. A: Representative Western blotting images of hepatocellular carcinoma cell lines and the normal hepatic cell line LO2. <sup>b</sup>*P* < 0.01 vs LO2; B: Representative Western blotting images of annexin A2 (ANXA2) silencing upon transfection of small hairpin RNA (shRNA). <sup>b</sup>*P* < 0.01 vs MHCC97-H; C: Representative immunofluorescence images of ANXA2 cellular distribution (× 400). DAPI: 4,6-diamidino-2-phenylindole.

(untransfected) cells. The levels detected in the MHCC97-H/control-shRNA cells and MHCC97-H cells were not significantly different. As shown in Figure 1C, ANXA2 (red fluorescence) expression in the MHCC97-H cells

was mainly localized to the cellular membrane and cytoplasm, and some localization was detected in the nucleus. The MHCC97-H/ANXA2-shRNA cells showed obviously lower density of the red immunofluorescent signal



**Figure 2** Effect of small hairpin RNA-mediated annexin A2 silencing on proliferation and cell cycling of MHCC97-H cells. A: Cellular proliferation assay. <sup>a</sup> $P < 0.05$  vs the  $A_{450}$  of MHCC97-H cells at 72 h; B: Cell cycle assay.  $P < 0.05$  for percentage of MHCC97-H/annexin A2 (ANXA2)-small hairpin RNA (shRNA) cells in S phase vs that of MHCC97-H cells.

**Table 1** Expression level of ANXA2 mRNA in hepatocellular carcinoma cells and normal hepatic cells

| Group     | n | C <sub>t</sub> ANXA2 | C <sub>t</sub> $\beta$ -actin | 2 <sup>-<math>\Delta\Delta</math>Ct</sup> |
|-----------|---|----------------------|-------------------------------|---|
| LO2       | 5 | 25.16 $\pm$ 0.09     | 20.86 $\pm$ 0.03              | 1.00                                      |
| HepG2     | 5 | 22.14 $\pm$ 0.15     | 20.66 $\pm$ 0.02              | 7.07 $\pm$ 0.35 <sup>b</sup>              |
| SMMC-7402 | 5 | 22.87 $\pm$ 0.15     | 20.80 $\pm$ 0.14              | 4.68 $\pm$ 0.31 <sup>b</sup>              |
| SMMC-7721 | 5 | 22.21 $\pm$ 0.12     | 20.72 $\pm$ 0.10              | 7.02 $\pm$ 0.19 <sup>b</sup>              |
| MHCC97-H  | 5 | 21.85 $\pm$ 0.26     | 20.78 $\pm$ 0.13              | 9.45 $\pm$ 0.53 <sup>b</sup>              |

<sup>b</sup> $P < 0.001$  vs the LO2 group.

in the cellular membrane and cytoplasm, as compared to both MHCC97-H and MHCC97-H/control-shRNA cells. Thus, the ANXA2-shRNA was capable of down-regulating ANXA2 expression. Intriguingly, the nuclei of the MHCC97-H/ANXA2-shRNA cells (blue fluorescence) showed signs of early apoptosis (fragmentation with condensed chromatin), as compared to the nuclei of the MHCC97-H cells or the MHCC97-H/control-shRNA cells.

### ANXA2 deficiency inhibits proliferation of MHCC97-H cells *in vitro*

The effects of ANXA2 deficiency on proliferation and cell cycling of MHCC97-H cells are shown in Figure 2. At 72 h post-transfection, the MHCC97-H/ANXA2-shRNA cells showed significantly lower proliferation potential than the MHCC97-H cells or MHCC97-H/control-shRNA cells ( $P < 0.05$ ) (Figure 2A). The proliferation rates of the MHCC97-H cells and the MHCC97-H/control-shRNA cells were not significantly different. The percentage of MHCC97-H/ANXA2-shRNA cells in S phase was significantly lower than that in the MHCC97-H cells (27.76% vs 36.14%,  $P < 0.05$ ), whereas the percentages of MHCC97-H/ANXA2-shRNA cells in G<sub>0</sub>-G<sub>1</sub> phase and G<sub>2</sub>-M phase were significantly higher than those in the MHCC97-H cells (both  $P < 0.05$ ) (Figure 2B). Thus, shRNA targeted suppression of ANXA2 inhibits the growth of MHCC97-H/shRNA cells *in vitro*.

**Table 2** Inhibition of ANXA2 mRNA expression by small hairpin in MHCC97-H cells

| Group                  | n | C <sub>t</sub> ANXA2 | C <sub>t</sub> $\beta$ -actin | 2 <sup>-<math>\Delta\Delta</math>Ct</sup> |
|------------------------|---|----------------------|-------------------------------|---|
| MHCC97-H               | 5 | 21.84 $\pm$ 0.11     | 20.77 $\pm$ 0.16              | 1.00                                      |
| MHCC97-H/control-shRNA | 5 | 21.83 $\pm$ 0.21     | 20.72 $\pm$ 0.10              | 0.97 $\pm$ 0.04                           |
| MHCC97-H/ANXA2-shRNA   | 5 | 24.24 $\pm$ 0.55     | 20.80 $\pm$ 0.14              | 0.20 $\pm$ 0.05 <sup>b</sup>              |

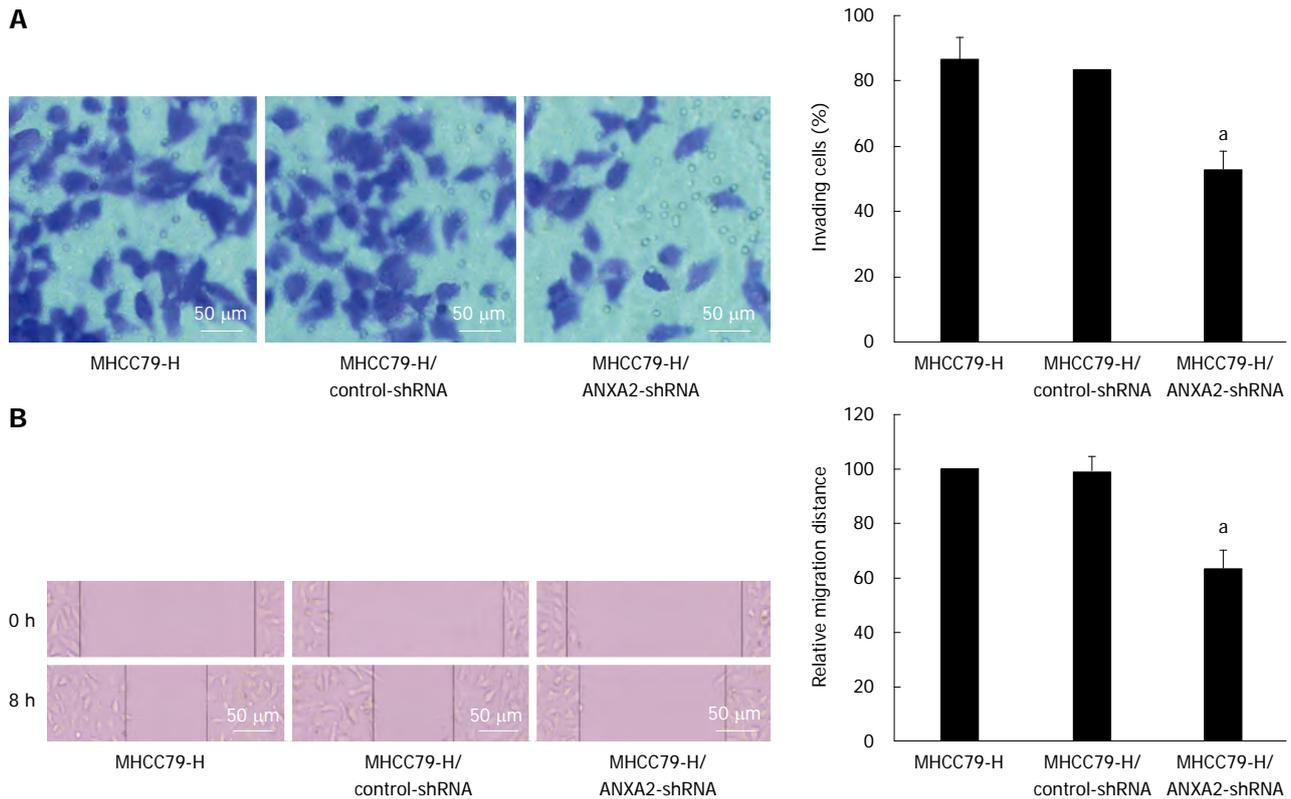
<sup>b</sup> $P < 0.001$  vs the MHCC97-H group. shRNA: Small hairpin RNA.

### ANXA2 silencing suppresses the invasion and migration potential of MHCC97-H cells *in vitro*

As shown in Figure 3, the invasive potential of MHCC97-H/ANXA2-shRNA cells was significantly lower than that of the MHCC97-H cells (52.16% vs 86.14%,  $P < 0.05$ ). The invasion inhibition rate in MHCC97-H/ANXA2-shRNA cells reached up to 39.45%. In addition, the migration potential of MHCC97-H/ANXA2-shRNA cells was significantly lower than that of the MHCC97-H cells (63.49% vs 100%,  $P < 0.05$ ). The migration inhibition rate in MHCC97-H/ANXA2-shRNA cells reached up to 36.51%. The migration inhibition rates of the MHCC97-H cells and the MHCC97-H/control-shRNA cells were not significantly different.

### Down-regulation of ANXA2 inhibits xenograft tumour growth *in vivo*

As shown in Figure 4A, the tumour volumes of MHCC97-H/ANXA2-shRNA xenograft group were remarkably smaller than those of the MHCC97-H xenograft group or the MHCC97-H/control-shRNA xenograft group. Unlike the non-xenografted group (Figure 4A), all three xenografted groups showed obvious emaciation, especially the MHCC97-H group and the MHCC97-H/control-shRNA group. As shown in Figure 4B, by day 21 after cell injection the average tumour weight for the MHCC97-H/ANXA2-shRNA group was distinctly lower than that for either the MHCC97-H group or the MHCC97-H/control-shRNA group (both  $P < 0.05$ ). The shRNA-mediated inhibition rate of xenografted tumours reached 38.2%. The



**Figure 3** Suppressive effect of small hairpin RNA-mediated annexin A2 silencing on the invasion and migration potential of MHCC97-H cells. A: Representative images of invasive cells (stained with crystal violet) from the MHCC97-H group; the MHCC97-H/control-small hairpin RNA (shRNA) group; the MHCC97-H/annexin A2 (ANXA2)-small hairpin RNA (shRNA) group; B: Representative images of cell migration. <sup>a</sup>*P* < 0.05 vs MHCC97-H cells.

**Table 3** Intensity of ANXA2 expression in xenograft tumours

| Group                  | n | ANXA2 intensity |   |    |     | Z                  |
|------------------------|---|-----------------|---|----|-----|--------------------|
|                        |   | -               | + | ++ | +++ |                    |
| MHCC97-H               | 4 | 0               | 0 | 0  | 4   |                    |
| MHCC97-H/control-shRNA | 4 | 0               | 0 | 1  | 3   | 1.000              |
| MHCC97-H/ANXA2-shRNA   | 4 | 1               | 3 | 0  | 0   | 2.530 <sup>a</sup> |

<sup>a</sup>*P* < 0.05 vs the MHCC97-H group. shRNA: Small hairpin RNA.

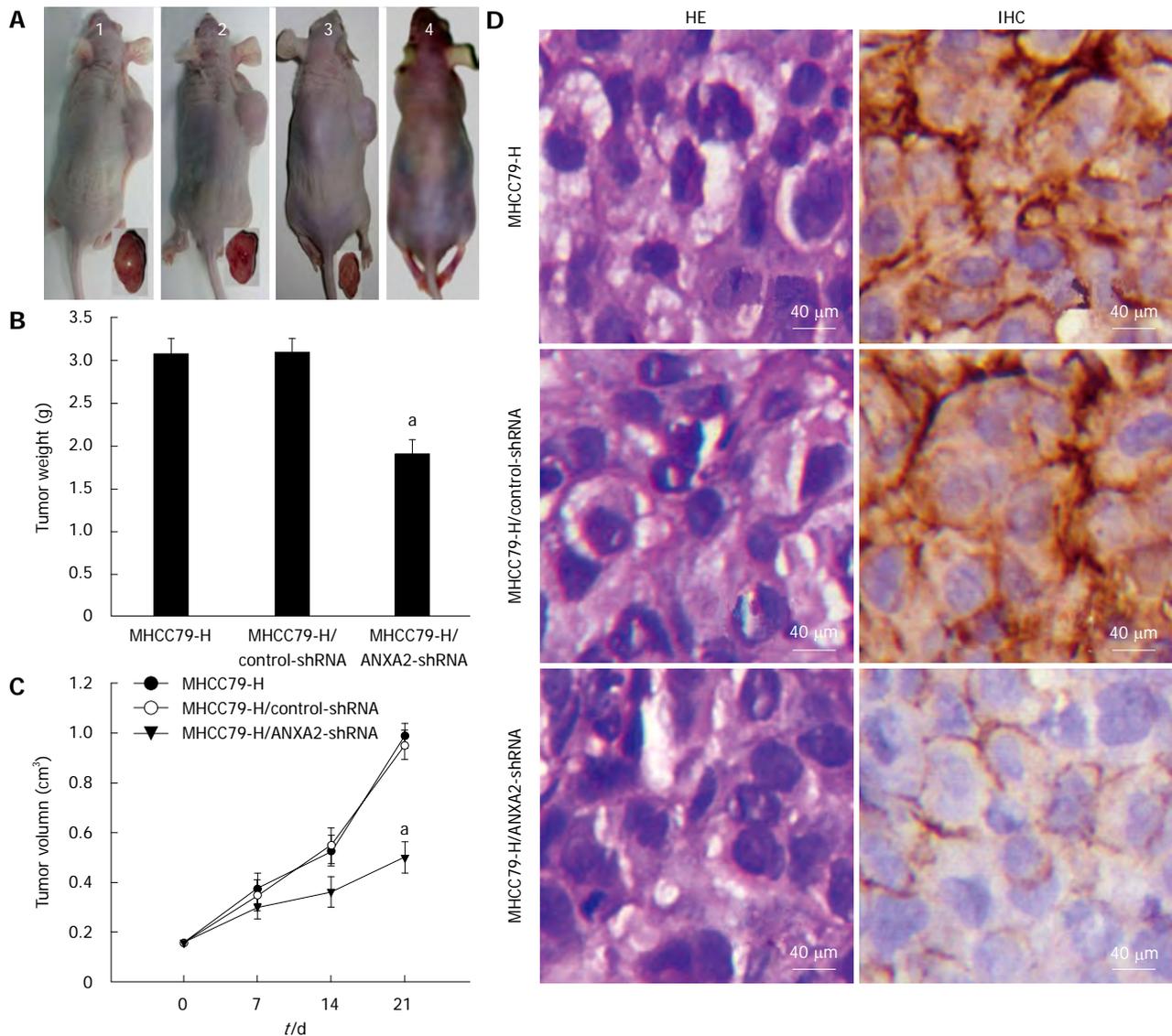
tumour growth curve over 21 d indicated that ANXA2 silencing in MHCC97-H cells reduced their tumorigenic potential *in vivo* (Figure 4C).

As shown in Figure 4D, the morphological characteristics of the xenograft tumours derived from MHCC97-H/ANXA2-shRNA cells were not fundamentally different from the tumours derived from the other cell types. Similar to the *in vitro* observations, ANXA2 expression was mainly localized to the cellular membrane in the MHCC97-H/ANXA2-shRNA tumour tissues and to both the cellular membrane and cytoplasm in the MHCC97-H tumour tissues and the MHCC97-H/control-shRNA tumour tissues. Moreover, the ANXA2 expression level in the xenograft tumours of the MHCC97-H/ANXA2-shRNA group was significantly lower than that in the xenograft tumours of the MHCC97-H group (Table 3). The ANXA2 expression levels in the xenograft tumours of the MHCC97-H group and the MHCC97-H/control-shRNA group were not significantly different.

## DISCUSSION

In the present study ANXA2 was found to be up-regulated in HCC cells, particularly those with high metastasis potential and invasion ability. Furthermore, shRNA-mediated silencing of ANXA2 was shown to inhibit the invasion, migration, and tumorigenic potential of hepatoma cells. These results not only provide further insight into the pathogenic mechanisms of HCC, but also suggest ANXA2 as a potential target of future molecular therapies.

Enhanced expression and activation of ANXA2 in cancerous tissues has been reported for many different tumour types, including HCC<sup>[14,24,25]</sup>, which led investigators to consider the potential anti-tumour benefit of inhibiting its gene expression or protein phosphorylation. Knockdown of ANXA2 in glioma cells led to a remarkable decrease in tumour size and suppression of tumour progression and proliferation<sup>[26]</sup>. Similarly, our results showed that the proliferation ability of MHCC97-H/ANXA2-shRNA cells was significantly suppressed both *in vitro* and *in vivo*. In another previous study of non-small cell lung cancer (NSCLC), ANXA2 knockdown in human adenocarcinoma A549 cells led to defective proliferation *in vitro* and reduced tumour growth *in vivo*; additionally, the knockdown cells were arrested at the G2 phase<sup>[27]</sup>. In the current study, MHCC97-H cells with shRNA-mediated silencing of ANXA2 showed a significantly lower percentage of cells in the S phase than the MHCC97-H cells



**Figure 4** Inhibitive effect of small hairpin RNA-mediated annexin A2 silencing on xenograft tumour growth *in vivo*. A: Representative images of xenografted and control mice and resected tumours. The MHCC97-H (untransfected) group (1); the MHCC97-H/control-small hairpin RNA (shRNA) group (2); the MHCC97-H/annexin A2 (ANXA2)-shRNA group (3); the blank control group (4). Tumorigenic nude mice appeared obviously emaciated, especially the those in the MHCC97-H group and MHCC97-H/control-shRNA group; B: Average tumour weights. <sup>a</sup> $P < 0.05$  vs MHCC97-H group; C: Tumour growth rates. <sup>a</sup> $P < 0.05$  vs MHCC97-H group at post-injection day 21; D: Representative immunohistochemical analysis and hematoxylin and eosin staining results ( $\times 400$ ). The density of ANXA2 staining (brown) in the cytoplasm of MHCC97-H/ANXA2-shRNA cells was obviously lower than that for the MHCC97-H cells or the MHCC97-H/control-shRNA cells. ANXA2 was mainly localized in the membrane of the MHCC97-H/ANXA2-shRNA cells, and localized in both the membrane and cytoplasm of the MHCC97-H cells and the MHCC97-H/control-shRNA cells. The morphological characteristics of subcutaneous xenograft tumours derived from MHCC97-H/ANXA2-shRNA cells were not fundamentally different from the other tumours.

with endogenously enhanced ANXA2. This results from both studies indirectly indicate a promising anti-proliferative effect for ANXA2 silencing.

While previous studies have shown that increased ANXA2 levels are associated with enhanced tumour cell proliferation and HCC development<sup>[21,27]</sup>, our study achieved a relatively low inhibition of tumour growth upon ANXA2 silencing. In the NSCLC study described above, the downstream effects of ANXA2 silencing were explored and a significant effect on p53 expression was discovered<sup>[27]</sup>. It is possible that ANXA2 may facilitate cell proliferation partly through the regulation of p53 *via* JNK/c-Jun in HCC, since disruption of the p53/

miRNA-34 axis has been shown to result in abnormal apoptosis and tumour progression<sup>[28]</sup>. Furthermore, since ANXA2 is only one of the increased proteins in HCC<sup>[1,2]</sup>, ANXA2 deficiency is expected to only partly, rather than wholly, suppress tumour growth.

Previous molecular studies have determined that ANXA2 promotes invasion and migration of human HCC cells *in vitro* *via* its interaction with HAB18G/CD147<sup>[18,21]</sup>. Depletion of HAB18G/CD147 produced a rounded morphology, which is associated with amoeboid movement, while depletion of ANXA2 resulted in an elongated morphology, which is associated with mesenchymal movement. HAB18G/CD147 was also found to promote

cell motility of HCC cells by regulating ANXA2-activated RhoA and Rac1 signalling pathways<sup>[18,21]</sup>. Plasmin, a downstream factor of ANXA2 activity, is essential for metastatic progression due to its activation of metalloproteinases and degradation of extracellular matrix components<sup>[10,29]</sup>. In another previous study of human adenomyosis, ANXA2 was shown to function as a key mediator of metastasis and proangiogenesis of endometrial cells<sup>[30]</sup>. Accumulating evidence has suggested that interactions between ANXA2 and its binding partners contribute to the tumour microenvironment, and together act to enhance metastasis<sup>[10]</sup>. Our observations of ANXA2 silencing suppressing the invasion and migration potential of hepatoma cells suggest that the invasion and migration potential of HCC cells are correlated with ANXA2 expression level. Furthermore, our finding that ANXA2 silencing is sufficient to inhibit invasion of HCC cells support the notion that ANXA2 is a potential therapeutic target for treating tumour development (through angiogenesis) and progression (through metastasis)<sup>[31,32]</sup>.

The effects of ANXA2 on p53 may modulate apoptotic processes, which are critically associated with cell survival and may also have effects on proliferation<sup>[33]</sup>. Additionally, ANXA2 has been characterized as one of the factor H ligands binding to apoptotic cells, and has been shown to promote cell apoptosis in systemic lupus erythematosus *via* this mechanism<sup>[34]</sup>. In the current study, obvious apoptosis was observed in MHCC97-H/ANXA2-shRNA, suggesting that ANXA2-shRNA could suppress the proliferation and invasion ability of hepatoma cells with high metastasis potential, possibly by promoting cell apoptosis. In a previous study of the expression characteristics and diagnostic value of ANXA2 in HCC, we discovered an intermediate state of ANXA2 level in HCC adjacent tissue<sup>[35]</sup>. In the current study, we defined the distribution profile of ANXA2 expression in HCC cells, with the primary localization occurring in the cell membrane and cytoplasm and some localization occurring in the nucleus.

In conclusion, the results presented herein suggest that ANXA2 is up-regulated in HCC cells with high metastasis potential and invasion ability, and demonstrated that shRNA-mediated silencing of ANXA2 inhibits the invasion, migration, and tumorigenic potential of HCC cells. Collectively, these data reveal a link between the level of ANXA2 expression and HCC metastasis, as well as suggest the potential utility of ANXA2 as a predictive biomarker for HCC prognosis and as a therapeutic target of molecular-based strategies. It, therefore, will be important to carry out further investigations to identify additional signalling modulators and to delineate pivotal regulatory mechanisms involving ANXA2 and HCC metastasis.

## COMMENTS

### Background

Cell invasion and tumour progression remain two of the major obstacles that must be overcome before hepatocellular carcinoma (HCC) is finally conquered.

Annexin A2 (ANXA2) is a 36-kDa calcium-dependent, phospholipid-binding protein expressed on various cell types. In addition, ANXA2 expression is up-regulated in various tumour types, where it plays multiple roles in tumorigenesis and development.

### Research frontiers

Up-regulated ANXA2 expression has been detected in clinical samples of HCC, and ANXA2 phosphorylation has been demonstrated as essential for invasion and metastasis of pancreatic cancer. Moreover, the mechanism by which ANXA2 promotes tumour metastasis has been defined as induction of the conversion of plasminogen to plasmin. However, the effect of small hairpin RNA targeting ANXA2 on biological behaviours of hepatoma cells has not yet been reported. In the present study, ANXA2 overexpression was found in MHCC97-H cells, a novel HCC cell line with high metastasis potential, which was then used to investigate the effects of ANXA2 silencing on cell invasion, migration, and tumorigenic potential of HCC.

### Innovations and breakthroughs

This study provides the first reported evidence of endogenous ANXA2 up-regulation in HCC cells with high metastasis potential and invasion ability, and of ANXA2 deficiency inhibiting the invasion, migration, and tumorigenic potential of HCC cells. Collectively, these data not only reveal a link between the level of ANXA2 expression and HCC metastasis, but also indicate the potential utility of this factor as a predictive biomarker for HCC prognosis and as a potential therapeutic target.

### Applications

The results provide further insight into the pathogenesis of HCC. These data provide foundational knowledge for further expansion in future studies to identify the additional signalling modulators involved in this pathogenic mechanism. Moreover, the newly identified tumorigenic roles of ANXA2 suggest its utility as a target of anti-metastatic and anti-proliferative therapies.

### Peer review

The paper is good design and illustration, deserved to be published.

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**P- Reviewers** Mazzanti R, Xu RY **S- Editor** Zhai HH

**L- Editor** A **E- Editor** Xiong L



## Role of activin A in carbon tetrachloride-induced acute liver injury

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Supported by The Natural Science Foundation of China, Grants No. 30801005, No. 81273199 and No. 81270513; and the Project of Science and Development of Jilin Province (to Liu ZH)

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Received: February 16, 2013 Revised: April 12, 2013

Accepted: May 8, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the expression and role of activin A in a mouse model of acute chemical liver injury.

**METHODS:** Acute liver injury in C57BL/6 male mice was induced by intraperitoneal injection with carbon tetrachloride (CCl<sub>4</sub>) (0.5 mL/kg, body weight) dissolved in olive oil (1:19 v/v). Mice were sacrificed 1, 3, 5 and 7 d after the treatment. The levels of alanine aminotrans-

ferase (ALT) and aspartate aminotransferase (AST) in serum were examined and pathological changes of liver observed by hematoxylin and eosin staining to evaluate the liver injury. Activin A protein levels in serum and hepatic tissue homogenate of mice were detected by enzyme-linked immunosorbent assay, and the expression pattern of activin A protein in livers of mice was examined by immunohistochemistry. Activin type II A receptor (ActR II A) and Smad3 expressions in the liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. In order to further investigate the role of activin A, we also utilized activin A blocking experiment by anti-activin A antibody (500 µg/kg, body weight) injection into mouse tail vein.

**RESULTS:** In CCl<sub>4</sub>-treated mice, serum ALT and AST levels were significantly increased, compared with that in control mice ( $P < 0.01$ ). Furthermore, the serious necrosis was observed around hepatic portal areas in CCl<sub>4</sub>-treated mice. Simultaneously, activin A levels in serum and hepatic tissue homogenate of mice treated with CCl<sub>4</sub> for 1, 3 and 5 d increased significantly, compared with that in control mice ( $P < 0.01$ ). Activin A protein expression in hepatocytes not within the necrotic area was also upregulated in mice following CCl<sub>4</sub> treatment. Not only activin A, but also ActR II A and activin signaling molecule Smad3 mRNA expressions in injury liver induced by CCl<sub>4</sub> were significantly higher than that in control liver. In addition, levels of serum ALT and AST in CCl<sub>4</sub>-treated mice were significantly decreased by injection of anti-activin A antibody to block endogenous activin A action, compared with that in CCl<sub>4</sub>-treated mice by injection of immunoglobulin G instead of anti-activin A antibody ( $P < 0.01$ ), and the severity of liver injury was also reduced remarkably.

**CONCLUSION:** These data show that activin A is involved in CCl<sub>4</sub>-induced acute liver injury. Blocking activin A actions may be a therapeutic approach for acute liver injury.

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**Key words:** Liver injury; Carbon tetrachloride; Activin A; Immunohistochemistry

**Core tip:** The objective of this study was to investigate the expression and role of activin A in acute liver injury. A carbon tetrachloride (CCl<sub>4</sub>)-induced acute liver injury mouse model was used. Liver injury effects were examined by measuring alanine aminotransferase and aspartate aminotransferase levels in serum and liver pathological changes. Activin A protein expression levels were detected by enzyme-linked immunosorbent assay and immunohistochemistry. Activin type II A receptor and Smad3 expressions in liver were analyzed by real-time quantitative reverse transcription-polymerase chain reaction. We found that activin A is involved in CCl<sub>4</sub>-induced acute liver injury, suggesting activin A could be a potential therapeutic option for acute liver injury diseases.

Wang DH, Wang YN, Ge JY, Liu HY, Zhang HJ, Qi Y, Liu ZH, Cui XL. Role of activin A in carbon tetrachloride-induced acute liver injury. *World J Gastroenterol* 2013; 19(24): 3802-3809 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3802.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3802>

## INTRODUCTION

Activin A, a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, has a wide range of biological roles<sup>[1-3]</sup>. It is distributed widely in various tissues and produced by numerous cells including macrophages, T-helper 2 cells and hepatocytes<sup>[4-6]</sup>. It plays important roles in regulation of pituitary hormone release, neuron survival, hematopoiesis and the early development of embryos<sup>[7-10]</sup>. The expression of activin A is closely related to liver diseases, and abnormal expression of activin A and its signal proteins are found during the development of virus hepatitis, hepatic fibrosis, liver cancer and other diseases<sup>[11-14]</sup>. Activin A affects the function of hepatocytes by inhibiting DNA synthesis while stimulating the synthesis of the extracellular matrix of hepatic stellate cells, which can result in the hepatic fibrosis<sup>[2,11,12]</sup>.

The liver is an important metabolic organ in the body, and can be injured by various factors, which include viral infection, trauma, and chemical reagents. Carbon tetrachloride (CCl<sub>4</sub>)-induced liver injury is a classic model of chemical liver injury in mice<sup>[15]</sup>. Activin A expression increased significantly in CCl<sub>4</sub>-induced chronic liver injury in mice, marking it as an important factor in liver fibrosis development<sup>[16]</sup>. However, it is still unclear whether activin A is involved in the process of CCl<sub>4</sub>-induced acute chemical liver injury.

In the present study, the expression and role of activin A were investigated in CCl<sub>4</sub>-induced acute chemical liver injury in mice. Further, the role of activin A in the process of acute liver injury was confirmed by *in vivo* blockade of activin A action.

## MATERIALS AND METHODS

### Reagents

CCl<sub>4</sub> was purchased from Beijing Chemical Factory (batch number 20050106). Olive oil was obtained from Beijing KeLipei Tsui olive oil Development Centers. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kit was provided by NJBI (Nanjing, China). Anti-activin A antibody was obtained from Sigma Company. Trizol reagent was provided by Invitrogen Corporation. SYBR fluorescence quantitative reverse transcription-polymerase chain reaction (PCR) kit was purchased from Takara Company.

### Animals

C57BL/6 male mice were provided by Animal Center of Jilin University (Changchun, China). All animal experiments were performed following an institutionally approved protocol in accordance with the Jilin University Guide for the care and use of laboratory animals.

### Preparation of CCl<sub>4</sub>-induced acute liver injury mouse model

C57BL/6 mice were randomly divided into the olive oil control group and the CCl<sub>4</sub> group ( $n = 24$ ). In the control group, mice were treated with olive oil (10 mL/kg) by intraperitoneal injection; in the CCl<sub>4</sub> group, mice were injected intraperitoneally with CCl<sub>4</sub> (0.5 mL/kg) + olive oil (9.5 mL/kg) (1:19 v/v). Mice were executed 1, 3, 5 and 7 d after the treatment, serum was collected for the determination of transaminases and activin A levels, and hepatic tissues were obtained for pathological examination and immunohistochemical staining.

### Determination of serum transaminases ALT and AST

The serum transaminases ALT and AST levels were detected by assay kit according to the manufacturer's protocol (NJBI, Nanjing, China). Briefly, 25  $\mu$ L AST or ALT substrates and 5  $\mu$ L serum were added into one well of polystyrene microtiter plates at 37 °C for 30 min. And then 25  $\mu$ L of 2,4-dinitrophenylhydrazine was added in to all wells at 37 °C for 30 min. Finally, 250  $\mu$ L of 0.4 mol/L sodium hydroxide was added to stop the reactions at room temperature for 15 min, and the absorbance at 510 nm in each well was measured with an enzyme-linked immunosorbent assay (ELISA) reader (BioRad Laboratories, Hercules, CA, United States). The levels of AST or ALT are expressed in U/L.

### Pathological examination

The right lobe of each mouse liver was collected, fixed with 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and sliced into a thickness of 3-4  $\mu$ m. The sections were deparaffinized and pathological liver change were examined by hematoxylin and eosin (HE) staining.

### Detection of activin A levels

To prepare the mouse hepatic tissue homogenate, 50 mg mouse liver was added to 1 mL lysis buffer (1% Triton

**Table 1** Primer sequences for polymerase chain reaction

| Genes     | Primers   | Sequences                     | Product size (bp) |
|-----------|-----------|-------------------------------|-------------------|
| GAPDH     | Sense     | 5'-GATTGTTGCCATCAACGACC-3'    | 371               |
|           | Antisense | 5'-GTGCAGGATGCATTGCTGAC-3'    |                   |
| Activin A | Sense     | 5'-GAGAGGAGTGAACGTGTGCT-3'    | 514               |
|           | Antisense | 5'-ATGACTGTGAGTGGAAAGG-3'     |                   |
| ActR II A | Sense     | 5'-ATTGCCAGCATCCATCTCTTG-3'   | 296               |
|           | Antisense | 5'-TGCCACCATCATAGACTAGATTC-3' |                   |
|           | Antisense | 5'-ACTCTGTGGCTCAATCCTGCT-3'   |                   |
| Smad3     | Sense     | 5'-CGGTCAAGAGCTGGTCAAGA-3'    | 241               |
|           | Antisense | 5'-TTGAAGCGAACTCACACAGC-3'    |                   |

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; ActR II A: Activin type II A receptor.

X-100, pH 7.5, 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L sodium fluoride, 4 µg/mL leupeptin, 1 µg/mL aprotinin). The lysate was centrifuged for 30 min at 12000 rpm at 4 °C and the supernatant was harvested. The levels of activin A in serum and hepatic tissue homogenates of mice were determined by ELISA according to the manufacturer's protocol (R and D).

#### Real-time quantitative reverse transcription-PCR

Total hepatic tissue RNA was extracted using the Trizol reagent according to the manufacturer's protocol (Invitrogen), and then activin βA, Activin type II A receptor (ActR II A) and Smad3 mRNA expressions were examined by SYBR real-time-PCR kit using a ABI PRISM 7700 sequence detection system (Perkin-Elmer Applied Biosystems) in a two-stage, single-tube reaction. The following reaction conditions were used: stage one, 95 °C, 10 s for 1 cycle; stage two, 95 °C, 5 s and 60 °C, 31 s for 40 cycles, collecting fluorescence in this phase; stage three, 95 °C, 15 s, 60 °C 1 min, 95 °C 15 s for 1 cycle. Primers were synthesized by BECL (Shanghai, China), and the sequences are shown in Table 1. The reverse transcription (RT)-PCR products were quantitatively analyzed according to a standard cDNA calibration curve<sup>[17]</sup>.

#### Immunohistochemical staining

The right lobe of the liver was fixed by 40 g/L paraformaldehyde for 24 h, embedded in paraffin, and then sliced into a thickness of 3-4 µm. The sections were deparaffinized, and 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>)-methanol was used to block endogenous peroxidase at room temperature for 30 min. Two percent of bovine serum albumin in 0.01 mol/L phosphate-buffered saline (PBS) was used to block nonspecific reactivity by a preincubation of section for 30 min. The sections were then incubated in anti-activin A antibody (1:400) at 4 °C overnight. The sections were washed with PBS, bound antibodies were detected with SP1 kit (ZSJQ, Beijing, China) and immunoreactive products were visualized in 0.05% diaminobenzidine and 0.03% H<sub>2</sub>O<sub>2</sub>. The sections were counterstained with hematoxylin, dehydrated, cleared, counted

and observed under an Olympus microscope (BX51). In control staining, the sections were incubated with normal mouse immunoglobulin G (IgG) instead of anti-activin A antibody<sup>[18]</sup>.

#### Blocking experiment

C57BL/6 mice adaptive feeding for 7 d were randomly divided into four groups (*n* = 12). The IgG control group (IgG Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with normal IgG (500 µg/kg); the antibody control group (Anti-A Cont), in which each mouse was injected intraperitoneally with olive oil (10 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 µg/kg); the CCl<sub>4</sub> group (IgG + CCl<sub>4</sub>), in which each mouse was injected intraperitoneally with CCl<sub>4</sub> (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with IgG (500 µg/kg); the antibody plus CCl<sub>4</sub> group (Anti-A + CCl<sub>4</sub>), in which each mouse was injected intraperitoneally with CCl<sub>4</sub> (0.5 mL/kg) + olive oil (9.5 mL/kg), 2 h later, injected by tail vein with anti-activin A antibody (500 µg/kg). Sera and livers of these mice were collected 3 d after CCl<sub>4</sub> injection for determination of serum ALT and AST levels and pathological examination of livers.

#### Statistical analysis

The statistical software SPSS 10.0 was used to analyze all data. *P* < 0.05 was considered statistically significant.

## RESULTS

#### The establishment of the acute liver injury mouse model

Change of serum transaminases ALT and AST levels is an important indicator of liver injury. Our results revealed that serum ALT and AST levels were significantly elevated on days 1, 3, 5 and 7 after the administration of CCl<sub>4</sub>, compared with that in control group, *P* < 0.01 (Figure 1A). Furthermore, lobular architecture in the control mouse liver was clear and the hepatic cells arranged in neat rows by HE staining. In mouse liver 1 d following CCl<sub>4</sub> treatment, there were round necrotic lesions around the hepatic lobule portal area, and 3 d after CCl<sub>4</sub> treatment, there was inflammatory cell infiltration in the hepatic lobule portal area (Figure 1B).

#### Increase of activin A levels in mice treated with CCl<sub>4</sub>

Activin A levels in serum and hepatic homogenates of experimental mice were determined by ELISA. The results showed that activin A levels increased significantly in serum and hepatic homogenate from CCl<sub>4</sub>-induced acute liver injury mice for 1, 3 and 5 d, compared with that in control group, *P* < 0.01 (Figure 2). These levels peaked on days 1 and 3, and returned to normal levels by day 7.

#### Elevation of activin A protein expression in liver of mice treated with CCl<sub>4</sub>

To explore the reason for increased activin A levels in the serum of CCl<sub>4</sub>-treated experimental mice and the

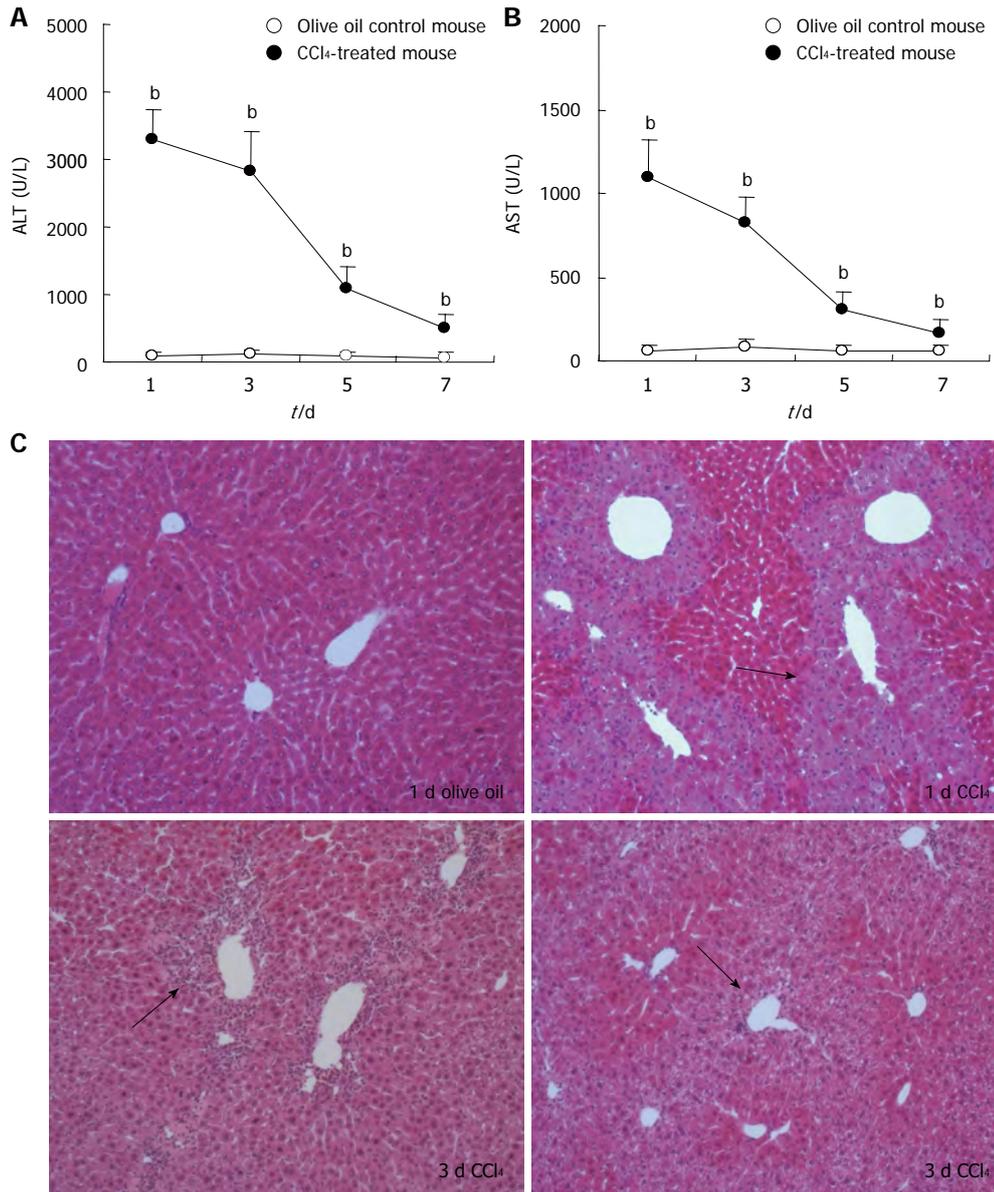


Figure 1 Examination of serum alanine aminotransferase and aspartate aminotransferase levels and pathological changes of liver in carbon tetrachloride-treated mice. A: Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected by enzyme-linked immunosorbent assay in olive oil control mouse and carbon tetrachloride (CCl<sub>4</sub>)-treated mouse. <sup>b</sup>*P* < 0.01 vs control; B: Pathological change of liver was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification, × 100).

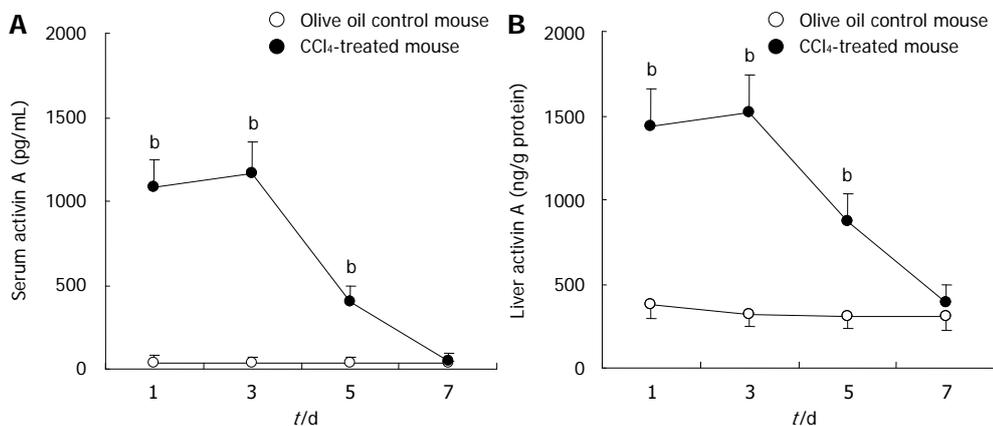
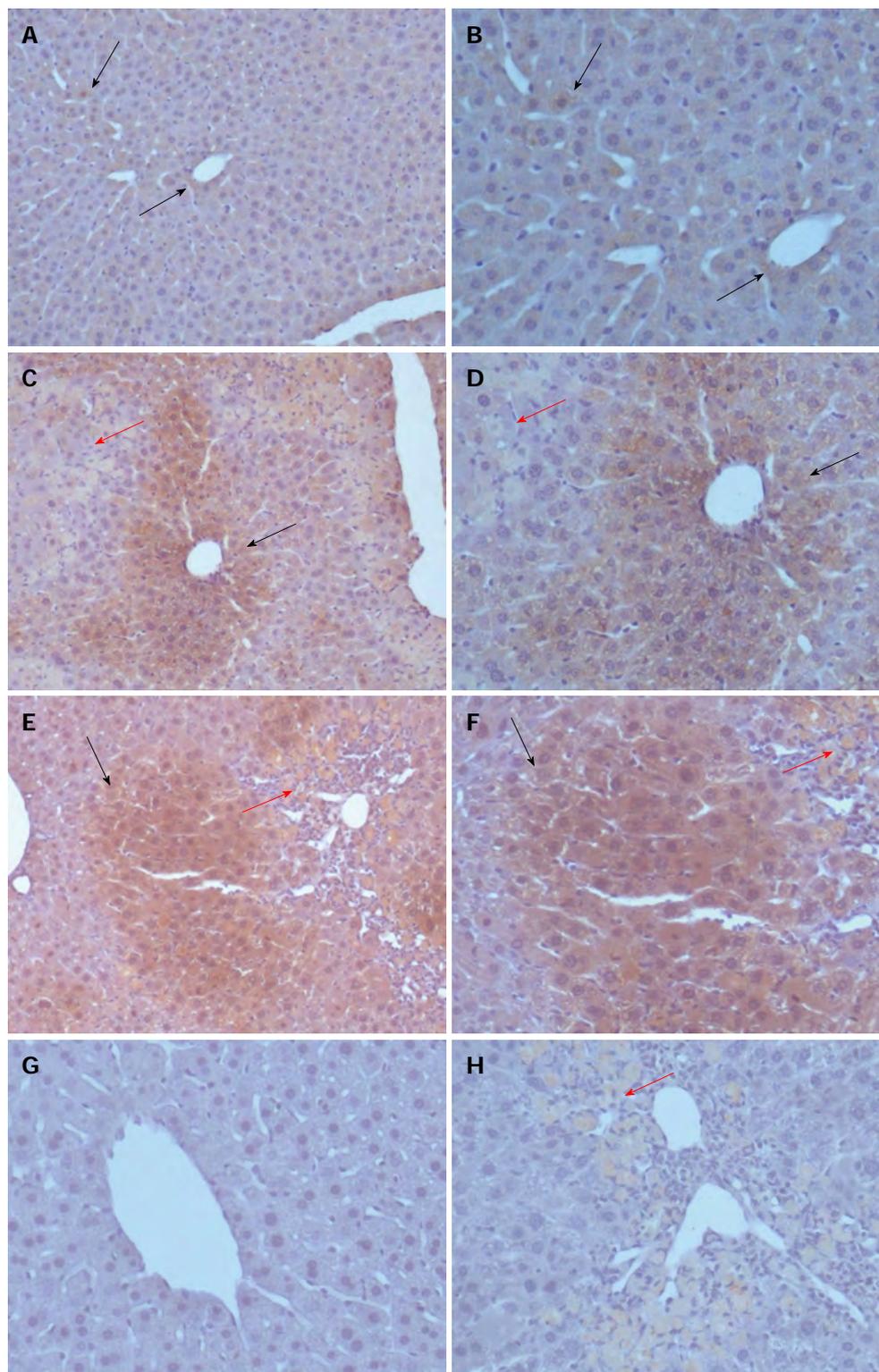


Figure 2 Detection of levels of activin A in serum and hepatic homogenates of mouse treated with carbon tetrachloride by enzyme-linked immunosorbent assay. CCl<sub>4</sub>: Carbon tetrachloride. <sup>b</sup>*P* < 0.01 vs control.



**Figure 3** Expression of activin A protein in liver of mouse assessed by immunohistochemical staining. A, B: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after olive oil treatment; C, D: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 1 after carbon tetrachloride (CCl<sub>4</sub>) treatment; E, F: Activin A expression was examined by using anti-activin A antibody in the same liver tissues on day 3 after CCl<sub>4</sub> treatment; G, H: The procedural background control staining was represented by using normal mouse immunoglobulin G instead of anti-activin A antibody in livers of olive oil-treated and CCl<sub>4</sub>-treated mice. Red arrows represent lesion area and black arrows represent positive staining for activin A. A, C, E: Magnification × 100; B, D, F, G, H: Magnification × 200.

expression pattern of activin A in acute liver injury, the expression of activin A protein was examined by immunohistochemical staining. The results revealed that activin A protein expression was detectable in the livers of olive

oil-treated mice and in injured livers from CCl<sub>4</sub>-treated mice. In addition, activin A protein expression in hepatocytes with non-necrotic area was up-regulated on days 1 and 3 after CCl<sub>4</sub> treatment (Figure 3).

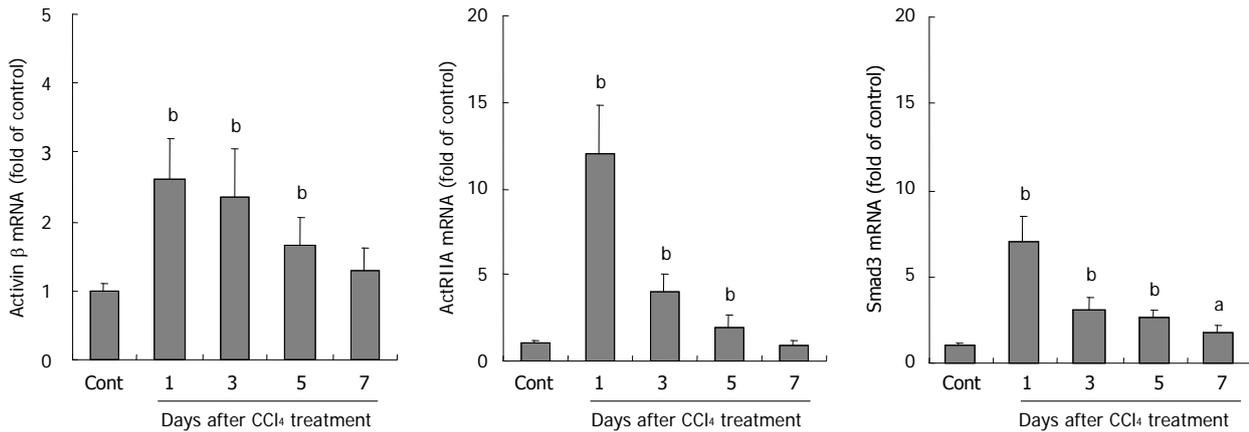


Figure 4 Assay of mRNA expressions of activin  $\beta$ A and activin signal molecules in liver of mouse by real-time quantitative reverse transcription-polymerase chain reaction. The mRNA levels in olive oil control group (Cont) were adjusted to 100%. All values (mean  $\pm$  SD) were expressed as % of that in control. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control.

### Up-regulation of activin $\beta$ A, ActRIIA and Smad3 mRNA expressions in liver of mice treated with CCl<sub>4</sub>

Activin A is a dimeric glycoprotein formed by two  $\beta$ A subunits. To further investigate activin and its signal molecule expression in liver injury, activin  $\beta$ A, ActR II A and Smad3 mRNA expressions were examined by real-time quantitative RT-PCR. The results showed that not only activin  $\beta$ A mRNA, but also ActR II A and intracellular signal protein Smad3 mRNA expressions were significantly increased in livers from CCl<sub>4</sub>-treated mice when compared with that in control group,  $P < 0.01$  (Figure 4).

### Protective effect of anti-activin A antibody on CCl<sub>4</sub>-treated mouse liver

In order to further confirm that activin A was involved in acute liver injury, pathological changes of livers in CCl<sub>4</sub>-treated mice were examined after injection of an anti-activin A antibody to block endogenous activin action. The results revealed that serum ALT and AST levels in mice treated with anti-activin A + CCl<sub>4</sub> were significantly lower than that in IgG + CCl<sub>4</sub> control mice (Figure 5A,  $P < 0.01$ ). Furthermore, the results showed that the necrotic severity around the hepatic lobule portal area in mice treated with anti-activin A + CCl<sub>4</sub> was remarkably reduced compared with that in IgG + CCl<sub>4</sub> control mice (Figure 5B).

## DISCUSSION

### Biologic activity of activin

Activin A as a multifunctional factor plays an important role in acute phase immune response, and its expression levels are increased in several inflammatory diseases such as septicemia, inflammatory bowel disease, and rheumatoid arthritis<sup>[19-22]</sup>. Recent studies have reported that activin A is closely related to the liver diseases hepatitis and hepatic fibrosis<sup>[3,14,16]</sup>. Activin receptors belong to serine/threonine kinase receptors of the TGF- $\beta$  superfamily, are divided into ActR I and ActR II. Activin binds directly to the type II receptor on the cell membrane, and then recruits the type I receptor<sup>[23,24]</sup>. Further, the activated

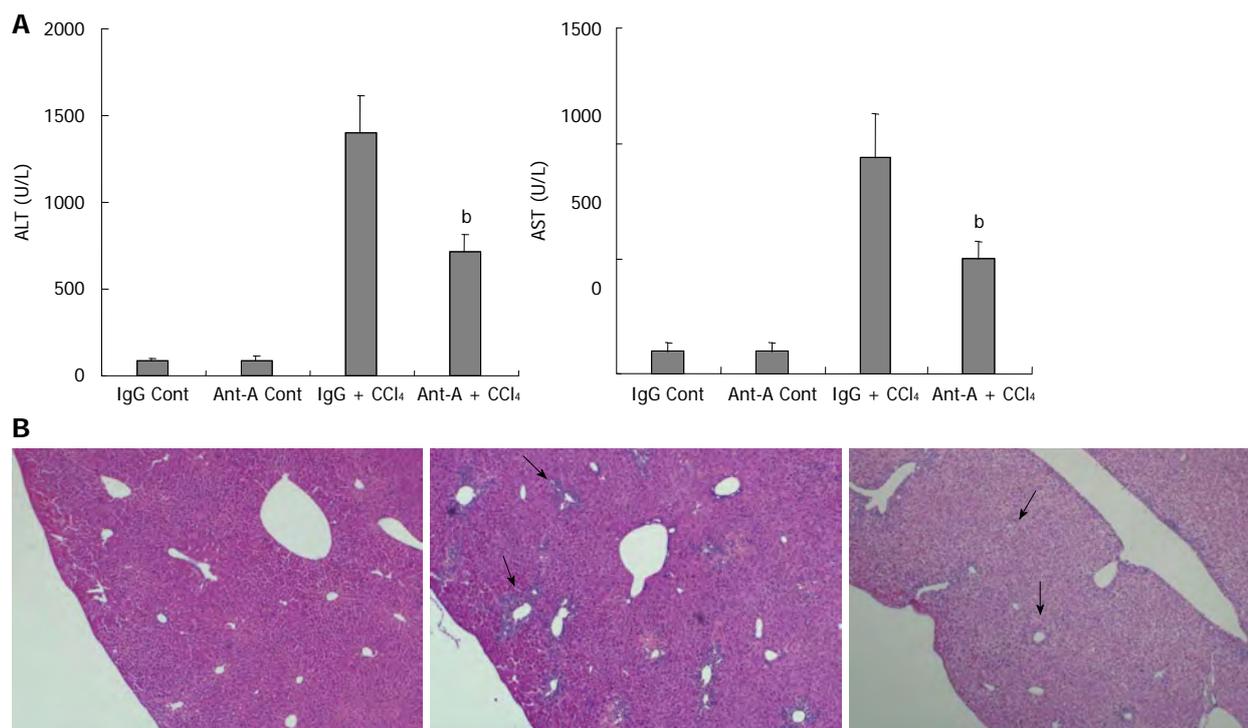
type I receptor propagates the signal through a cascade reaction elicited by downstream Smad proteins into the nucleus to activate target genes<sup>[25,26]</sup>.

### Damage effects of CCl<sub>4</sub> on the liver

CCl<sub>4</sub> is a chemical reagent that induces mouse liver injury, a classic chemical model for studying liver injury. The principle of injury is that in the function of cytochrome P450, CCl<sub>4</sub> generates chloromethyl free radicals (-CCl<sub>3</sub>), which enable the peroxidation of the membrane lipids on the liver cell membrane or subcellular structure causing the rise of membrane permeability that results in the release of large number of ALT in the cytoplasm into blood. Another possibility is covalent binding of -CCl<sub>3</sub> and hepatic microsomal lipids and proteins that damage the integrity of the structure and function of hepatic cell membranes<sup>[27-29]</sup>. Additionally, various factors can further aggravate CCl<sub>4</sub>-induced liver injury in mice, such as the increase of activin A expression in chronic liver injury<sup>[16]</sup>.

### Role of activin in liver injury

Abnormal expression of activin A is related to a variety of liver diseases, and activin A can directly induce hepatocyte apoptosis and inhibit hepatocyte proliferation and DNA synthesis<sup>[2,12,30]</sup>. To investigate whether activin A is involved in acute chemical liver injury, C57BL/6 mice were injected with CCl<sub>4</sub> through peritoneal cavity to establish acute liver injury model. The results showed that serum ALT and AST increased, and serious necrosis of hepatic tissue was observed by HE staining in CCl<sub>4</sub>-treated mice. Simultaneously, activin A levels increased significantly in serum and hepatic tissue homogenate of mice treated with CCl<sub>4</sub>. By immunohistochemical staining, the elevated expression of activin A protein was also observed in the injured livers of CCl<sub>4</sub>-treated mice. Since CCl<sub>4</sub> induced necrotic death of hepatocytes in large area, which necrotic hepatocytes could not release cytokines, but the decomposition product of necrotic tissues as inflammatory stimulator could induce hepatocytes activation to secrete activin A. Thus, activin A protein was expressed in hepatocytes around the necrotic area and the



**Figure 5** Effects of anti-activin A antibody *in vivo* on serum alanine aminotransferase and aspartate aminotransferase levels and pathological change of liver in carbon tetrachloride-treated mouse. **A:** Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were detected in mouse 3 d after carbon tetrachloride (CCl<sub>4</sub>) treatment. Immunoglobulin G (IgG) + Cont, IgG control group; Anti-A + Cont, anti-activin A control group; IgG + CCl<sub>4</sub>, IgG plus CCl<sub>4</sub> group; Anti-A + CCl<sub>4</sub>, anti-activin A plus CCl<sub>4</sub> group. <sup>b</sup>*P* < 0.01 vs IgG + CCl<sub>4</sub> group; **B:** Pathological change of liver in mouse 3 d after CCl<sub>4</sub> treatment was analyzed by hematoxylin and eosin staining. Arrows represent lesion area (magnification × 40).

activated hepatocytes have larger clear nuclei. In addition, the real-time quantitative RT-PCR results revealed that in CCl<sub>4</sub>-treated mice, activin A, ActR II A and intracellular activin signal protein Smad3 mRNA expressions significantly increased. In order to further confirm the role of activin A in CCl<sub>4</sub>-induced liver injury, pathological changes of livers in CCl<sub>4</sub>-treated mice were examined after injection with anti-activin A antibody to block endogenous activin action. These results revealed that activin A might participate in the process of CCl<sub>4</sub>-induced liver injury *via* inhibiting proliferation and DNA synthesis of hepatocytes to aggravate the CCl<sub>4</sub>-induced liver injury, and blockade of activin A actions by anti-activin A antibody could significantly reduce the levels of serum transaminases and the necrosis severity of hepatic tissue in CCl<sub>4</sub>-treated mice.

In summary, these data suggested that the activin A-ActR II A-Smad signal transduction cascade was induced in CCl<sub>4</sub>-treated mice. Further, activin A was involved in the process of CCl<sub>4</sub>-induced acute chemical liver injury of mice in an autocrine/paracrine manner. Activin A may be a potential therapeutic target for acute liver injury disease.

## COMMENTS

### Background

Activin A is an important mediator of liver cells, not only in proliferation of hepatocytes, but also in activation of hepatic stellate cells, secretion of liver extracellular matrix, as well as the formation and development of liver injury.

Carbon tetrachloride (CCl<sub>4</sub>)-induced liver injury is a classical model of chemical liver injury in mice. Previous studies reported that the expression of activin A increased significantly in CCl<sub>4</sub>-induced chronic liver injury in mice, and it was an important factor leading to liver fibrosis. The effect of activin A on the process of CCl<sub>4</sub>-induced acute chemical liver injury has not been reported.

### Research frontiers

Previous studies have demonstrated the effect of activin A on CCl<sub>4</sub>-induced chronic liver injury in mice, however, the effect and mechanism of activin A on CCl<sub>4</sub>-induced acute liver injury remains undefined.

### Innovations and breakthroughs

New therapies that aim to block biological role of activin A in liver injury diseases are being actively explored and evaluated. Authors believe this study to be the first to explore the effect of activin A on acute liver injury.

### Applications

The findings that block the biological action of activin A may be clinically relevant to potential liver injury therapeutics.

### Terminology

Activin is a multifunctional factor of transforming growth factor- $\beta$  superfamily. There are three types of activins formed by homo- or hetero-dimerization of two inhibin subunits ( $\beta$ A and  $\beta$ B), activin A ( $\beta$ A $\beta$ A), activin B ( $\beta$ B $\beta$ B) and activin AB ( $\beta$ A $\beta$ B). Activin A is an important mediator in CCl<sub>4</sub>-induced acute liver injury in mice, and blockade of biological action of activin A may be a potential therapeutics for acute liver injury diseases.

### Peer review

This is a good descriptive study in which authors analyze the role of activin A in carbon tetrachloride-induced acute liver injury in mouse. The results are interesting and suggest that activin A is involved in the process of CCl<sub>4</sub>-induced acute liver injury in an autocrine/paracrine manner. Further, the authors show that an antibody-mediated blockade of activin A biological action may provide insights into potential therapeutics.

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**P- Reviewers** Ding WX, Ohkohchi N, Shimizu Y, Tarantino G  
**S- Editor** Gou SX **L- Editor** A **E- Editor** Xiong L



## Quality of life following laparoscopic Nissen fundoplication: Assessing short-term and long-term outcomes

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Supported by EVO-funding of the Central Hospital of Central Finland

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Received: January 3, 2013 Revised: March 31, 2013

Accepted: April 9, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the quality of life following laparoscopic Nissen fundoplication by assessing short-term and long-term outcomes.

**METHODS:** From 1992 to 2005, 249 patients underwent laparoscopic Nissen fundoplication. Short-term outcome data including symptom response, side effects of surgery, endoscopy, and patient's perception of overall success were collected prospectively. Long-term outcomes were investigated retrospectively in patients with

a median follow-up of 10 years by assessment of reflux symptoms, side effects of surgery, durability of antireflux surgery, need for additional treatment, patient's perception of success, and quality of life. Antireflux surgery was considered a failure based on the following criteria: moderate to severe heartburn or regurgitation; moderate to severe dysphagia reported in combination with heartburn or regurgitation; regular proton pump inhibitor medication use; endoscopic evidence of erosive esophagitis Savary-Miller grade 1-4; pathological 24-h pH monitoring; or necessity to undergo an additional surgery. The main outcome measures were short- and long-term cure rates and quality of life, with patient satisfaction as a secondary outcome measure.

**RESULTS:** Conversion from laparoscopy to open surgery was necessary in 2.4% of patients. Mortality was zero and the 30-d morbidity was 7.6% (95%CI: 4.7%-11.7%). The median postoperative hospital stay was 2 d [interquartile range (IQR) 2-3 d]. Two hundred and forty-seven patients were interviewed for short-term analysis following endoscopy. Gastroesophageal reflux disease was cured in 98.4% (95%CI: 95.9%-99.6%) of patients three months after surgery. New-onset dysphagia was encountered postoperatively in 13 patients (6.7%); 95% reported that the outcome was better after antireflux surgery than with preoperative medical treatment. One hundred and thirty-nine patients with a median follow-up of 10.2 years (IQR 7.2-11.6 years) were available for a long-term evaluation. Cumulative long-term cure rates were 87.7% (81.0%-92.2%) at 5 years and 72.9% (64.0%-79.9%) at 10 years. Gastrointestinal symptom rating scores and RAND-36 quality of life scores of patients with treatment success were similar to those of the general population but significantly lower in those with failed antireflux surgery. Of the patients available for long-term follow-up, 83% rated their operation a success.

**CONCLUSION:** For the long-term, our results indicate decreasing effectiveness of laparoscopic antireflux

surgery, although most of the patients seem to have an overall quality of life similar to that of the general population.

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**Key words:** Laparoscopy; Nissen fundoplication; Long-term outcome; Antireflux; Gastrointestinal symptom rating scores; RAND-36

**Core tip:** Current evidence suggests that laparoscopic fundoplication is more effective than medical therapy for the short- and medium-term treatment of gastroesophageal reflux disease. This study examined short-term and long-term outcomes after laparoscopic Nissen fundoplication. Short-term outcomes were assessed by symptom response, side effects of surgery, endoscopy and the patient's perception of overall success. Long-term outcomes were examined by addressing multiple domains affected by the operation including reflux symptoms, side effects of surgery, durability of antireflux surgery, selective objective testing, need for additional medical or surgical treatment, the patient's perception of overall success, and long-term quality of life.

Kellokumpu I, Voutilainen M, Haglund C, Färkkilä M, Roberts PJ, Kautiainen H. Quality of life following laparoscopic Nissen fundoplication: Assessing short-term and long-term outcomes. *World J Gastroenterol* 2013; 19(24): 3810-3818 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3810.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3810>

## INTRODUCTION

Medical treatment with proton pump inhibitors (PPIs) heals esophagitis and suppresses heartburn in over 90% of patients<sup>[1-3]</sup>. Despite the fact that effective maintenance therapies do exist, patients often require lifelong medication, and compliance with long-term maintenance therapy is sometimes difficult to achieve<sup>[1-3]</sup>. Furthermore, concerns exist regarding long-term safety of PPI medication<sup>[4]</sup>. Current evidence suggests that laparoscopic fundoplication is more effective than medical therapy for the short- and medium-term treatment of gastroesophageal reflux disease<sup>[5-10]</sup>. In contrast to medical therapy that inhibits only acid reflux, antireflux surgery is designed to prevent reflux of all gastric content. This is achieved by creating a mechanical antireflux valve that increases lower esophageal sphincter pressure and markedly reduces the rate of spontaneous transient relaxations accompanied by reflux<sup>[11,12]</sup>. Side effects of Nissen fundoplication such as dysphagia, increased bloating and flatulence, and inability to belch or vomit may limit the success of antireflux surgery<sup>[12,13]</sup>. Patients may also need revision surgery to improve symptom control, or revert to the use of long-term medical therapy following recurrent symptoms.

Optimal management of chronic gastroesophageal

reflux disease is uncertain as treatment options include either modern medical therapy using proton pump inhibitors or antireflux surgery<sup>[5]</sup>. In addition, long-term quality of life studies are limited. This study examined short-term and long-term outcomes after laparoscopic Nissen fundoplication. Short-term outcomes were assessed by symptom response, side effects of surgery, endoscopy and the patient's perception of overall success. Long-term outcomes were examined by addressing multiple domains affected by the operation including reflux symptoms, side effects of surgery, durability of antireflux surgery, selective objective testing, need for additional medical or surgical treatment, the patient's perception of overall success, and long-term quality of life.

## MATERIALS AND METHODS

### Study population

From 1992 to 2005, a total of 249 patients underwent laparoscopic Nissen fundoplication for gastroesophageal reflux disease at the fourth Department of Surgery, Helsinki University Central Hospital (120 operations done by three surgeons: Kellokumpu I, Haglund C and Roberts PJ from 1992 to 1997) and at the Central Hospital of Central Finland (129 operations done by Kellokumpu I from 1998 to 2005). Indications for antireflux surgery were residual symptoms while on medical therapy or endoscopic esophagitis after at least three months of intensive acid suppression therapy, or both; dependence on continuous medication and expenses; and pathological 24-h esophageal pH monitoring in symptomatic patients without preoperative endoscopic signs of erosive esophagitis. Diagnosis of gastroesophageal reflux disease was based on upper gastrointestinal endoscopy on 24-h ambulatory pH measurement according to standard criteria as established by Johnson *et al*<sup>[14]</sup>, and on esophageal manometry to evaluate the lower esophageal resting pressure and pre-existing motility disorders<sup>[15]</sup>. Antisecretory medication was halted 7 d before assessment of esophageal function. Preoperative and short-term outcome data were entered in a prospective database. Long-term outcome data were collected retrospectively.

### Surgical technique

Laparoscopic floppy Nissen fundoplication was performed by the standard 5-trocar technique as described<sup>[16]</sup>. Every patient had prophylactic anticoagulation and compressive elastic stockings. The fundus was routinely mobilized by ligating and dividing the short gastric vessels (Kellokumpu I and Haglund C)<sup>[16]</sup>. The Rossetti-Hell modification<sup>[17]</sup> with mobilization of the fundus posteriorly to the upper pole of the spleen without division of short gastric vessels was preferred by Roberts PJ. A short (20 mm) fundic wrap was constructed around the distal esophagus with 2-0 non-absorbable sutures. Nasogastric tubes (18 F) and bougies (36 F) allowed calibration of the fundic wrap and closure of the hiatus. A posterior hiato-plasty was performed with 2-0 non-absorbable stitches

when the esophageal hiatus was enlarged. The left side of the wrap was anchored to the cardia with an additional 1-2 sutures. A nasogastric tube was left in place until the following morning when intake of liquids was started. Solid intake was usually started on the second or third postoperative day. All patients received dietary instructions before their discharge.

### Evaluation of short-term outcomes

Preoperative and short-term symptom assessment three months after surgery was based on a standardized questionnaire and interview about heartburn, regurgitation, and dysphagia according to the DeMeester-Johnson reflux scale<sup>[18]</sup>. Regurgitation was graded as: none = 0, mild = 1 (occasional after straining, large meal, or lying down), moderate = 2 (predictable with position change, straining, or lying down), severe = 3 (history of aspiration). Heartburn grades were none = 0, mild = 1 (recognizable symptom, no prior history of medical treatment), moderate = 2 (primary reason for medical visit or medical problem), severe = 3 (constant, marked disability in activities of daily living). Dysphagia grades were none = 0, mild = 1 (occasional with coarse foods), moderate = 2 (require liquids to clear), severe = 3 (history of meat impaction necessitating medical attention)<sup>[18]</sup>. In addition, the frequency of heartburn, regurgitation and dysphagia was rated as absent = 0, occasional (less than once every two weeks) as 1, frequent symptoms (more than once every two weeks but less than daily) as 2, and daily symptoms (at least one attack/day to constant symptoms) as 3<sup>[19]</sup>.

Side effects of surgery such as disturbed belching ability, bloating (defined as abdominal swelling), and flatulence were analyzed with the patients assessing their degree of disturbance by any postoperative decrease (belching) or increase (flatus, bloating) on a scale where 0 = no change, 1 = mild change, 2 = moderate change, and 3 = severe change.

Upper gastrointestinal endoscopy was routinely performed preoperatively and at three months after surgery. Endoscopic classification of esophagitis was based on the modified Savary-Miller 5-grade classification<sup>[20]</sup>. The state and localization of the fundic wrap and the presence of paraesophageal hernia were checked postoperatively.

Overall short-term success of the operation was assessed by the patient's subjective perception of the outcome after surgery compared to that with medical therapy and patient's willingness to undergo surgery again for similar preoperative conditions.

### Evaluation of long-term outcome

Long-term outcome was investigated in a subgroup of patients with a median follow-up of 10 years and was based on the same self-completed, standardized questionnaire about heartburn, regurgitation, and dysphagia<sup>[18,19]</sup>, and side effects of surgery as in the short-term. Patients also reported time to symptom recurrence and the use of PPI medication as well as the date of any re-fundoplication. Finally, long-term outcome of surgery was assessed

by a self-rated 7-point Likert scale describing outcome as good as expected (0), mildly (+1), moderately (+2) or markedly (+3) better or worse as -1, -2, -3 than expected and by willingness to undergo surgery again under similar preoperative conditions. During the long-term follow-up, objective testing including upper gastrointestinal endoscopy, barium examination, and esophageal function studies was not routinely performed.

Long-term quality of life assessment was done by the self-completed Gastrointestinal Symptom Rating Scale (GSRS)<sup>[21,22]</sup>, and overall quality of life by RAND-36 (SF-36)<sup>[23,24]</sup>. The GSRS is a disease-specific instrument comprising 15 items, each rated on a 7-point Likert scale from no discomfort to very severe discomfort, and further divided into five dimensions: abdominal pain syndrome (abdominal pain, hunger pain and nausea), reflux syndrome (heartburn and acid regurgitation), diarrhea syndrome (diarrhea, loose stools and urgent need for defecation), indigestion syndrome (borborygmus, abdominal distension, eructation and increased flatus), and constipation syndrome (constipation, hard stools and feeling of incomplete evacuation). The higher the GSRS score, the more the patient suffers from gastrointestinal symptoms. The Finnish RAND-36<sup>[23]</sup> covers eight areas of health status, including physical functioning, role limitation due to physical health problems, bodily pain, general health, energy, social functioning, role limitation due to personal emotional problems, and emotional well-being. The raw responses were recoded according to the original version of the RAND-36, each item being scored on a 0-to-100 scale with higher scores indicating better quality of life<sup>[23,24]</sup>. Questionnaires were sent to the patients in May 2006 and returned by November 2006. Incomplete answers were completed by telephone interview.

### Definition of failed antireflux surgery

Consistent with Lundell *et al.*<sup>[25]</sup>, laparoscopic Nissen fundoplication was considered failed if at least one of the following criteria was present: persistence or recurrence of moderate to severe heartburn or regurgitation<sup>[18]</sup> occurring more than once every two weeks (grade 2) or daily (grade 3)<sup>[19]</sup> or both; moderate to severe dysphagia<sup>[18]</sup> reported in combination with heartburn or regurgitation or both; the use of daily or weekly PPI medication; endoscopic evidence of erosive esophagitis Savary-Miller grade 1-4; pathological 24-h pH monitoring; and necessity to undergo redo surgery.

### Ethics

The Central Hospital of Central Finland ethics committee approved this study (K-Sshp Dnro 47/2005). The causes of death were obtained from the National Cause of Death Registry.

### Statistical analysis

The data are presented as mean  $\pm$  SD, as median with interquartile range (IQR), or as counts with percentages. The 95% CIs are given for the most important outcomes.

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

| Follow-up   | Study population ( <i>n</i> = 249) | Long-term ( <i>n</i> = 139) |
|---|------------------------------------|-----------------------------|
| Age, yr (mean ± SD)   | 51.2 ± 11.2                        | 51.5 ± 10.6                 |
| Sex, male/female  | 131/118                            | 76/62                       |
| ASA classification  |                                    |                             |
| I   | 130 (52.0)                         | 85 (61.2)                   |
| II  | 96 (38.4)                          | 40 (28.8)                   |
| III   | 23 (9.2)                           | 14 (10.1)                   |
| Duration of GERD in years, median (IQR)                                 | 6 (5.0-10.0)                       | 6 (4.0-10.0)                |
| Preoperative medication   |                                    |                             |
| PPI   | 222 (89.2)                         | 120 (86.3)                  |
| H2-antagonist and/or prokinetic   | 27 (10.8)                          | 19 (13.7)                   |
| Duration of medical treatment, mo, median (IQR)                         | 18 (8.0-36.0)                      | 18 (6.0-36.0)               |
| Preoperative <i>Helicobacter pylori</i> eradication                     | 36 (14.6)                          | 20 (14.4)                   |
| Preoperative symptom severity grade 1/2/3 as % <sup>1</sup>             |                                    |                             |
| Heartburn   | 0/49.8/48.6                        | 0/55.4/42.4                 |
| Regurgitation   | 5.6/87.6/4.4                       | 5.0/89.2/4.3                |
| Dysphagia   | 12.0/9.2/0                         | 12.9/11.5/0                 |
| pH < 4 of total time, median (IQR) as % <sup>2</sup>                    | 12.0 (8.0-18.7)                    | 11.5 (7.4-19.1)             |
| DeMeester score, median (IQR)   | 42.5 (28.4-71.3)                   | 41.0 (26.2-69.3)            |
| Lower esophageal sphincter pressure in mmHg <sup>3</sup> , median (IQR) | 11.0 (8.0-16.0)                    | 11.0 (7.5-17.0)             |
| Preoperative grading of esophagitis (Savary-Miller)                     |                                    |                             |
| None  | 68 (27.3)                          | 36 (25.9)                   |
| Grade 1 (single erosive lesion, one longitudinal fold)                  | 22 (8.8)                           | 16 (11.5)                   |
| Grade 2 (multiple erosive lesions, more than one fold)                  | 98 (39.4)                          | 57 (41.0)                   |
| Grade 3 (circumferential lesions)                                       | 13 (5.2)                           | 3 (2.2)                     |
| Grade 4 (chronic ulcer or stricture ± Gr. 1-3) <sup>4</sup>             | 5 (2.0)                            | 3 (2.2)                     |
| Grade 5 (Barrett's esophagus ± Gr. 1-3)                                 | 43 (17.3) <sup>5</sup>             | 24 (17.2) <sup>6</sup>      |

<sup>1</sup>DeMeester *et al*<sup>[18]</sup>; <sup>2</sup>24-h pH monitoring in 232 patients; <sup>3</sup>Esophageal manometry in 228 patients; <sup>4</sup>Grade 4 (4 strictures, 1 ulcer); <sup>5</sup>Barrett's esophagus only 18 (7.2%) and associated with Gr. 1-2 erosive changes in 24 patients (9.6%) and grade 3 in 1 (0.4%); <sup>6</sup>Barrett's esophagus only 10 (7.2%) and associated with Gr. 1-2 erosive changes in 14 patients (10.1%). GERD: Gastroesophageal reflux disease; PPI: Proton pump inhibitor; IQR: Interquartile range.

Groups were compared using the Mann-Whitney *U* test or the  $\chi^2$  test. The statistical significance between groups in HRQL measures was evaluated by bootstrap-type analysis of co-variance because of the violation of distribution assumptions. Repeated measures for dichotomous outcomes were analyzed by Cochran's *Q* test and the marginal homogeneity test. Time-to-failure analysis was based on the product limit estimate (Kaplan-Meier) of the cumulative "survival" function. The Finnish general population values<sup>[25]</sup> for the eight Rand-36 domains were weighted to match the gender- and age-distribution of the study population. Statistical analyses were performed with Stata statistical software, release 12.1 (StataCorp, College Station, TX, United States).

## RESULTS

Baseline clinical characteristics of the study population are shown in Table 1. Esophageal dilatations up to 18 mm in diameter were performed preoperatively in three of the four patients having esophageal stricture. Frequent or daily heartburn in 89.9% and regurgitation in 87.2% of the patients were the main presenting symptoms. DeMeester-Johnson severity grades are shown in Table 1, and operative data and surgical outcome in Table 2. While major intraoperative complications occurred in three patients, no deaths occurred. A distal esophageal perforation caused by diathermy scissors in one patient and by an inadvertent push of the calibration tube in

another patient with esophageal stricture were suture-repaired and covered by the fundic wrap. A small fundic perforation caused by Harmonic scissors-induced thermal injury occurred postoperatively in one patient and necessitated a laparotomy and suture repair. Overall, 30-d morbidity was 7.6% (95%CI: 4.7-11.7) (Table 2). Pleural empyema in one patient with esophageal injury necessitated a thoracotomy and decortication. Comparing the two treatment centers and time periods, 30-d morbidity was similar (10.0% *vs* 5.4%, *P* = 0.17), but operation time [median 135 min (IQR 116.3-180 min) *vs* 75 min (IQR 65-90 min), *P* < 0.001] and postoperative hospital stay [median 3 d (IQR 2-4 d) *vs* 2 d (IQR 1-2 d), *P* < 0.001] were shorter during the latter period.

### Short-term outcomes

Two of the 249 operated patients could not be contacted 3 mo after surgery, leaving 247 patients for short-term analysis. Based on symptom control and endoscopically-verified healing of erosive esophagitis, gastroesophageal reflux disease was cured in 243 of the 247 patients [98.4% (95%CI: 95.9%-99.6%)]. Only four patients reported short-term failures. Two had persisting reflux symptoms and erosive esophagitis (grade 2) with partly disrupted plication. One had erosive esophagitis (grade 1) and too distally placed and partly disrupted plication. Another one who had an apparently normal fundoplication had persistence of erosive esophagitis (grade 1). Three patients underwent a reoperation, and one was treated with

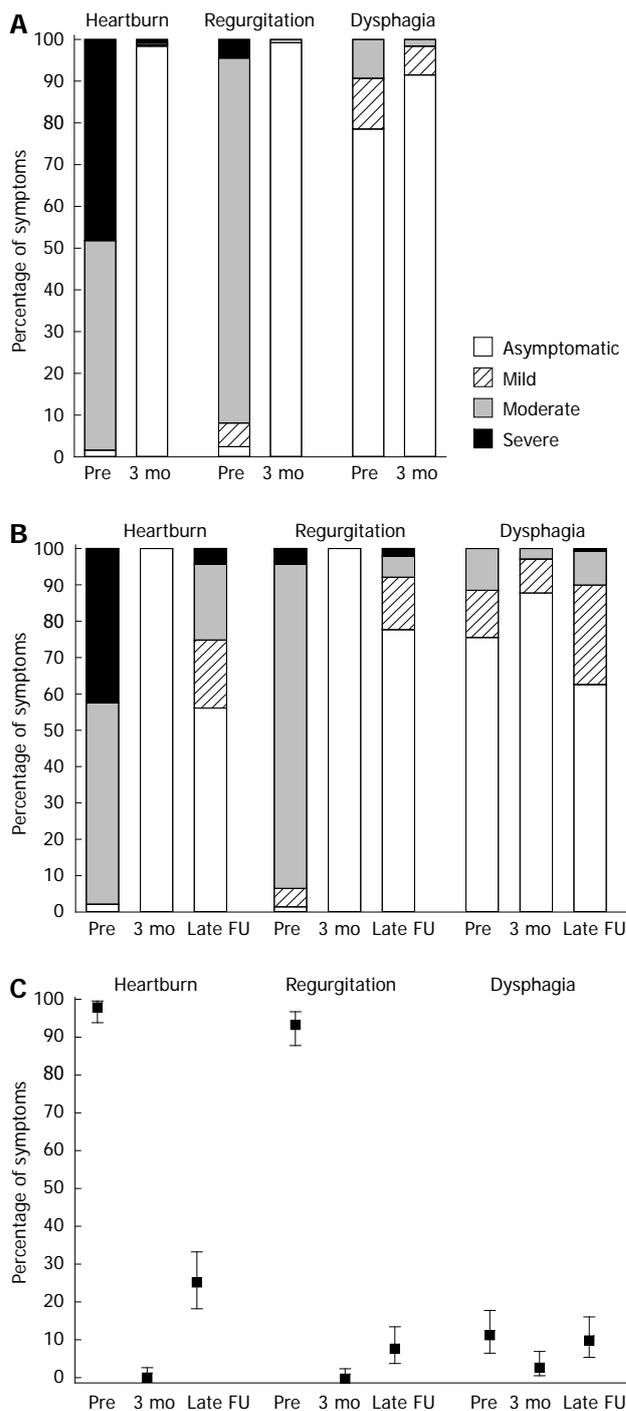
**Table 2 Operative data and short-term surgical outcome n (%)**

| Variables                                    | n = 249         |
|--|-----------------|
| Type of surgery                              |                 |
| Floppy Nissen fundoplication                 | 213 (85.5)      |
| Rosetti-Hell fundoplication                  | 36 (14.5)       |
| Hiatoplasty                                  | 235 (94.4)      |
| Conversion to open surgery <sup>1</sup>      | 7 (2.8)         |
| Operation time, min, median (IQR)            | 90 (70.3-135.0) |
| Mortality (30 d)                             | 0 (0.0)         |
| Intraoperative complications                 | 3 (1.2)         |
| Esophageal perforation                       | 2 (0.8)         |
| Burn injury of the fundus                    | 1 (0.4)         |
| Postoperative morbidity (30 d) <sup>2</sup>  | 19 (7.6)        |
| General                                      | 12 (4.8)        |
| Atelectasis + fever                          | 5 (2.0)         |
| Pneumonia                                    | 7 (2.8)         |
| Surgical                                     | 9 (3.6)         |
| Pleural empyema                              | 1 (0.4)         |
| Fundic perforation (burn injury)             | 1 (0.4)         |
| Port-site hernia and bowel obstruction       | 1 (0.4)         |
| Port-site bleeding                           | 2 (0.8)         |
| Excessive pain at port-site                  | 1 (0.4)         |
| Gastric dilatation                           | 1 (0.4)         |
| Wound infection                              | 1 (0.4)         |
| Urinary retention                            | 1 (0.4)         |
| Reoperation rate <sup>3</sup>                | 5 (2.0)         |
| Relaparotomy                                 | 4 (1.6)         |
| Thoracotomy and decortication                | 1 (0.4)         |
| Postoperative hospital stay, d, median (IQR) | 2 (2-3)         |

<sup>1</sup>Reasons for conversion: intraoperative esophageal perforation (n = 1), technical difficulties in dissection (n = 4), obesity (n = 1), problem with CO<sub>2</sub>-insufflation (n = 1); <sup>2</sup>Figures in the column include some patients with more than one complication; <sup>3</sup>Reoperations: bowel obstruction (n = 1), fundic perforation (n = 1), port-site bleeding (n = 2), thoracotomy and decortication for pleural empyema (n = 1). IQR: Interquartile range.

PPI-medication. None of the patients used PPI-medication at three months post-surgery. Barrett's esophagus without erosive changes was observed in 45 patients 3 mo after surgery and 2 patients had strictures, of which 1 was dilated. No cases of paraesophageal hernia were found. The short-term failure rate was similar between the two centers and time periods (1.7% vs 1.6%, P = 0.94). Two gastric ulcers and one case of acute gastritis were encountered in three patients with ulcer-like dyspepsia within 3-5 mo after surgery. All had biopsy-verified *Helicobacter pylori* infection and were treated by eradication therapy and followed-up by endoscopy and biopsies.

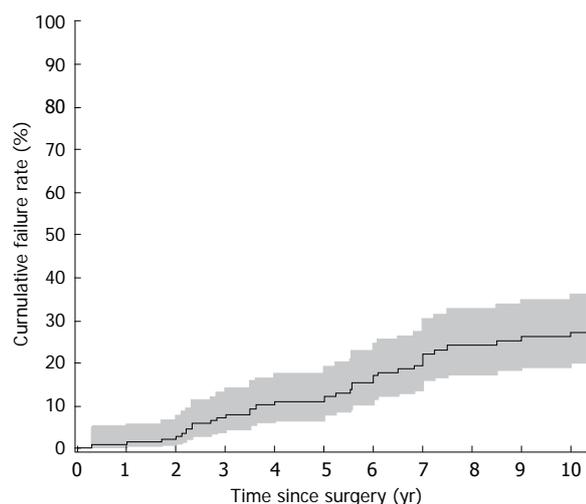
The patients observed typical reflux symptoms, especially heartburn and regurgitation, changed markedly after surgery. While 4 patients were asymptomatic for heartburn before and after surgery, 239 patients with reporting heartburn preoperatively [98.4% (95%CI: 95.8%-99.5%)] were completely symptom free (Figure 1A). Regurgitation was ameliorated in 239 [99.2% (97.0%-99.9%)] of the 241 patients, while 6 were asymptomatic before and after surgery (Figure 1A). Transitory new-onset dysphagia or worsening of preoperative dysphagia within 3 mo after surgery was reported by 176 patients (71.3%), but overall, dysphagia was significantly reduced from baseline. Dysphagia was alleviated in 45 [84.9% (72.4%-92.3%)] of the 53 patients having preoperative dysphagia. New onset



**Figure 1 Change in point prevalence and severity of typical reflux symptoms.** A: Patients were assessed for short-term symptoms (n = 247); B: Patients were assessed for long-term symptoms (n = 139); C: Measurement of the changes in long-term moderate to severe symptoms (n = 139). Differences between pre- and post-operative symptoms were significant for heartburn and regurgitation (P < 0.001), late dysphagia (P = 0.016). FU: Follow-up.

dysphagia (grade 1 in all) was reported by 13 of the 194 patients (6.7%) without preoperative dysphagia.

A decreased ability to belch was reported by 164 [66.4% (60.1%-72.3%)], increased flatulence by 183 [74.1% (68.2%-79.4%)] and increased bloating by 61 [24.7% (19.4%-30.0%)] of the 247 patients. The ability to vomit could not be evaluated at three months.



**Figure 2** Cumulative failure rates for laparoscopic Nissen fundoplication. Surgical failure is shown as a function of time after median follow-up of 10 years ( $n = 139$ ) (gray part shows 95%CI).

At their first clinical visit, 235 [95.1% (95%CI: 92.7%-98.0%)] patients reported better outcome after antireflux surgery than with preoperative medical treatment and were willing to have the procedure repeated in situations similar to preoperative ones.

### Long-term outcomes

A cohort of the first 180 consecutive patients operated on between 1992 and 2002 was selected to obtain a median follow-up time of 10 years until November 2006. Of these, 14 had died of the following non-gastroesophageal reflux disease (GERD), related causes: myocardial infarctions ( $n = 3$ ), intoxications ( $n = 2$ ), cerebral infarction, pulmonary embolism, aortic rupture, gastric cancer, pneumonia, necrotizing fasciitis, chemotherapy-related sepsis, cerebral trauma and intracranial hemorrhage, and severe myasthenia gravis. In addition, 1 patient had severe dementia, 1 had undergone esophageal resection for Barrett's esophagus-related adenocarcinoma, 3 returned incomplete questionnaires, and 22 patients could not be contacted, leaving 139 available for evaluation of late outcomes [median follow-up time 10.2 years (IQR 7.2-11.6 years), response rate 85%]. During the long-term follow-up, 24-h pH monitoring and endoscopy was done in 32 patients; 6 showed endoscopically verified disrupted fundoplication and pH < 4 median 25.1% (IQR 13.0%-42.8%) of total time, and 26 had intact fundoplication and pH median 0.6% (IQR 0.1%-2.4%).

The crude long-term failure rate was 24.5% (34 of 139 patients): 6 patients (4.3%) underwent reoperation for symptomatic and objective recurrence caused by defective plication, 16 (11.5%) had heartburn, regurgitation, dysphagia and PPI medication on a daily/weekly basis, 11 patients (7.9%) not using PPI medication reported frequent/daily symptoms, and 1 was asymptomatic while on medication (Figure 1B and C). The cumulative 5-year failure rate was 12.3% (95%CI: 7.8%-36.0%) and the 10-year

failure rate 27.1% (20.1%-36.0%) (Figure 2), and cure rates 87.7% (81.0%-92.2%) and 72.9% (64.0%-79.9%), respectively. One patient underwent a reoperation for paraesophageal hernia and one for late trocar-site hernia.

Decreased belching ability in the long-term was reported by 61.2% (52.5%-69.3%), increased rectal flatulence by 91.4% (85.4%-95.5%) and bloating by 71.9% (63.7%-79.2%). Total inability to vomit was reported by 43 patients (30.9%).

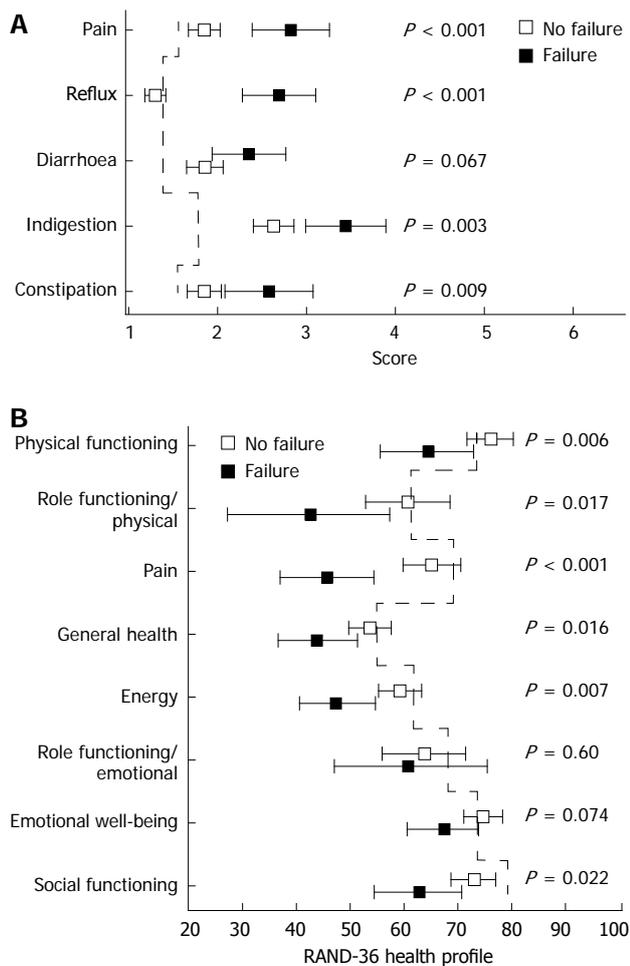
The mean GSRS score describing overall discomfort related to gastrointestinal symptoms was 1.9 (95%CI: 1.8-2.0) in patients cured by antireflux surgery and 2.8 (2.4-3.1) in those with failed antireflux surgery ( $P < 0.001$ ). Mean scores for all GSRS dimensions in comparison to a healthy control population are shown in Figure 3A. The failure of antireflux surgery and post-fundoplication side effects were reflected in the reflux syndrome score and indigestion syndrome score. Mean values of RAND-36 QoL scores were similar to the scores of the Finnish age- and gender-matched reference population for patients without treatment failure but were significantly impaired for those with failed antireflux surgery (Figure 3B).

Long-term outcomes were subjectively graded as good as expected (0) by 18.0%, moderately better (+2) by 14.4%, markedly better (+3) by 50.4%, and worse (-1) 5.8%, moderately worse (-2) 8.6% and markedly worse (-3) by 2.9%. At the time of this survey, 118 patients [84.9% (95%CI: 77.8%-90.4%)] were willing to undergo surgery again under similar preoperative conditions, whereas 21 patients (15.1%) chose medical treatment as a better initial treatment option.

## DISCUSSION

Laparoscopic Nissen fundoplication has become the method of choice in antireflux surgery providing good short-term outcome in over 90% of patients, an associated morbidity rate of less than 10%, and only a 5% incidence of new-onset dysphagia<sup>[5-10,12,13]</sup>. Here, we show that laparoscopic Nissen fundoplication effectively abolished heartburn and regurgitation, alleviated most preoperative dysphagia, and cured erosive esophagitis in most patients in the short term. This was achieved with low morbidity, a short hospital stay, a low incidence of new-onset dysphagia, and good patient satisfaction, with approximately 95% of the patients reporting a better outcome after surgery compared with non-surgical medical therapy. Mechanical side effects of total fundoplication may lead to a functional obstruction in the gastroesophageal junction and to an inability to vent air from the stomach<sup>[12]</sup>. As a result, we observed common postoperative side effects including inability to belch, abdominal bloating, and increased flatulence. However, these mild side effects did not seem to limit the success of antireflux surgery.

Long-term outcomes after laparoscopic antireflux surgery in this study were examined in multiple domains including symptom response, side effects of surgery, durability of the antireflux surgery, patient's subjective



**Figure 3** Comparing successful vs failed laparoscopic Nissen fundoplication by rating common gastrointestinal symptoms. A: Gastrointestinal symptom rating scores (GSRS) according to treatment success or failure (dotted line = healthy controls<sup>[22]</sup>); B: RAND-36 scores according to treatment success or failure (dotted line = age-matched and sex-matched general population<sup>[23]</sup>). P values were age- and sex-adjusted for treatment success vs failure.

perception of the overall success of the operation, and quality of life. In addition, treatment failure was defined according to composite criteria in contrast to previous studies<sup>[26-30]</sup> expressing the clinical outcome as the point prevalence of reflux-associated symptoms and patient satisfaction. However, our 5-year (88%) and 10-year (73%) cure rates compare well to previous studies reporting long-term control of reflux in 74%-90% of patients<sup>[26-31]</sup>. Our results also compare well to the nationwide long-term outcome after laparoscopic antireflux surgery in Sweden demonstrating a treatment failure in 25%-29% of the patients<sup>[32]</sup>. Only one randomized study comparing medical and surgical therapies for GERD and including patients with good response to PPI therapy, has reported similar remission rates at five years (85% *vs* 92%) between laparoscopic antireflux surgery and modern antisecretory medication<sup>[9]</sup>. Similar outcomes after laparoscopic Nissen fundoplication were obtained in our study despite the inclusion of patients reporting residual symptoms while on medical antisecretory treatment.

Patient well-being or quality of life are among the most important outcome measures in the field of functional surgery. The GSRS is a useful patient-related scale for evaluating the outcome of treatment for GERD<sup>[21]</sup>. In our study, the GSRS reflected well the long-term success of antireflux surgery as well as the side effects of antireflux surgery; it differentiated patients experiencing treatment failure from those being cured and from healthy controls<sup>[22]</sup>. Certain side effects, including increased bloating and rectal flatulence, manifest as indigestion syndrome and seem to be inevitable for most patients following fundoplication.

Overall quality of life was evaluated with RAND-36 (SF-36) because it is well validated in Finnish<sup>[23]</sup>. Consistent with previous studies<sup>[26,30]</sup> our results revealed that the quality of life of patients with long-term treatment success is similar to that of an age- and sex-matched general population. On the other hand, failed antireflux surgery and symptom recurrence significantly worsened quality of life in most dimensions.

Patients were also given a chance to evaluate the result of their surgery in the long-term. Here, 83% of the patients rated their surgical result to be as good as or better than expected, while only 17% felt that their surgical result was only fair or poor. Similarly, 118 patients (85%) were willing to undergo surgery again under similar pre-operative conditions.

A major challenge with this study and other similar studies was its retrospective nature and the lack of systematic long-term assessment by endoscopy and ambulatory 24-h pH monitoring<sup>[31]</sup>. The significance of the use of antireflux medication in the long term is also debatable without objective measurements and may not be a reliable marker of surgical failure. On the other hand, we used standardized symptom questionnaires, composite criteria, and quality of life assessment to define treatment failures. Furthermore, it is the symptom response experienced by patients that ultimately determines the success or failure of antireflux surgery and quality of life, not the change in objective measurements. It is also well known that the outcome after antireflux surgery is dependent on surgeon's experience and quality of the surgery. Satisfactory short-term outcomes indicate, however, that the quality of surgery has been as good in this study as elsewhere. The long-term outcome was investigated in 139 of the first 180 consecutive patients selected for the 10-year follow-up. The compliance in this study compares favorably with that in earlier studies with 85% of the eligible patients returning the questionnaires for long-term evaluation.

In the short-term, laparoscopic antireflux surgery effectively alleviated symptoms of gastroesophageal reflux disease and cured erosive esophagitis. Postoperative adverse effects were usually mild and patient satisfaction was good. For the long-term, our results indicate decreasing effectiveness of laparoscopic antireflux surgery, although most of the patients seem to have an overall quality of life similar to that of the general population.

## COMMENTS

### Background

Optimal management of chronic gastroesophageal reflux disease (GERD) is uncertain because treatment options can include either modern medical therapy that uses proton pump inhibitors or antireflux surgery.

### Research frontiers

Only one randomized study comparing medical and surgical therapies for GERD, has reported similar remission rates at 5 years. Data on long-term (10-year) cures and quality of life after antireflux surgery vs medical therapy are limited.

### Innovations and breakthroughs

To assess long-term outcomes, the authors examined several domains affected by the operation and defined treatment failures based on composite criteria and quality of life assessment. The results show that the excellent short-term results after laparoscopic Nissen fundoplication deteriorate over time. Quality of life analysis with the Gastrointestinal Symptom Rating Scale reflected well the side effects of antireflux surgery and differentiated patients having treatment failure from those being cured and from healthy controls. Overall quality of life (RAND-36) of patients with long-term treatment success was similar to that of an age- and sex-matched general population. Failed antireflux surgery and symptom recurrence significantly worsened the quality of life in most dimensions.

### Applications

By defining treatment outcomes with composite criteria and understanding how quality of life is affected by failed antireflux surgery, this study represents a potential new strategy for assessment of the long-term outcome after antireflux surgery.

### Peer review

The authors examined the short- and long-term outcome and quality of life after laparoscopic fundoplication. By addressing several domains affected by the operation and using composite criteria to define treatment failure, they show the excellent short-term results after laparoscopic Nissen fundoplication deteriorate over time. They also show failed antireflux surgery is associated with reduced quality of life. Use of composite criteria and quality of life assessment to define treatment failure represents a potential new strategy for the assessment of long-term outcomes following antireflux surgery.

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**P-Reviewers** Clark J, Detry O **S-Editor** Wen LL **L-Editor** A  
**E-Editor** Li JY



## Is alcoholic pancreatitis associated with enteroviral infection?

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Received: April 24, 2012 Revised: September 29, 2012

Accepted: October 30, 2012

Published online: June 28, 2013

### Abstract

**AIM:** To investigate whether enteroviral infection might trigger acute pancreatitis in patients made susceptible due to high alcohol consumption.

**METHODS:** Patients with alcohol-induced acute pancreatitis were analyzed for signs of simultaneous or preceding enteroviral infection. We studied the serum samples of 40 patients hospitalized for alcohol-induced acute pancreatitis and 40 controls recruited from an alcohol detoxification center. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect enterovirus RNA and diagnose acute viremia. Immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) enteroviral antibodies were measured using enzyme immunoassay to detect subacute and

previous infections. The samples were considered positive when the antibody titers were  $\geq 15$  IU. Furthermore, using RT-PCR, we studied pancreatic biopsy samples obtained during surgery from nine patients with chronic pancreatitis, one patient with acute pancreatitis and ten control patients with pancreatic carcinoma for evidence of persisting enteroviral RNA in the pancreatic tissue.

**RESULTS:** No enterovirus RNA indicating acute viremia was detected by RT-PCR in the serum samples of any patient or control. A high incidence of positive antibody titers was observed in both study groups: IgM antibodies had positive titers in 5/40 (13%) vs 4/40 (10%),  $P = 0.723$ ; IgG in 15/40 (38%) vs 19/40 (48%),  $P = 0.366$ ; and IgA in 25/40 (63%) vs 33/40 (83%),  $P = 0.045$ , patients and controls, respectively. Ten (25%) patients had severe pancreatitis and two (5%) required treatment in intensive care. The median length of hospitalization was 7 d (range: 3-47 d). The severity of acute pancreatitis or the length of hospitalization was not associated with enteroviral IgM, IgG or IgA antibodies. Five pancreatic biopsy samples tested positive with RT-PCR, three (8%) in the control group and two (5%) in the patient group ( $P = 0.64$ ).

**CONCLUSION:** The rate of enteroviral infection is not increased in patients with alcohol-induced acute pancreatitis when compared to alcoholics with similar high alcohol use.

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**Key words:** Pancreatitis; Alcoholic; Pancreatitis; Acute necrotizing; Enterovirus

Khan J, Nordback I, Seppänen H, Lappalainen-Lehto R, Järvinen S, Oikarinen S, Tauriainen S, Rätty S, Hyöty H, Sand J. Is alcoholic pancreatitis associated with enteroviral infection? *World J Gastroenterol* 2013; 19(24): 3819-3823 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3819.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3819>

## INTRODUCTION

Although heavy alcohol consumption is known to be associated with the development of acute pancreatitis, surprisingly little is known of the actual mechanism behind this association. Furthermore, only a small proportion of heavy drinkers ever develop acute pancreatitis even during long-term follow up<sup>[1]</sup>. Excessive alcohol consumption has been reported to cause 9%-70% of all cases of acute pancreatitis<sup>[2-7]</sup>, being predominant in some countries (*e.g.*, United States, Hungary and Finland), whereas gallstones are predominant in many other countries such as China, Greece and Italy. While alcohol remains a clear risk factor for acute pancreatitis, a multitude of other factors that may be genetic or environmental could be involved in triggering or modulation of the disease.

One previously suggested co-factor possibly associated with the induction of acute alcohol-associated pancreatitis is enteroviral infection. Human enteroviruses typically cause mild respiratory or gastrointestinal infections, but are also associated with myocarditis and aseptic meningitis. Over 100 enterovirus serotypes have been identified, including the polio virus. Other enteroviruses are classified as *coxsackie A* and *B* viruses, *enteric cytopathogenic human orphan* viruses or as numbered serotypes (*e.g.*, *enterovirus 70*). The evidence suggesting an association between enteroviruses and acute pancreatitis is mostly derived from case reports<sup>[8-12]</sup> and historical serological studies<sup>[13,14]</sup>. Evidence of enterovirus infection in the pancreatic beta cells has been reported by several authors<sup>[15-17]</sup>. More recently, Ozsvár *et al.*<sup>[18]</sup> reported a significant rise in *coxsackie B* virus antibody titers in acute and chronic pancreatitis patients. Recent animal studies further support a possible connection between enteroviral infection and pancreatitis<sup>[19-22]</sup>. Jerrells *et al.*<sup>[23]</sup> reported that mice on an alcohol diet and infected with a strain of *coxsackie B* virus developed more severe pancreatitis than control mice, and that even typically avirulent strains produced severe pancreatitis in these mice. Clemens *et al.*<sup>[24]</sup> showed that the pancreas of mice on an alcohol diet had impaired regeneration potential compared to control mice which may be associated with the severity of acute pancreatitis and the development of chronic pancreatitis. These studies suggest that enteroviruses may play a triggering role in at least a portion of human alcoholic pancreatitis.

To the best of our knowledge, there are no studies addressing the association between enteroviral infection and alcohol-associated acute pancreatitis in humans, where the alcohol intake of the non-pancreatitis controls has been comparable. The aim of this study was to ascertain whether patients suffering from alcohol-associated acute pancreatitis show evidence of simultaneous or preceding enteroviral infection in greater numbers than control subjects with similar recent alcohol consumption, but no previous or current pancreatitis. In addition, we analyzed pancreatic biopsy samples obtained from chronic pancreatitis patients and control patients during surgery to evaluate whether chronic pancreatitis speci-

mens showed signs of persistent enteroviral genome in the pancreas.

## MATERIALS AND METHODS

This study was a retrospective analysis of previously collected serum samples from a prospective study<sup>[25]</sup>. The study patients were recruited between January 2001 and November 2005. The samples for the first group, 40 patients hospitalized due to their first alcohol-associated acute pancreatitis, were collected during the first days of hospitalization. The samples for the control group, 40 alcoholics recruited from an alcohol detoxification center, were collected during their stay in the center. The patients were diagnosed with acute pancreatitis when they met the following criteria: acute epigastric pain that led to hospitalization, clinical signs consistent with acute pancreatitis together with serum amylase activity of at least three times the upper normal range, elevated serum inflammation markers (C-reactive protein concentration and leukocyte count), and/or the findings of acute pancreatitis on imaging. Alcohol was considered the probable etiology when the patient reported high alcohol intake during the alcohol use disorders identification test (AUDIT) or in a thorough interview of the patient or the family and other etiologies were excluded by laboratory testing and imaging<sup>[26]</sup>. Heavy alcohol consumption was similarly identified in the control subjects. Previously diagnosed pancreatitis or any acute illness were exclusion criteria when recruiting the control subjects.

The length of hospitalization, the development of complications and the need for and duration of treatment in the intensive care unit in alcohol-associated acute pancreatitis patients were recorded together with basic information such as body mass index (BMI), age and gender. Acute pancreatitis was considered severe when it met the Atlanta criteria<sup>[27]</sup>. The AUDIT questionnaire, amount of alcohol consumption (g/wk) preceding hospitalization and amount of smoking were elicited by a person specialized in addiction problems. The control group was matched according to age and reported amount of alcohol consumption. Thirty-two (80%) of the patients were male with a median age of 47 years (range: 18-73 years) and median BMI of 26 kg/m<sup>2</sup> (range: 19-34 kg/m<sup>2</sup>). In the control group, 25 (63%) patients were male with a median age of 46 years (range: 22-66 years) and median BMI of 26 kg/m<sup>2</sup> (range: 16-34 kg/m<sup>2</sup>). The median AUDIT scores were 22 (range: 5-38) in the patient group and 29 (range: 15-36) in the control group.

The biopsy samples were collected from 20 patients who underwent pancreatic surgery: one with alcohol-associated acute pancreatitis, nine with chronic pancreatitis and ten with pancreatic carcinoma. The development of chronic pancreatitis was alcohol associated in five and idiopathic in four patients. None of the patients with pancreatic carcinoma had a history of acute pancreatitis or excessive alcohol consumption. They were operated on between December 2001 and March 2006. The biopsy

samples were analyzed for the presence of enteroviral RNA using a highly sensitive reverse transcription-polymerase chain reaction (RT-PCR) method which amplifies a sequence common to all known enterovirus serotypes. The details of this method have been described earlier<sup>[28]</sup>. Frozen tissue samples were disrupted and homogenized using the TissueRuptor homogenizator (Qiagen, Hilden, Germany). RNA was extracted from the homogenized sample using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions.

The serum samples were stored at -70 °C during the interval between their acquisition and analysis. Evidence of enteroviral infection was analyzed by detecting immunoglobulin G (IgG), immunoglobulin A (IgA) and immunoglobulin M (IgM) class antibodies by enzyme immunoassay and by detecting enteroviral-RNA using the RT-PCR method described above. IgM class enterovirus antibodies were measured against a mixture of three enterovirus antigens (coxsackie virus B3, coxsackie virus A16 and echovirus 11) using a capture enzyme immunoassay as previously described<sup>[29]</sup>. IgG and IgA class antibodies were measured against a synthetic enterovirus peptide antigen (sequence KEVPALTAVETGAT-C derived from an immunodominant region of capsid protein VP1, which is a common epitope for several enteroviruses) as described earlier<sup>[30-32]</sup>. The samples were considered positive when the antibody titers were  $\geq 15$  EIU.

### Statistical analysis

Statistical testing was performed with SPSS statistical software using Pearson's correlation,  $\chi^2$  test, Mann-Whitney *U*-test and Fisher's Exact test. *P* values  $\leq 0.05$  were considered statistically significant. This study was performed according to the Helsinki Declaration and was approved by the Ethics Committee of Tampere University Hospital.

## RESULTS

Ten (25%) patients had severe pancreatitis according to the Atlanta criteria. Of these, six patients had necrotizing pancreatitis, one developed infected necrosis and three developed pseudocysts. Two patients required treatment in intensive care. The median length of hospitalization was 7 d (range: 3-47 d).

No enterovirus RNA was detected by RT-PCR in any patient or control subject. IgM antibodies had positive titers in 5/40 (13%) *vs* 4/40 (10%), *P* = 0.723; IgG in 15/40 (38%) *vs* 19/40 (48%), *P* = 0.366; and IgA in 25/40 (63%) *vs* 33/40 (83%), *P* = 0.045, patients and controls, respectively. The severity of acute pancreatitis or the length of hospitalization was not associated with enteroviral IgM, IgA or IgG antibodies.

Three pancreatic biopsy samples from patients with pancreatic carcinoma and two biopsy samples from patients with chronic pancreatitis tested positive for enteroviral RNA. The etiology of chronic pancreatitis was alcohol consumption in both patients. The tissue specimen

from the patient with alcohol-induced acute pancreatitis was negative for enteroviral RNA.

## DISCUSSION

In this study, we ascertained whether patients hospitalized for their first alcohol-induced acute pancreatitis had evidence of simultaneous or preceding enteroviral infection. In animal studies, enterovirus infection has been found to cause pancreatitis and, furthermore, simultaneous consumption of alcohol has been found to exacerbate the pancreatic insult. We hypothesized that enteroviral infection might be the triggering factor in at least some of the patients with their first alcohol-induced acute pancreatitis.

All the samples analyzed in this study were stored frozen. To the best of our knowledge, no studies have been reported on the possible adverse effects of prolonged storage and thawing of samples of enteroviral antibodies or on RT-PCR sensitivity. In general, repeated freezing and thawing may slightly alter the results observed, but the cycles generally do not affect samples to any clinically significant extent<sup>[33-35]</sup>.

No evidence of acute viremia was found in any of the patients. Positive IgM antibodies reflect subacute disease and 13% of our patients tested positive, with a similar rate in the control group. We also report a relatively high number of patients with positive IgA and IgG antibody titers. However, this was also the case in the control group. IgG antibodies remain elevated long after the infection, while IgA antibodies usually disappear within a few months. Therefore, we suspect that this finding reflects the fact that our patients and controls were of lower socio-economic background with a tendency to acquire such infections at an increased rate when compared to the general population. An association between lower socio-economic status and increased enteroviral infection rate has previously been reported<sup>[36,37]</sup>. Thus, our findings do not suggest a role for enteroviral infection in the pathogenesis of alcohol-induced acute pancreatitis in humans, at least to a clinically significant extent. In fact, IgG and IgA class enterovirus antibodies tended to be at lower levels in the pancreatitis group, which may reflect the general immunosuppression associated with this disease.

A surprisingly high percentage of pancreatic tissue samples obtained during surgery, from patients operated on either for chronic pancreatitis or carcinoma of the pancreas, tested positive for enteroviral RNA in RT-PCR. In an earlier study, Lászik *et al*<sup>[38]</sup> studied pancreatic tissue specimens obtained during surgery for acute pancreatitis using *in situ* hybridization and reported no evidence of enteroviral infection in any of the samples. In the present study, we did not investigate whether enteroviral genome was present in the acini or the islets of Langerhans in the pancreas. Recent studies suggest a role for enteroviral infection in the genesis of type 1 diabetes<sup>[39]</sup>, and, furthermore, direct beta cell involvement<sup>[15-17]</sup>. It is therefore possible, although not certain, that the high percentage

of enteroviral genome observed in the tissue samples in our study also came from the islets of Langerhans in this patient material.

In conclusion, we report no evidence of an increased rate of enteroviral infection in patients hospitalized for their first alcohol-induced acute pancreatitis when compared to alcoholics with similarly heavy alcohol consumption, but with no history or signs of acute or chronic pancreatitis. The rate of positive results in pancreatic tissue samples was clearly higher in our study than reported elsewhere, although the sample size was small.

## COMMENTS

### Background

Although heavy alcohol consumption is known to be associated with the development of acute pancreatitis, surprisingly little is known of the actual mechanism behind this association. Furthermore, only a small proportion of heavy drinkers ever develop acute pancreatitis even during long-term follow up.

### Research frontiers

One previously suggested co-factor possibly associated with the induction of acute alcohol-associated pancreatitis is enteroviral infection. Human enteroviruses typically cause mild respiratory or gastrointestinal infections, but are also associated with myocarditis and aseptic meningitis.

### Innovations and breakthroughs

There are no studies addressing the association between enteroviral infection and alcohol-associated acute pancreatitis in humans, where the alcohol intake of the non-pancreatitis controls has been comparable.

### Applications

The aim of this study was to ascertain whether patients suffering from alcohol-associated acute pancreatitis show evidence of simultaneous or preceding enteroviral infection in greater numbers than control subjects with similar recent alcohol consumption, but no previous or current pancreatitis.

### Peer review

This study partially answered a question in etiology of acute pancreatitis. It was well designed retrospective study.

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**P- Reviewers** Muniraj T, Sezgin O **S- Editor** Gou SX  
**L- Editor** Webster JR **E- Editor** Xiong L



## Characteristics of allergic colitis in breast-fed infants in the absence of cow's milk allergy

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Supported by OTKA-K105530, -K81117 and ETT-028-02; the János Bolyai Research Grant, to Veres G; and the János Bolyai Research Scholarship of the Hungarian Academy of Sciences

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Received: October 4, 2012 Revised: February 5, 2013

Accepted: March 23, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the characteristics of mucosal lesions and their relation to laboratory data and long-term follow up in breast-fed infants with allergic colitis.

**METHODS:** In this study 31 breast-fed infants were prospectively evaluated (mean age, 17.4 wk) whose rectal bleeding had not ceased after a maternal elimination diet for cow's milk. Thirty-four age-matched and breast-fed infants (mean age, 16.9 wk) with no rectal bleeding were enrolled for laboratory testing as controls. Laboratory findings, colonoscopic and histological characteristics were prospectively evaluated in infants with rectal bleeding. Long-term follow-up with different nutritional regimes (L-amino-acid based formula or breastfeeding) was also included.

**RESULTS:** Iron deficiency, peripheral eosinophilia and

thrombocytosis were significantly higher in patients with allergic colitis in comparison to controls ( $8.4 \pm 3.2 \mu\text{mol/L}$  vs  $13.7 \pm 4.7 \mu\text{mol/L}$ ,  $P < 0.001$ ;  $0.67 \pm 0.49 \text{ G/L}$  vs  $0.33 \pm 0.17 \text{ G/L}$ ,  $P < 0.001$ ;  $474 \pm 123 \text{ G/L}$  vs  $376 \pm 89 \text{ G/L}$ ,  $P < 0.001$ , respectively). At colonoscopy, lymphonodular hyperplasia or aphthous ulceration were present in 83% of patients. Twenty-two patients were given L-amino acid-based formula and 8 continued the previous feeding. Time to cessation of rectal bleeding was shorter in the special formula feeding group (mean, 1.4 wk; range, 0.5-3 wk) when compared with the breast-feeding group (mean, 5.3 wk; range, 2-9 wk). Nevertheless, none of the patients exhibited rectal bleeding at the 3-mo visit irrespective of the type of feeding. Peripheral eosinophilia and cessation of rectal bleeding after administration of elemental formula correlated with a higher density of mucosal eosinophils.

**CONCLUSION:** Infant hematochezia, after cow's milk allergy exclusion, is generally a benign and probably self-limiting disorder despite marked mucosal abnormality. Formula feeding results in shorter time to cessation of rectal bleeding; however, breast-feeding should not be discouraged in long-lasting hematochezia.

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**Key words:** Rectal bleeding; Breast-feeding; Allergic colitis; Colonoscopy; Amino-acid formula

**Core tip:** Rectal bleeding is a common problem in otherwise healthy breast-fed infants; our primary aim was to find characteristic lesions at colonoscopy and determine the cessation of rectal bleeding when administering different nutritional regimes (L-amino-acid based formula or breast-feeding). Our secondary aim was to find correlations between laboratory data, severity of mucosal lesions and cessation of rectal bleeding in allergic colitis infants with no cow's milk allergy.

Molnár K, Pintér P, Gyórfy H, Cseh Á, Müller KE, Arató A, Veres G. Characteristics of allergic colitis in breast-fed infants in the absence of cow's milk allergy. *World J Gastroenterol* 2013; 19(24): 3824-3830 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3824.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3824>

## INTRODUCTION

Rectal bleeding is a common problem in otherwise healthy breast-fed infants<sup>[1]</sup>. The differential diagnosis of this condition includes anal fissures, infectious colitis, congenital bleeding disorders, inflammatory bowel disease (IBD) and, most frequently, allergic colitis (AC)<sup>[2]</sup>. Currently there is no reliable diagnostic test available for AC, and the diagnosis is often made presumptively in healthy infants with rectal bleeding who show no anal fissures or infectious colitis<sup>[3]</sup>. AC is believed to be a hypersensitive gastrointestinal disorder to allergens present in breast milk, and formula is regarded as a form of food allergy in infancy<sup>[4]</sup>. As a resulting first-line therapeutic intervention, cow's milk (CM) is generally eliminated from the maternal diet<sup>[5]</sup>.

Recent guidelines state that the elimination diet for the mother should be continued for a minimum of at least 2 wk, and up to 4 wk in cases of AC<sup>[6]</sup>. According to the guidelines, if an infant has no cessation of rectal bleeding after 4 wk of maternal CM-free diet then the patient has no CM allergy. It is of interest that the majority of infants with rectal bleeding, despite the general belief, have no CM allergy as an underlying cause of hematochezia. This phenomenon was supported by recent studies showing that even in breast-fed infants with rectal bleeding, CM allergy is more uncommon than previously believed<sup>[7]</sup>. Only 18% of 40 infants with rectal bleeding had proven CM allergy, none had positive specific immunoglobulin E (IgE) to cow's milk, egg or wheat on admission, and only 5% had a positive skin-prick test to cow's milk<sup>[8]</sup>. Nevertheless, an atopy patch test was useful to identify sensitization for cow's milk (50%), soy (28%), egg (21%), rice (14%) and wheat (7%) in 14 AC infants with multiple food allergy<sup>[9]</sup>.

Lymphonodular hyperplasia (LNH), patchy granularity and aphthous lesions were found at colonoscopy in patients with AC; however, most patients showed no abnormal mucosal lesions<sup>[5,7,8]</sup>. These studies included patients with short-term rectal bleeding which may not have shown the development of characteristic lesions at colonoscopy. Therefore, in our study, there was a minimum 4-wk timeline between onset of rectal bleeding and the procedure of colonoscopy. We hypothesized that 4 wk would be enough to find a typical mucosal abnormality in otherwise healthy breast-fed infants with rectal bleeding not caused by CM allergy.

Taken together, our primary aim was to find characteristic lesions at colonoscopy and determine the cessation of rectal bleeding when administering different nutritional

regimes (L-amino-acid based formula or breast-feeding). Our secondary aim was to find correlations between laboratory data, severity of mucosal lesions and cessation of rectal bleeding in AC infants with no CM allergy.

It should be noted that there is no previous, prospective study in AC patients with no CM allergy.

## MATERIALS AND METHODS

### Patients

During a 4-year period (January 2006-February 2010) at the First Department of Pediatrics, Semmelweis University, breast-fed infants were invited to join the present study. Inclusion criteria were age less than 6 mo, exclusive breast-feeding, normal stool cultures (*Salmonella*, *Shigella*, *E. coli*, *Yersinia*, *Campylobacter*), and no cessation of rectal bleeding after introduction of a maternal elimination diet for CM for a minimum of 4 wk. All subjects were evaluated by history and physical examination for signs of fissure, infection and allergic diseases. Rectal bleeding as bloody spots or streaks with or without mucus was confirmed macroscopically before the colonoscopy. A complete blood count with differential, C-reactive protein, prothrombin time, activated partial thromboplastin time, serum albumin, serum IgE, and specific IgE to common food antigens including CM, egg, wheat, rye, soy, fish, nuts, peanuts, sesame, almond, tomato, banana, celery, carrots, apple, peach, lemon, and orange were obtained. Thirty-four age-matched and breast-fed infants (mean age, 16.9 wk; range, 4-25 wk; girls, 15) with no rectal bleeding (apnea, 10; gastro-esophageal reflux, 12; minor trauma, 7; minor surgical intervention, 5) were enrolled as controls for the laboratory testing. Characteristics of patients and controls are summarized in Table 1.

The Institutional Ethical Committee approved the study; written parental informed consent was obtained.

### Evaluation of colonoscopy

To characterize allergic colitis and exclude other rare forms of rectal bleeding in infancy such as angiodysplasia, hemangioma, polyp, IBD or blue rubber bleb syndrome, colonoscopy with multiple biopsies was performed using a flexible pediatric gastroscope with narrow band imaging technique included (Olympus GIF-Q180, Olympus Hungary KFT). Ambulatory colonoscopy was done by the same gastroenterologist (Veres G) with the assistance of a trained endoscopy nurse. The procedure was done under general anesthesia. The colon was gently cleansed by a single water enema (5 mL/kg) before the procedure.

### Evaluation of histology

Using standard biopsy forceps, 4 biopsies were taken from the sigmoid colon (about 10 cm from the anal verge) targeted to areas with gross endoscopic findings. Biopsy samples were fixed in 4% buffered formalin for 24 h then embedded into paraffin. Hematoxylin eosin (HE) stain was performed on 3-4  $\mu$ m thin slides. The biopsy was ex-

**Table 1** Clinical characteristics and parameters related to atopy in infants with allergic colitis and control subjects *n* (%)

| Characteristic                              | AC ( <i>n</i> = 30) | Controls ( <i>n</i> = 34) |
|---|---------------------|---------------------------|
| Gender (F:M)                                | 19:11               | 19:15                     |
| Age at presentation (wk, mean)              | 17.2                | 16.9                      |
| Family history of atopy                     | 17 (57)             | 13 (38)                   |
| Atopic dermatitis                           | 8 (27)              | 7 (21)                    |
| Duration of hematochezia (wk, mean)         | 8.6                 | -                         |
| Increased level of IgE (> 5 U/mL)           | 3                   | Not done                  |
| Positivity to specific IgE to food antigens | 0                   | Not done                  |

AC: Allergic colitis; M: Male; F: Female; IgE: Immunoglobulin E.

amined for routine histology by a pathologist (Gyórfy H). Eosinophils were counted in 10 high-power fields (HPF) and the average number of cells was recorded. Although there are no standard accepted criteria for the diagnosis of AC, several studies have demonstrated eosinophilic infiltration ( $\geq 6$ /HPF) in the lamina propria of the left colon or rectum<sup>[10-12]</sup>. Based on previous studies<sup>[11,13]</sup>, patients with AC were subdivided according to the number of eosinophils on biopsy specimens. AC1 consisted of patients with eosinophils between 6 and 19/HPF, and AC2 with eosinophils  $\geq 20$ /HPF. Using these criteria, AC2 patients fulfill the diagnostic criteria for eosinophilic colitis.

#### Treatment and follow-up

All subjects were followed up for 6 mo to assess outcome, including visits after one week following colonoscopy, after 3 mo, and after 6 mo. Elemental L-amino acid formula (Neocate; SHS Int., Liverpool, United Kingdom) was offered to the parents to treat their children. Assessment of rectal bleeding during the follow-up period was qualitative and assessed by parental self-report. In addition, if macroscopic bleeding eased, parents were asked to provide feces to exclude occult bleeding (HSV10; Diagnosticum Zrt, Budapest, Hungary). Because macroscopic bleeding was the main inclusion criteria, the cessation of macroscopic bleeding was considered to be the endpoint.

Up to 1 year of age, patients received AA formula or continued breast-feeding. At the age of 1 year, CM was introduced to both groups with no macroscopic bleeding in the follow-up. After 4 wk of the challenge, parents were asked to provide feces to exclude occult bleeding (HSV10; Diagnosticum Zrt, Budapest, Hungary). In addition to CM, parents were asked to avoid eggs, wheat, soya, nuts and fish ("six food elimination diet") up to one year of age. After CM introduction, other foods were introduced gradually (wheat, soya, eggs, fish and nuts).

At the age of 6 mo, introduction of supplemental feeding (except the 6 foods listed above) was recommended irrespective of whether the patient was on the formula or the breast-feeding arm. Subjects with worsening of symptoms or having a severe form of colitis underwent repeat colonoscopy with biopsy. Six month time-point was chosen for this procedure to allow enough time for healing in the latter group.

#### Statistical analysis

Our data followed normal distribution, therefore parametric statistical tests were implicated and data were expressed as means with standard deviation. For comparison of datasets, unpaired *t* test and analysis of variance (ANOVA) with Bonferroni's post hoc tests were used. For analyses of contributing effects, factorial ANOVA, logistic regression and Pearson's correlation were used. The level of significance was 5% ( $P < 0.05$ ) and Bonferroni's adjustment for multiple comparisons was introduced where needed. Statistical analysis was performed with Statistica 8 (Statsoft, Tulsa, OK, United States).

## RESULTS

#### Clinical characteristics of infants with rectal bleeding

Thirty-one healthy, breast-fed infants (mean age, 17.4 wk; range, 5-26 wk; 12 girls) were enrolled. The mean (range) duration of bloody stools before colonoscopy was 8.7 (4-16) wk (Table 1). All mothers strictly followed a CM-free diet with the help of a trained dietician. In addition to CM-free diet, 7 mothers had an elimination diet for egg and 4 had an exclusion diet for egg, fish, wheat, nuts and soy. In all patients, rectal bleeding was the main symptom that prompted the request for gastroenterological evaluation. In addition to bloody stools, watery (42%) or mucous stools (68%) were common. None of the patients had fissures at rectal examination. Stool frequency averaged 4.2 (range, 1-7) bowel movements per day. Three patients had a classic history of infantile colic. One patient had hemangioma on the thorax with no involvement of the liver. One patient had transient hypothyroidism. The others had no previous hospitalization or chronic disease. Growth was reported as normal in all patients whose weight and height charts were reviewed for an objective assessment of their growth pattern.

During follow-up, one subject had failure to thrive and was subsequently diagnosed with infantile Crohn's disease. She was 24 wk old at study entry with a history of rectal bleeding for 12 wk. Her data were excluded from the study (final study group consisted of 30 infants).

#### History of atopy, analysis of IgE-mediated hypersensitivity

Eight patients (27%) had atopic dermatitis which is comparable with the percentage of control subjects (21%). Seventeen patients (57%) had a positive family history of atopy among first-degree relatives which was higher than in controls (38%). Only 3 patients had an elevated level of serum IgE (> 5 kU/L). Specific IgE to the most common food antigens was determined in 25 of 31 patients (81%) with negative results (Table 1).

#### Laboratory investigation

The laboratory abnormalities are presented in Table 2. The mean iron level was significantly decreased in patients ( $8.43 \pm 3.2 \mu\text{mol/L}$ ) in comparison to controls ( $13.7 \pm 4.7 \mu\text{mol/L}$ ,  $P < 0.001$ ). We found a markedly increased thrombocyte count in patients ( $474 \pm 123 \text{ G/L}$ )

**Table 2** Laboratory data on admission in infants with allergic colitis, allergic colitis 1, allergic colitis 2 and control subjects (mean  $\pm$  SD)

| Characteristic      | AC<br>(n = 30)               | AC1<br>(n = 19)            | AC2<br>(n = 11)              | Controls<br>(n = 34) |
|---------------------|------------------------------|----------------------------|------------------------------|----------------------|
| Hemoglobin (g/L)    | 115 $\pm$ 13                 | 114 $\pm$ 12               | 115 $\pm$ 14                 | 113 $\pm$ 10         |
| Iron ( $\mu$ mol/L) | 8.4 $\pm$ 3.2 <sup>b</sup>   | 8.9 $\pm$ 2.9 <sup>b</sup> | 7.6 $\pm$ 4.0 <sup>b</sup>   | 13.7 $\pm$ 4.7       |
| Thrombocytes (G/L)  | 474 $\pm$ 123 <sup>b</sup>   | 458 $\pm$ 126 <sup>b</sup> | 497 $\pm$ 122 <sup>b</sup>   | 376 $\pm$ 89         |
| Eosinophils (G/L)   | 0.67 $\pm$ 0.49 <sup>b</sup> | 0.51 $\pm$ 0.45            | 0.91 $\pm$ 0.47 <sup>a</sup> | 0.33 $\pm$ 0.17      |

<sup>a</sup>*P* < 0.05 vs allergic colitis (AC), <sup>b</sup>*P* < 0.01 vs controls. Allergic colitis 1 (AC1) consisted of patients with eosinophils between 6 and 19/ high power field (HPF), and allergic colitis 2 (AC2) with eosinophils  $\geq$  20 eosinophils/HPF.

**Table 3** Frequency of laboratory abnormalities in infants with allergic colitis on admission and at 6-mo visit *n* (%)

| Characteristic                            | On admission | 6-mo visit | ND |
|---|--------------|------------|----|
| Thrombocytosis ( $> 450 \times 10^9/L$ )  | 16 (60)      | 5          | 2  |
| Iron deficiency ( $< 9 \mu\text{mol/L}$ ) | 15 (50)      | 4          | 2  |
| Eosinophilia ( $> 5\%$ of leukocytes)     | 12 (40)      | 5          | 1  |

The number of patients who had previous abnormal parameters and no control analysis depicted in the last column. ND: Not done.

when compared with controls (376  $\pm$  89 G/L, *P* < 0.001). Similarly, patients exhibited increased peripheral eosinophilia (6.7  $\pm$  4.9 G/L) when compared with controls (3.3  $\pm$  1.7 G/L, *P* < 0.001). On the other hand, 60% of patients had thrombocytosis, 50% showed low iron level, and 40% demonstrated eosinophilia (Table 3). There was no difference concerning hemoglobin level between patients and controls.

Patients were subdivided into two groups (AC1 and AC2: eosinophilic colitis) according to the number of eosinophils determined by histology on biopsies (see above). Iron level, hemoglobin and thrombocytes were comparable in AC1 and AC2. However, peripheral eosinophils were significantly elevated in AC2 (9.1  $\pm$  4.7 G/L) when compared with AC1 (5.1  $\pm$  4.5 G/L, *P* < 0.01).

Only one patient had marked anemia which required blood transfusion. This patient was the only one who had low albumin level ( $< 35$  g/L). Her other characteristics (including macroscopic and microscopic mucosal abnormalities) were not different from those of the other subjects. Laboratory data in patients who were subsequently diagnosed with Crohn's disease showed iron deficiency (4 mol/L) without anemia (113 g/L), and thrombocytosis (524  $\times 10^9/L$ ).

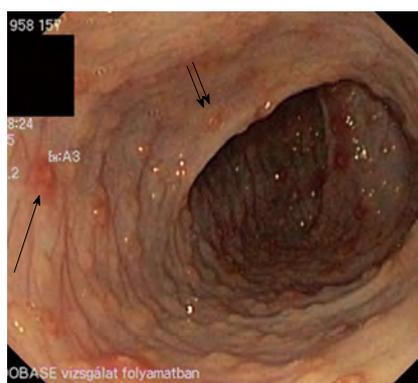
### Characterization of colonoscopy

At colonoscopy, the cecum was reached in 23 (74%) patients. In all other subjects the colonic mucosa was visualized from the anus to the splenic flexure, two of them to the hepatic flexure. Three patients had normal visual colonoscopy findings. Twenty-seven (90%) showed abnormal mucosal lesions such as LNH, aphthous ulceration, and marked erythema (2 patients). LNH or aphthous ulceration was present in 25/30 patients (83%)

**Table 4** Frequency of colonoscopic abnormalities and number of eosinophils on histology/high power field of infants with allergic colitis 1 and allergic colitis 2 *n* (%)

| Characteristic                | AC1 (n = 19) | AC2 (n = 11) |
|-------------------------------|--------------|--------------|
| LNH                           | 15 (79)      | 7 (64)       |
| Aphthous ulceration           | 8 (42)       | 6 (55)       |
| LNH and aphthous ulceration   | 6 (32)       | 5 (45)       |
| Marked erythema               | 1 (5)        | 1 (9)        |
| No. of eosinophils/HPF (mean) | 12.2         | 29.5         |

LNH: Lymphonodular hyperplasia; AC1: Allergic colitis 1; AC2: Allergic colitis 2; HPF: High power field.



**Figure 1** Endoscopic findings of allergic colitis in a breastfed infant. Lymphonodular hyperplasia with circumscribed erythema (arrow) and tiny bleeding spot (double arrow) in the descending colon in a breast-fed infant with allergic colitis.

(Figure 1). LNH was found in 73% of patients (22/30) and aphthous ulceration in 47% of the patients (14/30). Subdividing the patients with regard to the number of eosinophils on biopsies, LNH was seen in 15 patients with AC1 (79%) and in 7 patients with AC2 (64%). Aphthous ulceration was visualized in 8 patients with AC1 (42%) and in 6 patients with AC2 (55%). More patients with AC2 (45%) depicted both phenomenon, which were present in 32% of subjects with AC1. However, the discrepancies failed to reach significance (Table 4).

LNH and aphthous ulceration without any specific signs for IBD were present in the patient with Crohn's disease at her first colonoscopy. She was the only patient with no cessation of rectal bleeding at the 3-mo visit. Deep ulceration in the transverse colon and non-caseating granulomas on histology established the diagnosis of Crohn's disease at her second colonoscopy (after 6-mo visit). Abnormal findings at colonoscopy are depicted in Table 4. There were no adverse events recorded under general anesthesia and colonoscopy.

### Characterization of histology

Light microscopy revealed normal mucosal architecture in all patients studied, including the first biopsy of the patient with Crohn's disease which showed normal mucosal architecture with an elevated number of eosinophils (29/HPF). All patients had more than 6 eosinophils/HPF on histol-

**Table 5** Baseline characteristics and cessation of rectal bleeding in patients with amino acid-based formula feeding and breast-feeding [mean (range)]

| Characteristic                             | Amino-acid based formula feeding | Breast-feeding            |
|--|----------------------------------|---------------------------|
| No. of patients                            | 22                               | 8                         |
| Age at presentation (wk)                   | 16.0 (5-26)                      | 20.6 (16-25) <sup>a</sup> |
| Rectal bleeding at baseline (wk)           | 7.9 (4-14)                       | 10.5 (6-16)               |
| Cessation of rectal bleeding (wk)          | 1.4 (0.5-3)                      |                           |
| Rectal bleeding at 3-mo visit ( <i>n</i> ) | 0                                | 0                         |

<sup>a</sup>*P* < 0.05 vs formula feeding.

ogy (Table 4). All three patients with normal macroscopic findings at colonoscopy showed an elevated eosinophil count on biopsies (6, 20 and 39 cells/HPF, respectively). Nineteen patients had an eosinophil count between 6 and 19/HPF (AC1) and 11 patients had more than 20/HPF (AC2). Mean number of eosinophils was 12.2/HPF in AC1 and 29.5/HPF in AC2 (eosinophilic colitis).

#### Follow-up and clinical outcome, laboratory data

Based on marked mucosal abnormalities and the long-lasting rectal bleeding, we decided to treat patients with special formula. At the first visit one week after colonoscopy, elemental L-amino acid formula was offered to the parents to treat their children. Parents refused the formula feeding in 8 cases (5 in AC1 and 3 in AC2) and continued the previous feeding type. Twenty-two patients received the special formula (14 in AC1 and 8 in AC2). There were no significant differences concerning baseline characteristics between the 2 groups.

The duration of bleeding was significantly shorter in the special formula feeding group (mean, 1.4 wk; range, 0.5-3 wk) when compared with the breast-feeding group (mean, 5.3 wk; range, 2-9 wk). Nevertheless, none of the patients exhibited gastrointestinal complaints or visible rectal bleeding at the 3-mo visit irrespective of the type of feeding (Table 5). Three patients with serum iron level below 5 µmol/L were treated with iron-containing syrup (Maltofer; dosage, 2 drops/kg) for 6 wk. At 6-mo visit, control blood count and iron level were analyzed in patients who had abnormal levels on admission.

#### Follow-up and colonoscopy, histology

Due to the cessation of bleeding, only four parents agreed for a control colonoscopy to be performed. At baseline all of them had LNH and aphthous ulceration. One patient was in AC1 (13 eosinophils/HPF) and 3 patients were in AC2 (eosinophils > 30/HPF). At control colonoscopy none of the patients had aphthous ulceration; only scattered LNH without bleeding spots were visible. The number of eosinophils normalized in AC1 patients (5/HPF) and markedly decreased in AC2 patients (8-17/HPF).

#### Correlation analysis and risk factors

Correlation analysis revealed that the time to stop the bleeding was negatively correlated with the administration of elemental formula (*P* < 0.001, *r* = -0.762). In ad-

dition, the number of eosinophils on biopsy specimens at baseline correlated negatively with elemental formula concerning cessation of rectal bleeding (*P* = 0.025, *r* = -0.667). There was no correlation between abnormal serum laboratory data (thrombocytes, eosinophils, iron) and the ending of rectal bleeding. In patients with AC1 and AC2, we found similar correlations between elemental diet and the time to stop the bleeding.

## DISCUSSION

The elimination of CM from the diet of the lactating mother is a commonly used and recommended practice<sup>[10-12]</sup> for infants with rectal bleeding. However, recent studies have suggested that there are significant proportions of infants with rectal bleeding with no CM allergy<sup>[8,9,13]</sup>. Based on these results we focused on AC patients with no cessation of rectal bleeding after a minimum of 4 wk of maternal CM-free diet. Four weeks is a long enough time-span to exclude rare forms of surgical diseases, to cure an undetected viral gastrointestinal disorder or for elimination of CM protein from patients<sup>[6,8,14]</sup>.

In our study, frequency of atopic dermatitis, measurement of serum IgE, and specific IgE to different foods were not significant factors in the diagnostic procedure, as has been suggested by previous studies<sup>[14,15]</sup>. On the other hand, a recent Italian study suggested a delayed-type immunogenic mechanism in this process, hence the atopy patch test was useful in the diagnostic procedure in 14 AC infants with multiple gastrointestinal food allergy<sup>[9]</sup>.

In our study, iron deficiency, peripheral eosinophilia and thrombocytosis were significantly higher in AC patients in comparison to controls. Surprisingly, no previous study has analyzed iron levels in patients with AC. Peripheral eosinophilia and thrombocytosis were reported previously<sup>[7,8,15]</sup>. To determine the risk factors for the clinical outcome, none of the 3 abnormal laboratory findings correlated with the cessation of rectal bleeding.

Theoretically, colonoscopy would have detected other rare causes of rectal bleeding such as polyps, hemanangioma, blue rubber bleb syndrome and angiodysplasia. However, all but 3 patients showed mucosal abnormalities such as LNH, aphthous ulceration and marked focal erythema. As a bleeding source, LNH with circumscribed erythema or/and central pit-like bleeding spots (Figure 1) or aphthous ulceration were present in 25 patients with AC (83%). This is in contrast to previous studies, which reported a much lower percentage of characteristic abnormalities at colonoscopy. In a prospective evaluation of 34 infants with rectal bleeding, only 10 (29%) showed LNH including three of them with normal histology<sup>[5]</sup>. Xanthakos *et al*<sup>[7]</sup> followed 22 infants younger than 6 mo of age with hematochezia. Only 12 patients showed gross endoscopic abnormalities at colonoscopy including diffuse erythema, friability, LNH and aphthous ulceration. In addition, a recent study followed 40 infants with visible rectal bleeding where 41% of patients had normal mucosa, 33% had aphthous ulceration, and 51% depicted focal erythema with no report of LNH<sup>[8]</sup>. One of the ex-

planations for the high rate of LNH and aphthous ulceration found in our study is the long-lasting rectal bleeding (minimum 4 wk). LNH has been described as a sign of food allergy in children with CM allergy<sup>[16-18]</sup>. In our patients with no CM allergy, LNH was also a characteristic abnormality at colonoscopy. We do not think that LNH is a specific sign of food allergy or AC, just an indicator of a focal inflammation beneath the epithelium. However, LNH with circumscribed erythema or/and central pit-like bleeding spots and aphthous ulceration may have a role in the diagnostic procedure of AC.

The number of eosinophils on biopsies in patients with AC is a question of debate<sup>[19]</sup>. Previous studies<sup>[20,21]</sup> have suggested, as a diagnostic criteria for AC, eosinophilic infiltration in the lamina propria of  $\geq 6$ /HPF, whereas Machida *et al*<sup>[5]</sup> suggested for this entity of  $\geq 20$ /HPF. All patients in the present study had  $\geq 6$  eosinophils/HPF in the lamina propria. To find a possible difference of laboratory, colonoscopy and clinical outcome, patients were subdivided to moderate (AC1) or high (AC2) mucosal density of eosinophils. It should be noted that AC2 patients fulfill the present criteria for eosinophilic colitis ( $\geq 20$  eosinophils/HPF)<sup>[22]</sup>. Peripheral eosinophilia and cessation of rectal bleeding after administration of elemental formula correlated with the higher number of eosinophils on biopsies. In conclusion, these data suggest that both groups represent similar entities, and a cut-off  $> 20$  eosinophils/HPF for eosinophilic colitis may be artificial.

Due to the marked mucosal abnormality and the long-lasting rectal bleeding, elemental formula was given to the patients. It was refused by 8 cases who continued their previous feeding. Elemental diet shortened the time to cessation of hematochezia; nevertheless, it should be emphasized that none of the patients exhibited gastrointestinal complaints or rectal bleeding at the 3-mo visit irrespective of the type of feeding. This result suggests that the underlying cause could be food-related other than CM, hence AA formula shortened the resolutions of symptoms. Probably, our study population may have multiple food allergy; at least, we cannot exclude this because the nursing mothers conducted only limited types of food elimination diet (beside CM).

The beneficial effect of amino acid-based formula was reported previously<sup>[23]</sup>, suggesting an allergy-related mechanism even in AC patients with no CM allergy.

On the other hand, we cannot exclude the possibility that our patients had no food allergy at all. This phenomenon is similar to infantile eczema<sup>[24]</sup> or eosinophilic esophagitis in infancy, where considerable proportions of patients have no food allergy but an allergic-immunologic component does exist<sup>[25]</sup>. Exclusive amino acid formula-based dietary trials resulted in more than 90% remission in children with eosinophilic esophagitis. Similarly to current guidelines in AC, empiric elimination diets of avoidance of foods commonly known to cause hypersensitivity reactions resulted in 50%-74% disease remission in eosinophilic esophagitis<sup>[26]</sup>. Taken together, previous studies and the beneficial effect of AA formula in the present study suggest that our study population probably has

multiple food allergy. One of the most interesting points of data in our study is that patients were able to tolerate a non-restricted diet after a period of elimination diet (AA-based formula) or after a more prolonged persistence of symptoms on breast-feeding. Acquired tolerance may explain this finding, which is supported by our previous study where depleted FOXP3 regulatory T cells normalized after feeding of AA formula<sup>[27]</sup>.

Nevertheless, the only patient who had not ceased the rectal bleeding was subsequently diagnosed with infantile Crohn's disease and she was excluded from the study. As reported previously<sup>[7,28,29]</sup>, at baseline, none of her laboratory, colonoscopy or histology parameters were different from the patients with AC.

In conclusion, rectal bleeding in infancy, even after exclusion of CM, is generally benign and is probably a self-limiting disorder, despite the marked mucosal abnormality. Iron deficiency, peripheral eosinophilia and thrombocytosis are characteristic findings in these patients. At colonoscopy, LNH and aphthous ulceration may be characteristic signs. Administration of amino acid formula shortened the timeline of the rectal bleeding in patients with AC; however, breast-feeding should not be discouraged in this population with long-lasting hematochezia.

## COMMENTS

### Background

Allergic inflammation of the large intestine is believed to be a hypersensitive gastrointestinal disorder to allergens present in breast milk, and formula is regarded as a form of food allergy in infancy. As a resulting first line therapeutic intervention, cow's milk is generally eliminated from the maternal diet; however, significant numbers of infants with rectal bleeding, despite the general belief, have no cow's milk allergy as an underlying cause of hematochezia. Currently there is no reliable diagnostic test available for allergic colitis, and the diagnosis is often made presumptively in healthy breast-fed infants with rectal bleeding who show no anal abnormalities or infectious colitis.

### Research frontiers

A research group in Hungary investigated the characteristics of endoscopic findings in colonic mucosal lesions and their relation to laboratory data in breast-fed infants with allergic colitis. Long-term follow-up with different nutritional regimes (L-amino acid-based formula or breast-feeding) was also included. According to the literature there is no previous, prospective study in allergic colitis patients with no cow's milk allergy.

### Innovations and breakthroughs

In this study, 31 breast-fed infants were prospectively evaluated whose rectal bleeding had not ceased after a maternal elimination diet for cow's milk allergy. As controls, 34 age-matched and breast-fed infants without rectal bleeding were enrolled for the laboratory testing. In the laboratory findings, iron deficiency and elevation of peripheral eosinophilic cell and platelet counts were significantly higher in patients with allergic colitis in comparison to controls. At colonoscopy, increments of lymphoid tissue appearing as a lump (called lymphonodular hyperplasia) or aphthous ulceration were present in 83% of patients. Twenty-two patients were given L-amino acid-based formula and 8 continued the previous feeding. Time to cessation of rectal bleeding was shorter in the special formula feeding group when compared with the breast-feeding group. Nevertheless, none of the patients exhibited rectal bleeding at the 3-mo visit irrespective of the type of feeding. The elevated peripheral eosinophilic cell count and cessation of rectal bleeding after administration of elemental formula correlated with the higher density of mucosal eosinophilic cells in the colon.

### Applications

Administration of amino acid formula shortened the timeline of rectal bleeding in patients with allergic colitis; however, breast-feeding should not be discouraged in this population with long-lasting rectal bleeding. These data suggest that the significance of real food allergy in breast-fed infants with allergic colitis

should be revised in the future. This phenomenon is similar to infantile eczema or eosinophilic esophagitis in infancy where considerable proportions of patients had no food allergy but an allergic-immunologic component does exist. It may indicate that the "food allergy" theory is currently much less applicable, and probably a significant number of nursing mothers continue an unnecessary restriction diet worldwide.

### Terminology

Allergic colitis is explained as an allergic condition of the gastrointestinal tract in breast-fed infants that results from an immune response to the allergens present in breast milk.

### Peer review

This is a very interesting article from a teaching institution in Hungary. It is well written and conclusions are valid.

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P-Reviewer Camilleri-Brennan J S-Editor Wen LL  
L-Editor Logan S E-Editor Li JY



## Active treatments are a rational approach for hepatocellular carcinoma in elderly patients

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Received: January 4, 2013 Revised: February 10, 2013

Accepted: April 28, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To determine whether an active intervention is beneficial for the survival of elderly patients with hepatocellular carcinoma (HCC).

**METHODS:** The survival of 740 patients who received various treatments for HCC between 1983 and 2011 was compared among different age groups using Cox regression analysis. Therapeutic options were principally selected according to the clinical practice guidelines for HCC from the Japanese Society of Hepatology. The treatment most likely to achieve regional control capability was chosen, as far as possible, in the following order: resection, radiofrequency ablation, percutaneous ethanol injection, transcatheter arterial chemoembolization, transarterial oily chemoembolization, hepatic arterial infusion chemotherapy, systemic chemotherapy including molecular targeting, or best supportive care.

Each treatment was used alone, or in combination, with a clinical goal of striking the best balance between functional hepatic reserve and the volume of the targeted area, irrespective of their age. The percent survival to life expectancy was calculated based on a Japanese national population survey.

**RESULTS:** The median ages of the subjects during each 5-year period from 1986 were 61, 64, 67, 68 and 71 years and increased significantly with time ( $P < 0.0001$ ). The Child-Pugh score was comparable among younger (59 years of age or younger), middle-aged (60-79 years of age), and older (80 years of age or older) groups ( $P = 0.34$ ), whereas the tumor-node-metastasis stage tended to be more advanced in the younger group ( $P = 0.060$ ). Advanced disease was significantly more frequent in the younger group compared with the middle-aged group ( $P = 0.010$ ), whereas there was no difference between the middle-aged and elderly groups ( $P = 0.75$ ). The median survival times were 2593, 2011, 1643, 1278 and 1195 d for 49 years of age or younger, 50-59 years of age, 60-69 years of age, 70-79 years of age, or 80 years of age or older age groups, respectively, whereas the median percent survival to life expectancy were 13.9%, 21.9%, 24.7%, 25.7% and 37.6% for each group, respectively. The impact of age on actual survival time was significant ( $P = 0.020$ ) with a hazard ratio of 1.021, suggesting that a 10-year-older patient has a 1.23-fold higher risk for death, and the overall survival was the worst in the oldest group. On the other hand, when the survival benefit was evaluated on the basis of percent survival to life expectancy, age was again found to be a significant explanatory factor ( $P = 0.022$ ); however, the oldest group showed the best survival among the five different age groups. The youngest group revealed the worst outcomes in this analysis, and the hazard ratio of the oldest against the youngest was 0.35 for death. The survival trends did not differ substantially between the survival time and percent survival to

life expectancy, when survival was compared overall or among various therapeutic interventions.

**CONCLUSION:** These results suggest that a therapeutic approach for HCC should not be restricted due to patient age.

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**Key words:** Hepatocellular carcinoma; Population aging; Survival; Life expectancy; Active intervention

**Core tip:** Progressive population aging worldwide demands consensus development for decision making to treat elderly patients. A simple comparison of survival days is confounded by aging; therefore, age compensation is mandatory to evaluate survival benefits among different age groups. In this study, age difference was compensated by life expectancy in a hepatocellular carcinoma cohort. The authors suggested that age itself might not be a critical determinant for the selection of a therapeutic option. This study emphasizes the importance of clarifying risk determinants specific for elderly patients with respect to individual aspects and medical economy.

Suda T, Nagashima A, Takahashi S, Kanefuji T, Kamimura K, Tamura Y, Takamura M, Igarashi M, Kawai H, Yamagiwa S, Nomoto M, Aoyagi Y. Active treatments are a rational approach for hepatocellular carcinoma in elderly patients. *World J Gastroenterol* 2013; 19(24): 3831-3840 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3831.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3831>

## INTRODUCTION

The Japanese population is aging more rapidly than that of any other nation in the world. In 1990, approximately one in eight people in Japan were aged 65 years or older<sup>[1]</sup>. This level was already the highest in Asia, although somewhat lower than in most developed European countries. Since then, the population of Japan has aged because of an increasing life expectancy and a falling birth rate<sup>[2]</sup>. As a result, as many as one in four of the Japanese population will be at least 65 years old by the year 2025<sup>[3]</sup>, which is a much higher ratio than that predicted for any other country.

There is much controversy concerning medical interventions for elderly patients. Zhang *et al*<sup>[4]</sup> reported that an interventional scheme should not be changed on the basis of the age of patients facing the treatment of myocardial infarction, whereas Teo *et al*<sup>[5]</sup> reported that the addition of percutaneous coronary intervention to optimal medical therapy did not improve the clinical outcomes in patients 65 years of age or older. An active treatment is recommended for elderly patients suffering from subarachnoid hemorrhage<sup>[6,7]</sup>. However, a conservative treatment was reported to be superior for elderly patients in the management of traumatic dental axis frac-

ture<sup>[8]</sup>. Although some benefit of active treatment for hepatocellular carcinoma (HCC) in elderly patients has been suggested, the debate still continues as to how fast patients suffering from HCC are actually getting older and whether the survival benefit offered by active interventions is comparable between the young and the elderly.

In this report, aging trends among patients actively treated for HCC were evaluated over 25 years from 1986 to 2011 at a single institution in Japan. To compare the survival benefits between the relatively young and the elderly, survival was compared after adjusting the absolute survival time or life expectancy. Finally, a case presentation of an elderly patient who was successfully managed is used to illustrate the risks and benefits of active interventions for HCC in elderly patients.

## MATERIALS AND METHODS

### Patients

Clinicopathological data were retrospectively analyzed for 918 patients who were admitted between 1983 and 2011 for the first time for management of HCC in our hospital. The subjects' basic characteristics are shown in Table 1. To compare the ages of patients during each 5-year interval of the overall study period, 840 patients were selected who were admitted between 1986 and 2010. Only 740 patients who had already died or who had been followed for longer than a year in our hospital were included in survival analyses using actual survival time. Among these 740 cases, 504 could be allocated a life expectancy because these data are available for each age and gender since 1996 in Japan. To compare survival among different age groups, patients were classified into five groups according to their ages; 49 years of age or younger (-49), 50-59 years of age (50s), 60-69 years of age (60s), 70-79 years of age (70 s) and 80 years of age or older (80+).

Hepatic nodules were radiographically diagnosed as HCC when they fulfilled at least one of the following criteria based on dynamic computed tomography (CT)/magnetic resonance imaging and/or CT during hepatic arteriography/CT during arterial portography: (1) the typical hemodynamics of classical HCC, with a substantial arterial phase enhancement followed by a washout with a corona-like peripheral enhancement in an equilibrium phase; or (2) with similar characteristics as coexisting nodules that had already been diagnosed as HCC. Otherwise, a histological diagnosis was made.

Therapeutic options were principally selected according to the clinical practice guidelines for HCC from the Japanese Society of Hepatology, 2009<sup>[9]</sup>. The treatment most likely to achieve regional control capability was chosen, as far as possible, in the following order: resection, radiofrequency ablation (RFA), microwave coagulation (MWC), percutaneous ethanol injection (PEI), transcatheter arterial chemoembolization (TACE), transarterial oily chemoembolization (TOCE), hepatic arterial infusion chemotherapy (HAIC), systemic chemotherapy including molecular targeting or best supportive care. Each treatment was used alone or in combination, such as RFA af-

**Table 1 Basic characteristics**

|   |                               |
|---|-------------------------------|
| Age (yr)                                | 67.0 (60.0-73.0) <sup>1</sup> |
| Gender (male/female)                    | 647/271                       |
| HBsAg (-/+ /ND)                         | 582/196/140                   |
| anti-HCV (-/+ /ND)                      | 278/534/106                   |
| AIH/PBC/alcohol/NASH/BCS                | 10/6/85/16/2                  |
| Child-Pugh (A/B/C/ND)                   | 704/172/36/6                  |
| Size (mm)                               | 28.0 (19.0-45.0) <sup>1</sup> |
| Stage ( I / II / III / IV) <sup>2</sup> | 163/306/287/162               |
| Therapy (Loco/IVR/Cx/others/BSC)        | 459/332/78/11/38              |

<sup>1</sup>Median and interquartile range; <sup>2</sup>General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan. ND: Not determined; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; NASH: Nonalcoholic steatohepatitis; BCS: Budd-Chiari syndrome; Loco: Logo-regional treatments, including resection, radio-frequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization, and transarterial oily chemoembolization; Cx: Chemotherapy including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; BSC: Best supportive care; HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus.

ter TACE or TACE following HAIC, with a clinical goal of striking the best balance between functional hepatic reserve and the volume of the targeted area. Stereotactic radiotherapy was considered when loco-regional treatments were indicated but not applicable, whereas liver transplantation was selected by an exclusive decision process. Treatments were classified into four groups: (1) loco-regional including resection, RFA, MWC and PEI; (2) interventional radiology (IVR) including TACE and TOCE; (3) chemotherapy (Cx) including HAIC and systemic chemotherapy; and (4) other, including stereotactic radiotherapy, proton beam and liver transplantation, which were applied to only 11 patients in total. If pleural treatments were added as an adjunct, the case was classified into a group according to the applied treatment with the highest regional control capability.

Measuring hepatitis B surface antigen (HBsAg), anti-hepatitis C virus (HCV), anti-mitochondrial, anti-M2 and anti-nuclear antibodies serologically defined background liver diseases. A habitual daily alcohol intake of more than 60 g was considered alcohol abuse. Nonalcoholic steatohepatitis was diagnosed on the basis of histological findings, whereas Budd-Chiari syndrome was diagnosed angiographically. Patients who were negative for all of the above criteria were considered not definitive for a background liver disease. The institutional review board of our institution, which did not require informed consent for a retrospective study using medical records or imaging examinations, approved the present study, which conformed to the ethical guidelines of the 2008 Declaration of Helsinki.

### Serum biochemistry and histological examination

HBsAg and anti-HCV antibodies were detected by a chemiluminescence immunoassay using the ARCHITECT HBsAg QT and ARCHITECT HCV kits (Abbott

Japan Co. Ltd., Chiba, Japan), respectively. Serum anti-mitochondrial and anti-M2 antibodies were quantified using the commercial kits AMA FluoroAID-1 and Me-sacup mitochondria M2 (MBL Co. Ltd., Nagoya, Japan), respectively. Total and *Lens culinaris* agglutinin A-reactive  $\alpha$ -fetoprotein (AFP) serum concentrations were quantified with a liquid-phase binding assay system (LiBASys; Wako Pure Chemical Industries Ltd., Osaka, Japan). L3 was calculated as a percentage of *Lens culinaris* agglutinin A-reactive species against total AFP. Serum des- $\gamma$ -carboxy prothrombin (DCP) was measured using an electro-chemiluminescence immunoassay (Wako Pure Chemical Industries Ltd, Osaka, Japan). Other blood biochemistries were routinely measured in the clinical laboratories of our hospital.

Two expert histologists independently rendered histological diagnoses based on microscopic observations of tissues stained with hematoxylin and eosin, silver, iron, periodic acid-Schiff, periodic acid-Schiff with diastase digestion, and azan. When there was any discordance between the two histologists, the specimen was reviewed to reach a consensus diagnosis.

### Life expectancy and percent life expectancy

The Japanese life expectancy per year for each gender at a specific age is available for 1996 onwards and was downloaded from the Ministry of Health, Labour and Welfare<sup>[1]</sup>. The life expectancy for our cohort was plotted in three dimensions using O-Chart Standard software (ONO SOKKI Co., Ltd., Yokohama, Japan). The survival time for each case was divided by the life expectancy to obtain the percent life expectancy (%LE).

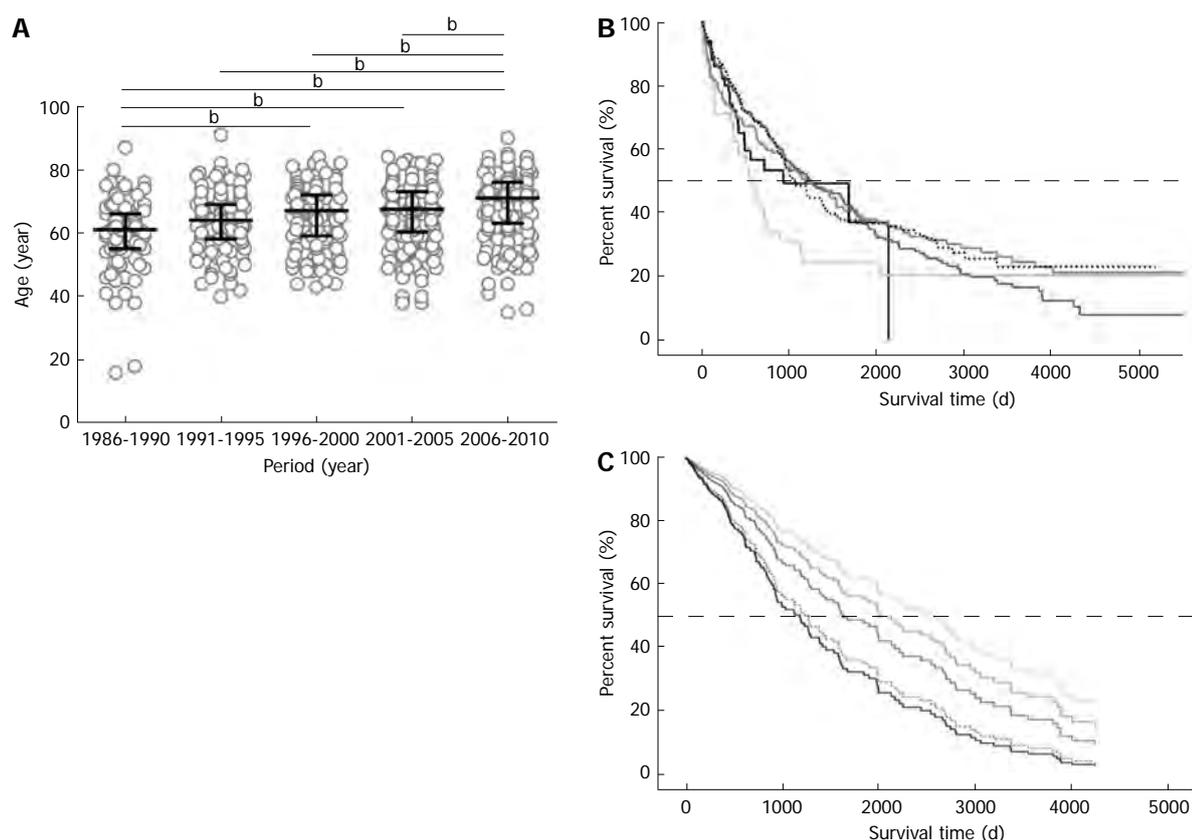
### Statistical analysis

Patient ages were compared using the Kruskal-Wallis test, and Dunn's multiple comparison tests were used to compare the different periods in 5-year intervals. The influence of multiple factors on survival was evaluated using Cox regression analysis. The comparisons of categorical data were performed with the Fisher's exact test or the  $\chi^2$  test among three or two different age groups, respectively. Overall survival was demonstrated by calculating Kaplan-Meier survival fractions. All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, United States), except for the multivariate analysis, which was performed using PASW statistics 17.0 (SPSS Inc., Chicago, United States). A two-tailed *P* value less than 0.05 was considered statistically significant after Bonferroni correction.

## RESULTS

### Patients receiving active treatments for HCC are continuously aging in Japan

The median ages for each 5-year interval steadily increased from 61 (interquartile range: 55-66) years of age (from 1986 to 1990) to 71 (63-76) years of age (from 2006 to 2010), as shown in Figure 1A. The median age was sig-



**Figure 1** Age distribution in different periods and the survival of patients with hepatocellular carcinoma. **A:** The age of patients who were admitted for the management of hepatocellular carcinoma was plotted for each 5-year interval since 1986; the median ages were significantly different among the different periods ( $P < 0.0001$ ); **B:** The overall survival of 740 patients in five age groups who have already died or have been followed for longer than 1 year was calculated on the basis of Kaplan-Meier survival fractions: the median survival time of all cases was 1094 d; **C:** Overall survival was compared among the different age groups after compensation for background characteristics using a Cox proportional hazard model and was significantly different among age groups ( $P = 0.020$ ). The solid black and dotted lines are the survival curves of the 80 years of age or older and 70-79 years of age groups, respectively. The other lines are 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, indicated in colors ranging from dark to pale. <sup>a</sup> $P < 0.01$ . The horizontal bars (A) indicate the median and interquartile range. The dotted horizontal lines (B and C) indicate a position of 50% survival.

nificantly different among the periods ( $P < 0.0001$ ), and the median age of these patients increased by 10 years in the last 20 years. The patients admitted from 2006 to 2010 were significantly older than the patients who were hospitalized during any other periods ( $P < 0.001$  *vs* 1986-1990, 1991-1995, 1996-2000; and  $P < 0.01$  *vs* 2001-2005).

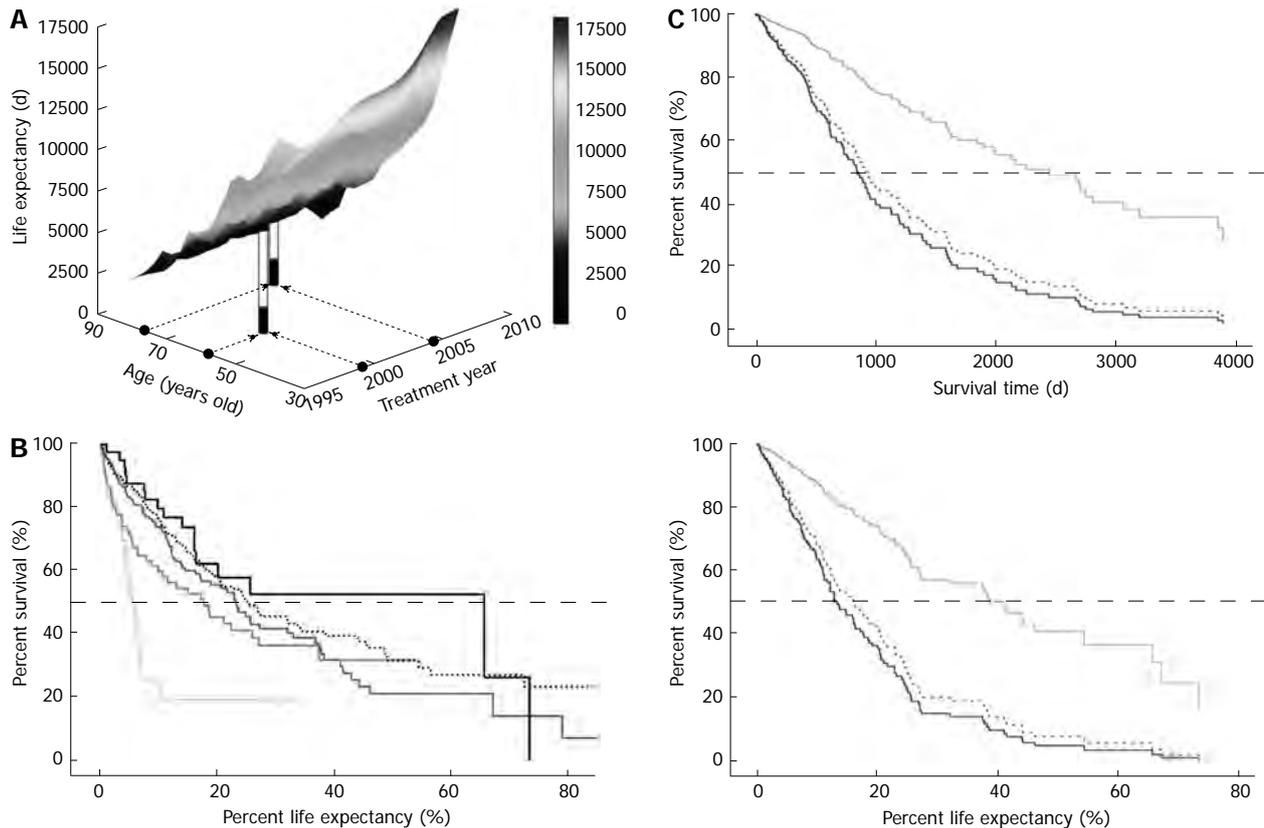
**Aging is a significant factor affecting overall survival time in HCC**

The median survival time for 740 patients who were deceased or were followed longer than 1 year was calculated from the Kaplan-Meier survival fractions as 1094 d. When the patients' ages were categorized into the five groups to test the dependence of survival on age, there was no significant survival difference among the groups ( $P = 0.41$ ), and the least median survival time was 553 d in the -49 group, as shown in Figure 1B. For the Cox regression analysis, 379 cases were included because they were not missing values for the 10 explanatory variables: (1) age (years); (2) gender (male/female); (3) HBsAg (-/+); (4) anti-HCV (-/+); (5) AFP ( $\log_{10}$ ); (6) L3 (%); (7) DCP ( $\log_{10}$ ); (8) Child-Pugh class (A/B/C); (9) HCC stage (I / II / III / IV); and (10) therapy (loco-regional/IVR/Cx/

other). Among these 10 variables, age, AFP, DCP, HBsAg, anti-HCV, Child-Pugh class, HCC stage and therapy were determined to be significant factors that influenced survival time (Table 2). The impact of age on survival time was found to be significant ( $P = 0.020$ ), with a hazard ratio of 1.021, suggesting that a 10-year-older patient has a 1.23-fold higher risk of death. When the survival differences among the five age groups were estimated with the Cox proportional hazards model on the basis of the above 10 explanatory factors, overall survival was poorer with age and was the worst in the 80+ group, as shown in Figure 1C. The risk of death in the 80+ group was 2.41-times higher when compared with that of the -49 group.

**Fractional life expectancy is an indicator of survival benefit adjusted for age**

It may be reasonable to assume that older patients will have shorter survival times irrespective of effective treatments, preserved functional hepatic reserve, or other factors, simply because of their shorter residual length of life. To compare the survival from the point of aging, survival was normalized by life expectancy. A ratio



**Figure 2** Life expectancy and percent life expectancy of patients with hepatocellular carcinoma. **A:** Life expectancy (LE) for each case was plotted in a three-dimensional space. The percent LE (%LE) was defined as the ratio between survival time and LE and is shown for representative cases. The LE of a male at 59 years of age in the year 1999 is 7928 d, whereas the LE is 3760 d for a 77-year-old male in the year 2004 (white piles). Both patients survived for 1779 d, as indicated by the black piles, with %LE values of 22.4% and 48.6%, respectively; **B:** A survival proportion was expressed in %LE in the five different age groups, and the median %LE of all 504 cases was 22.9%. The solid black and dotted lines are the survival curves of 80 years of age or older and 70-79 years of age group, respectively. The other lines represent 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, in colors ranging from dark to pale, respectively; **C:** In a cohort of 328 patients for whom LE is available, the survival among patients receiving loco-regional, interventional radiology (IVR), or chemotherapy (Cx) treatments was evaluated on the basis of absolute time (upper panel) or %LE (lower panel). The solid black and dotted lines are survival curves for Cx and IVR, respectively, and the gray line represents the loco-regional group. The dotted horizontal lines indicate a position of 50% survival.

of survival days to the expected residual life length is defined as the %LE. Life expectancy data for each age and gender are available from 1996 onward in Japan; therefore, life expectancy was plotted for the 504 cases in our cohort (Figure 2A). Overall survival based on %LE revealed a median survival percentage of 22.9%. When the survival based on %LE was compared among the five different age groups, the median survival was significantly different among the groups (Figure 2B,  $P < 0.0001$ ), ranging from 5.4% in the <49 group to the best rate of 65.7% in the 80+ group.

Among 504 patients, 174 cases were excluded from further analyses because a therapeutic intervention was never performed or because one or more of the 10 explanatory candidate factors was not measured. Among the remaining 330 cases, only two patients received therapies that were categorized in “other”. Finally, 328 cases were subjected to Cox regression analysis to investigate the survival differences associated with different therapeutic modalities. As shown in Figure 2C, the survival curves were very similar to the evaluations based on survival time (upper) and %LE (lower). Both analyses

showed that loco-regional therapies far surpassed IVR and Cx in terms of survival benefit.

When the relationship between survival time and %LE was evaluated in each case, however, it became clear that the two survival indicators were not consistent. For example, the same survivals of 1779 d for males at 59 and 77 years of age in 1999 and 2004 gave rise to very different %LE values of 22.4% and 48.6%, respectively, as indicated in Figure 2A. In another example, a 69-year-old female in 2002 and a male of the same age in 1999 survived 2693 and 1980 d, respectively. Because their life expectancies were 7125 and 5168 d, respectively, the shorter absolute survival value for the male surpassed the female’s longer survival in terms of %LE at 37.8% and 38.3%, respectively. Taken together, %LE is a potential alternative for evaluating survival benefit in HCC patients among different age groups.

### ***Elderly patients survive for the highest percentage of their life expectancy after receiving active treatments for HCC***

Although the overall survival trends and survival benefits

**Table 2** Cox regression analysis for survival time

| Variable                 | Significance | HR    | 95%CI for HR |        |
|--------------------------|--------------|-------|--------------|--------|
|                          |              |       | Lower        | Upper  |
| Age                      | 0.020        | 1.021 | 1.003        | 1.040  |
| Gender                   | 0.652        | 1.079 | 0.775        | 1.503  |
| HBsAg                    | 0.029        | 1.598 | 1.051        | 2.432  |
| anti-HCV                 | 0.036        | 1.503 | 1.027        | 2.198  |
| AFP                      | 0.000        | 1.314 | 1.135        | 1.521  |
| L3                       | 0.288        | 1.004 | 0.997        | 1.012  |
| DCP                      | 0.005        | 1.216 | 1.061        | 1.395  |
| Child-Pugh class         |              |       |              |        |
| A                        | 0.000        |       |              |        |
| B                        | 0.000        | 2.307 | 1.562        | 3.408  |
| C                        | 0.001        | 3.373 | 1.617        | 7.035  |
| Tumor stage <sup>1</sup> |              |       |              |        |
| I                        | 0.000        |       |              |        |
| II                       | 0.148        | 1.504 | 0.865        | 2.616  |
| III                      | 0.051        | 1.751 | 0.997        | 3.074  |
| IV                       | 0.000        | 5.715 | 2.985        | 10.939 |
| Therapy category         |              |       |              |        |
| Loco-regional            | 0.000        |       |              |        |
| IVR                      | 0.000        | 2.567 | 1.800        | 3.661  |
| Chemotherapy             | 0.000        | 2.861 | 1.675        | 4.889  |
| Others                   | 0.000        | 6.151 | 2.505        | 15.107 |

<sup>1</sup>General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan. HR: Hazard ratio; AFP:  $\alpha$ -fetoprotein; L3: Percentage of fucosylated fraction in AFP; DCP: Des- $\gamma$ -carboxy prothrombin; Loco-regional: Therapies including resection, radiofrequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization and transarterial oily chemoembolization; Chemotherapy: Therapies including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

of different therapies were consistent between the analyses using dependent variables of survival time and %LE, the two analyses indicated substantially different survival benefits when the survival was compared among the different age groups. A comparison based on survival time in 330 cases revealed that the 80+ group had the worst survival (Figure 3A upper panel), consistent with a similar finding in the initial cohort of 760 patients (Figure 1C). However, when the survival benefit was evaluated on the basis of %LE, age was again found to be a significant explanatory factor ( $P = 0.022$ ), but the 80+ group showed the best survival among the five different age groups, as shown in the lower panel of Figure 3A. Intriguingly, the -49 group revealed the worst outcomes in this analysis. The hazard ratio of the 80+ group against the -49 group was 0.35 for death (Table 3). The other significant explanatory factors for %LE were HBsAg and anti-HCV, AFP, DCP, Child-Pugh score, tumor-node-metastasis (TNM) stage, and therapeutic options, and the maximal hazard ratios for each variable were 1.71, 1.60, 1.35, 1.24, 2.14, 3.88 and 3.37, respectively, suggesting that age is one of the most powerful determinants of %LE.

### Tumors were more advanced in the young

Functional hepatic reserve and anatomical tumor extent

**Table 3** Cox regression analysis for percent life expectancy

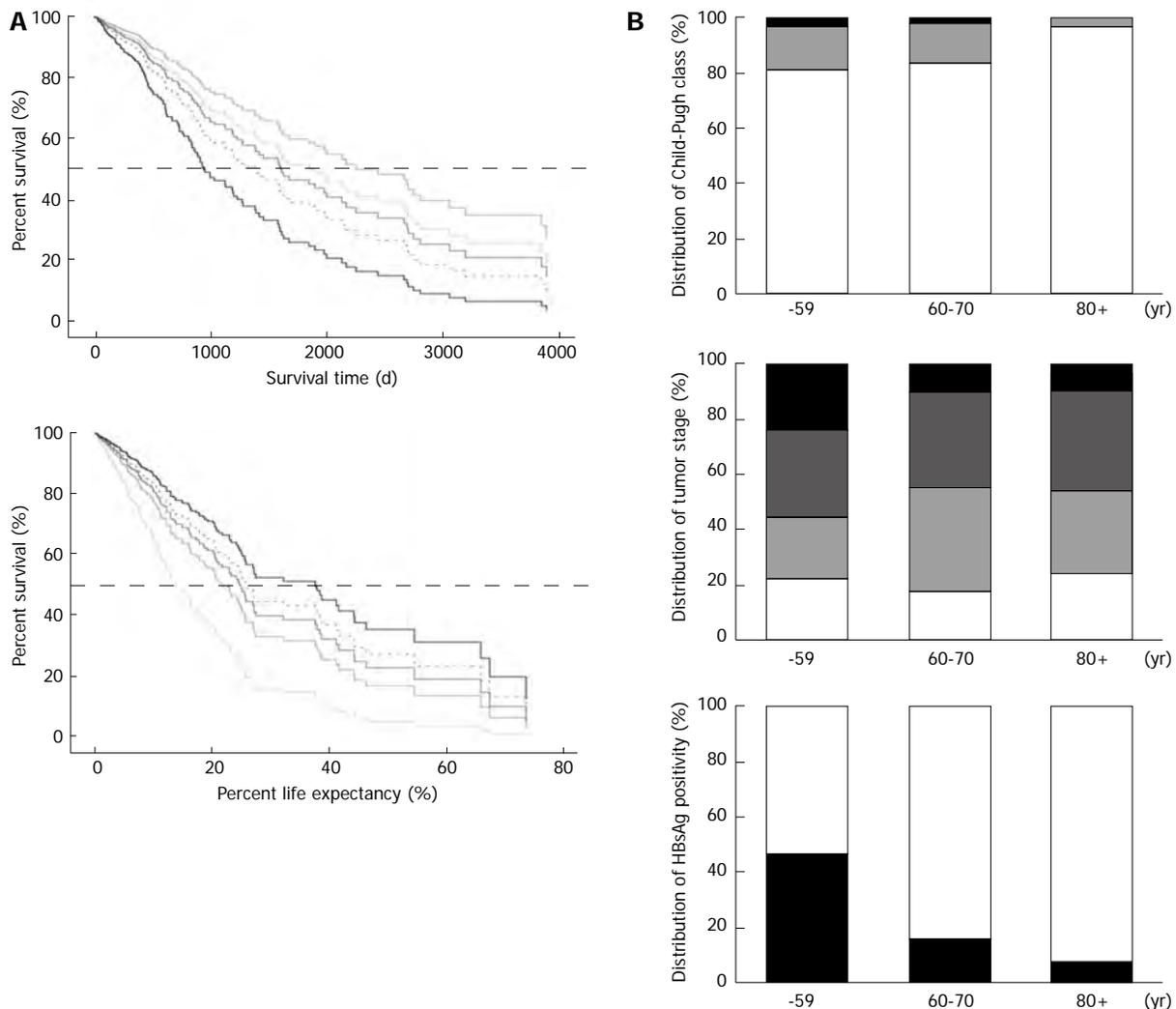
| Variable                 | Significance | HR    | 95%CI for HR |        |
|--------------------------|--------------|-------|--------------|--------|
|                          |              |       | Lower        | Upper  |
| Gender                   | 0.072        | 0.717 | 0.499        | 1.030  |
| HBsAg                    | 0.043        | 1.709 | 1.018        | 2.869  |
| anti-HCV                 | 0.043        | 1.597 | 1.015        | 2.511  |
| AFP                      | 0.000        | 1.348 | 1.145        | 1.587  |
| L3                       | 0.421        | 1.004 | 0.995        | 1.012  |
| DCP                      | 0.015        | 1.243 | 1.043        | 1.482  |
| Child-Pugh class         |              |       |              |        |
| A                        | 0.001        |       |              |        |
| B                        | 0.001        | 2.144 | 1.393        | 3.300  |
| C                        | 0.084        | 2.375 | 0.891        | 6.332  |
| Tumor stage <sup>1</sup> |              |       |              |        |
| I                        | 0.000        |       |              |        |
| II                       | 0.554        | 1.216 | 0.637        | 2.320  |
| III                      | 0.180        | 1.554 | 0.816        | 2.960  |
| IV                       | 0.000        | 3.879 | 1.871        | 8.045  |
| Therapy category         |              |       |              |        |
| Loco-regional            | 0.000        |       |              |        |
| IVR                      | 0.000        | 2.755 | 1.816        | 4.179  |
| Chemotherapy             | 0.000        | 3.365 | 1.884        | 6.010  |
| Others                   | 0.246        | 3.359 | 0.435        | 25.971 |
| Age group                |              |       |              |        |
| 49 years/younger         | 0.240        |       |              |        |
| 50s                      | 0.242        | 0.598 | 0.253        | 1.415  |
| 60s                      | 0.107        | 0.496 | 0.211        | 1.164  |
| 70s                      | 0.047        | 0.436 | 0.192        | 0.990  |
| 80 years/older           | 0.041        | 0.348 | 0.126        | 0.958  |

<sup>1</sup>General rules for the clinical and pathological study of primary liver cancer from Liver Cancer Study Group of Japan; HR: Hazard ratio; AFP:  $\alpha$ -fetoprotein; L3: Percentage of fucosylated fraction in AFP; DCP: Des- $\gamma$ -carboxy prothrombin; Loco-regional: Therapies including resection, radiofrequency ablation, microwave coagulation and percutaneous ethanol injection; IVR: Interventional radiology including transcatheter arterial chemoembolization, and transarterial oily chemoembolization; Chemotherapy: Therapies including hepatic arterial infusion chemotherapy, systemic chemotherapy and molecular targeting therapy; Others: Other therapies including stereotactic body radiation, proton beam and liver transplantation; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen.

were compared among three groups: a younger group of -49 and 50s, a middle-aged group consisting of 60s and 70s, and an elderly group of 80+. As shown in the upper panel of Figure 3B, the functional hepatic reserve, as assessed by the Child-Pugh class, did not differ among the three groups ( $P = 0.34$ ), while the TNM stage tended to be more advanced in the younger group (Figure 3B middle panel,  $P = 0.060$ ). Advanced disease was significantly more frequent in the younger group as compared to the middle-aged group ( $P = 0.010$ ), whereas there was no difference between the middle-aged and elderly groups ( $P = 0.75$ ). A similar trend was observed for HBsAg positivity ( $P < 0.0001$ ). In the younger group, HBsAg was positive in 45.7% of patients, while it was positive in only 14.8% and 6.7% of patients in the middle-aged and elderly groups, respectively, which led to a significant difference between the younger and middle-aged groups ( $P < 0.0001$ ), but no significant difference between the middle-aged and elderly groups ( $P = 0.23$ ).

### Case

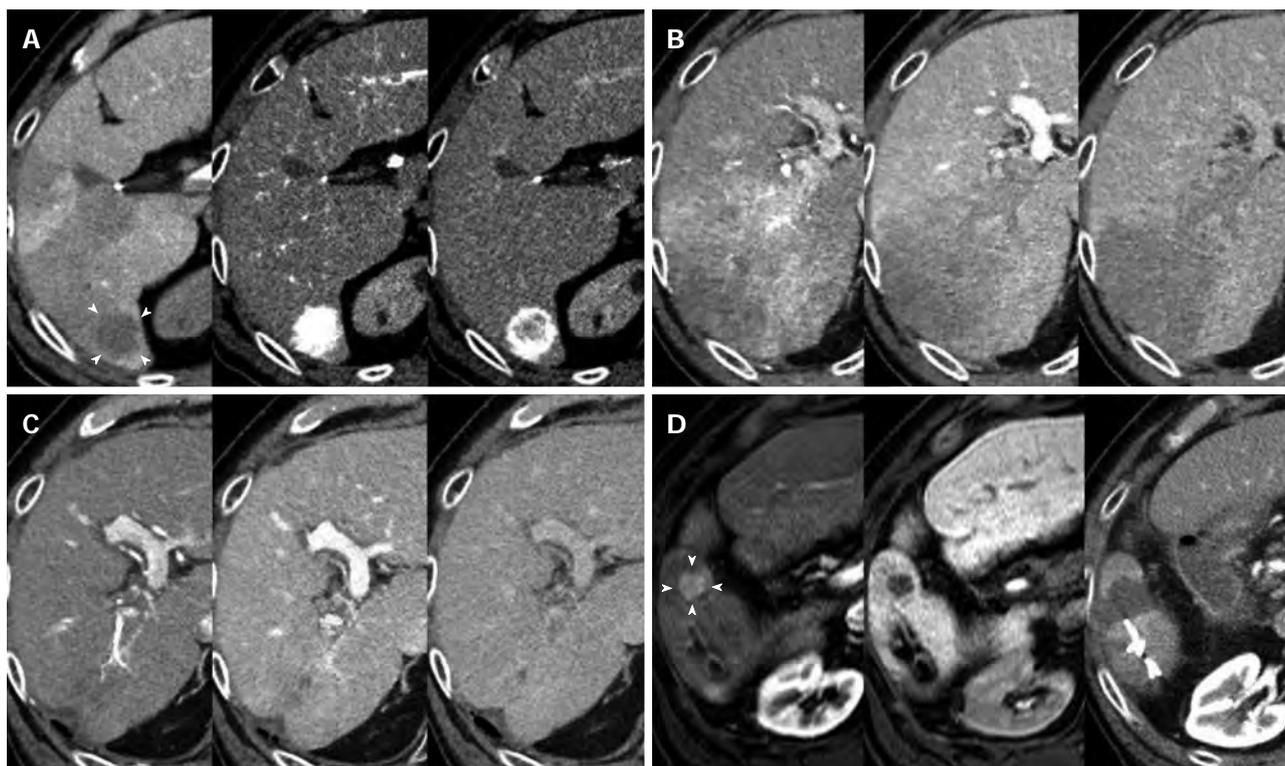
This case is an example of an 85-year-old Japanese male



**Figure 3** Differences in survival and background characteristics by age groups. A: In a cohort of 330 patients for whom life expectancy (LE) data are available, the survival of five different age groups was evaluated on the basis of absolute time (upper panel) or percent LE (lower panel). The solid black and dotted lines are the survival curves of the 80 years of age or older and 70-79 years of age groups, respectively. The other lines are 60-69 years of age, 50-59 years of age and 49 years of age or younger groups, indicated in colors ranging from dark to pale. The oldest group showed the worst survival in days but the best in percent LE; significantly better than that of the youngest group ( $P = 0.041$ ); B: The distributions of Child-Pugh class (upper), tumor stage (middle), and hepatitis B surface antigen (HBsAg) positivity (lower) among three age groups: 59 years of age or younger, 60-79 years of age, and 80 years of age or older. For the hepatic reserve, the white, grey and black columns indicate Child-Pugh A, B and C classes, respectively, whereas tumor stages from I to IV are represented in order from white to black. The black column reveals that HBsAg was positive in the lower graph. There was no significant difference in terms of functional hepatic reserve among the three groups, although anatomical tumor extent and frequency of positive reaction for HBsAg were significantly higher in the youngest group as compared with the middle-aged group ( $P = 0.010$  and  $P < 0.0001$ , respectively). The dotted horizontal lines indicate a position of 50% survival.

who was admitted to our hospital on April 2009 for the treatment of an HCC that was approximately 20 mm in diameter and located in segment 6 (Figure 4A). He suffered from HCV infection and diabetes mellitus, for which he had used insulin by injection for more than a decade, and he had one prior hepatic resection for HCC. His life expectancy upon admission was 6.27 years. RFA was completed after TACE through the right intercostal space under ultrasound guidance. Twenty-five months after the ablation, however, a follow-up CT revealed that the HCC had spread to wide areas of segments 6 and 7 with portal vein tumor thrombus that extended up to the right main trunk (Figure 4B). Relying on the Child A-class preserved hepatic functional reserve, HAIC was our recommendation at this stage, delivered through a

catheter that was implemented and connected to a port under the skin. After receiving written informed consent from the patient, 125 mg of 5-fluorouracil and 5 mg of cis-diamminedichloroplatinum were infused over 23 h and 60 min, respectively, through the common hepatic artery, and repeated for 5 consecutive days. After 2 d of no drug administration, the same schedule was performed for the following 2 wk. The 5-d HAIC was then repeated every 2-3 mo. The tumor and portal vein tumor thrombi gradually disappeared to reveal an enormous tumor reduction by July 2012 (Figure 4C). A new lesion appeared in segment 5 and gradually enlarged to 15 mm in diameter; therefore, RFA was performed again on August 2012 (Figure 4D). In September 2012, the patient turned 89 years old and has survived for 42 mo (55.8% of LE)



**Figure 4** Representative follow-up images of successfully treated hepatocellular carcinoma in an elderly patient. A: A classical hepatocellular carcinoma (HCC) was detected in segment 6 of the liver on April 12, 2009 as demonstrated by (1) a lower intensity up on computed tomography (CT) during arterial portography, indicated by arrowheads (left); (2) vigorous staining during the arterial phase of the CT during hepatic arteriography (middle); and (3) washout with a corona-like peripheral enhancement during the equilibrium phase of the CT during hepatic arteriography (right); B: A dynamic CT 25 mo after the initial radiofrequency ablation revealing recurrent HCCs, which had spread to large areas of segments 6 and 7 and extended to the main trunk of the right portal vein. The images were obtained during arterial, portal and equilibrium phases of the dynamic CT study, shown in order from left to right; C: After hepatic arterial infusion chemotherapy *via* a catheter using 5-fluorouracil and cis-diamminedichloroplatinum for 15 mo, an enormous tumor reduction, including the portal vein tumor thrombus, was achieved. The images were obtained during arterial, portal, and equilibrium phases of the dynamic CT study, shown in order from left to right; D: A new 10-mm lesion appeared in segment 6 during hepatic arterial infusion chemotherapy treatment, and a second radiofrequency ablation (RFA) was applied 41 mo after the initial RFA. Magnetic resonance imaging study using a contrast medium of gadolinium ethoxybenzyl diethylene-triamine-pentaacetic-acid showing the arterial supply (left, arrowheads) and a defect in the hepatobiliary phase (middle) of the study. An arterial phase image of the dynamic CT (right) obtained one day after RFA revealing that the ablated area of lower intensity included the target.

since the initial RFA. He is in good shape with a PS of 0 and without severe complaints.

## DISCUSSION

In this study, we introduced a new indicator, the %LE, to evaluate whether active treatments for HCC are beneficial for patients over 80 years of age. Considering that morbidity, mortality and other health-related outcomes are generally compared in populations after adjusting for age structures, the age should also be normalized when survival is compared among different age groups. Based on the assumption of a community downscaling to an individual, the life expectancy adjustment among individuals should correspond to age adjustment among communities. As shown in Figure 1B and C, there is a large difference in survival curves between the Kaplan-Meier fractions and the Cox hazards after compensation using 10 explanatory factors. The worst survival of the -49 group in the Kaplan-Meier analysis was the best survival in the Cox regression. In contrast, when %LE was used, the order of survival was consistent between the Kaplan-

Meier and Cox regression analyses (Figures 2B and 3A lower panel). The explanatory factors for survival time and %LE in the Cox regression analyses were consistent, and the analyses using survival time or %LE revealed similar survival curves among the different therapeutic approaches (Figure 2C); therefore, it is suggested that the factor that explains the large difference between the Kaplan-Meier and Cox regression analyses using survival time is age. Therefore, it is assumed that the impact of age on survival irrespective of liver pathophysiology can be normalized using %LE instead of survival time. The worst survival of the -49 group in the %LE analysis concurs with the common clinical experience in Japan of higher HBsAg positivity in the younger generation of HCC, leading to the onset of HCC at advanced stages<sup>[10]</sup>. Taken together, these data suggest that the %LE can be a useful factor to compare survival benefit, especially between cohorts of patients with large differences in age.

It is controversial whether active intervention is beneficial to elderly patients, and inconsistent recommendations have been reported for various diseases, including HCC. Studies that demonstrate an adverse influence of

aging on survival in HCC were all reported more than 20 years ago<sup>[11-14]</sup>, whereas recent reports emphasized the benefit of active treatments in the elderly<sup>[15-18]</sup>, suggesting improvements of the medical and social environments over the past decades. Unfortunately, however, all recent reports discussed survival benefits in the elderly principally based on Kaplan-Meier survival fractions. As shown in Figure 1B, a simple comparison of survival time does not reveal survival differences among various age groups. However, one should not conclude that age is not a significant factor for survival on the basis of Kaplan-Meier analysis, because survival varies widely among the different age groups in the Cox regression analysis (Figure 1C). Although our conclusion is consistent with previous reports that a therapeutic scheme should not be changed because of a patient's age, this study explains the rationale of active intervention for HCC in the elderly more theoretically. However, our conclusions are based on the study from a single institution and a limited number of patients. To establish the best approach for the elderly patients, a multicenter study should be conducted using a larger cohort.

Generally, our institution applies the same process to decide a treatment strategy irrespective of the patient's age, and this approach led to the best survival in the elderly in terms of %LE, as shown in this study. We also presented the case of a patient over 80 years of age, for whom active interventions such as RFA, TACE or HAIC were safely applied and provided survival benefit. Although it is important to thoroughly assess the risks and to obtain written informed consent, because aging is associated with a progressive deterioration of organ function that reduces the functional reserve to recover from stress/complications<sup>[19]</sup>, our data suggested that age itself should not be a reason to change a treatment strategy. The number of elderly patients in one institution is generally limited, as is the case in this study; therefore, it is difficult to clarify risk determinants that are specific for elderly patients. Future trials should include efforts to enroll the elderly and to clarify factors specific for them that determine outcomes, not only for physical function, but also for quality of life and independence. Facing the world's highest proportion of elderly people, Japanese society should play a primary role in the application of guidelines for the elderly subset by achieving maximal benefits while minimizing risks.

## COMMENTS

### Background

Increasing life expectancy and a falling birth rate have led to population aging worldwide, especially in developed countries. As a result, the proportion of elderly patients is steadily rising in many diseases, including hepatocellular carcinoma (HCC). There is much controversy concerning medical interventions for elderly patients; therefore, it is important to clarify a treatment strategy specific for the elderly in terms of both survival benefit and medical resources.

### Research frontiers

Aging itself has a significant impact on survival days; therefore, a simple comparison of survival days among different age groups may not be suitable for evaluating survival benefit with regard to aging. Another approach is required to compensate for the effect of aging on survival.

## Innovations and breakthroughs

To avoid the confounding effect between survival days and aging, the research team led by Takeshi Suda from Division of Gastroenterology and Hepatology, Niigata University introduced percent life expectancy instead of survival days as a novel indicator for the evaluation of survival benefit. Using percent life expectancy, the authors finally concluded that a therapeutic approach for HCC should not be restricted because of patient age.

## Applications

Facing progressive population aging, medical society should play a primary role in the application of guidelines for the elderly subset by achieving maximal benefits while minimizing risks. In future trials with larger cohorts, percent life expectancy should play an important role in evaluating survival benefits associated with aging.

## Terminology

Computed tomography (CT) during hepatic arteriography and CT during arterial portography are one of the best ways to evaluate perfusion characteristics of a nodular lesion in the liver, and provide the diagnostic rationale for HCC. Radiofrequency ablation therapy and percutaneous ethanol injection are puncture-based locoregional approaches for HCC. Both are usually applied percutaneously under ultrasound guidance, and utilize radiofrequency waves and anhydrous ethanol, respectively, to degenerate cancer cells. Transcatheter arterial chemoembolization and transarterial oily chemoembolization are interventional radiology including the embolization of hepatic arteries feeding HCC. These techniques employ gelatin and oil for embolization, respectively, and gelatin achieves a prolonged obstruction. The tumor markers of  $\alpha$ -fetoprotein, L3, and des- $\gamma$ -carboxy prothrombin are commonly measured as useful independent indicators for the biological malignant potential of HCC.

## Peer review

Facing the world highest elderly ratio in Japan, it is necessary to clarify whether a medical decision process that is applied for the general population provides similar benefits for elderly patients in terms of survival. For this purpose, the authors introduced a new indicator, percent life expectancy, to normalize aging effects, and evaluated the survival benefit among different age groups. Many developed countries, such as United States and China, are also facing population aging; therefore, it is important to clarify risk determinants specific for the elderly in terms of individual aspects and medical economy. This manuscript will have a significant impact and the hepatology community will recognize the importance of the gerontological aspect.

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P- Reviewer Hwang SG S- Editor Wen LL  
L- Editor Stewart GJ E- Editor Li JY



## Anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis for low rectal cancer: Experience from a Chinese cohort

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Supported by Grants from the National Natural Science Foundation of China, No. 81041025 and 81000189

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Received: May 14, 2013 Revised: May 24, 2013

Accepted: June 1, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the safety and efficacy of anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis (TCMA) for low rectal cancer.

**METHODS:** From August 1993 to October 2012, 420 patients including 253 males and 167 females with low rectal cancer underwent transabdominal and transanal anterior resection, followed by TCMA. The distance between the anus and inferior margin of the tumor ranged from 5 to 7 cm, and was 5 cm in 6 patients, 6 cm in 127, and 7 cm in 287 patients. Tumor-node-metastasis staging showed that 136 patients had stage I, 252 had stage II and 32 had stage III. Fifty-six patients with T3 or over received preoperative neoadjuvant chemoradiotherapy.

**RESULTS:** The postoperative follow-up rate was 91.9% (386/420) with a median time of 6.4 years. All 420 patients underwent radical resection. No postoperative

death occurred. Postoperative complications included anastomotic leakage in 13 (3.1%) patients and anastomotic stenosis in 7 (1.6%). The local recurrence rate after surgery was 6.2%, the hepatic metastasis rate was 13.2% and the pulmonary metastasis rate was 2.3%. The 5-year survival rate was 74.0% and the disease-free survival rate was 71.0%. Kirwan classification showed that continence was good in 94.4% of patients with stage I when scored 12 mo after resection.

**CONCLUSION:** TCMA for patients with low rectal cancer leads to better quality of life and satisfactory defecation function, and lowers anastomotic leakage occurrence, and might be one of the safe operative procedures in anus-preserving rectectomy.

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**Key words:** Low rectal cancer; Rectectomy; Telescopic colorectal mucosal anastomosis; Reconstruction; Abdominoperineal resection

**Core tip:** Li *et al* developed the telescopic colorectal mucosal anastomosis technique based on the experiences and lessons from several sphincter-preserving operations under preconditions of low rectal resection, which improved the anastomotic stoma and alleviated tension. With this modified technique used over the past 20 years, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence.

Li SY, Chen G, Bai X, Zuo FY, Chen G, Du JF, Wei XJ, Cui W. Anus-preserving rectectomy *via* telescopic colorectal mucosal anastomosis for low rectal cancer: Experience from a Chinese cohort. *World J Gastroenterol* 2013; 19(24): 3841-3846 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3841.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3841>

## INTRODUCTION

Abdominoperineal resection (APR) is thought to be the gold standard in the treatment of low rectal cancer less than 5 cm from the anal verge<sup>[1,2]</sup>. However, over the last 50 years, surgeons realized that permanent colostomy led to inconvenience in terms of the social life of patients and mental health issues<sup>[3,4]</sup>. Dixon developed the operative procedure of anterior resection, in which end-to-end anastomosis of the sigmoid colon and rectum was performed after radical resection of low rectal cancer. However, it was difficult to perform this operation in overweight patients with a narrow pelvis<sup>[5]</sup>. Parks *et al*<sup>[6]</sup> proposed a colon-anal anastomosis, which was modified by Bacon's operation with the preservation of both internal and external anal sphincters. Due to satisfactory clinical results, this type of operation was popular in European countries, however, a temporary diverting stoma was routinely required to ensure healing of the anastomotic stoma<sup>[7]</sup>. Heald *et al*<sup>[8]</sup> reported total mesorectal excision (TME) for the first time, involving resection of the mesorectum more than 5 cm from the distal margins of the tumor, which reduced the local recurrence rate, and improved the survival rate of patients with rectal cancer<sup>[9,10]</sup>. However, potential ischemia of the distal bowel during such surgery could lead to an increased rate of anastomotic leakage.

Li *et al*<sup>[11]</sup> developed the telescopic colorectal mucosal anastomosis (TCMA) based on the experiences and lessons from several sphincter-preserving operations under preconditions of low rectal resection, which improved the anastomotic stoma and alleviated tension. With this modified technique, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence<sup>[11-13]</sup>. In this study, we summarized the influential factors for high-incidence anastomotic leakage after sphincter-preserving surgery in radical rectal resection (8.1%-18.0%), including anastomotic skills, blood supply and tension of the anastomotic stoma<sup>[6,8,14-16]</sup>.

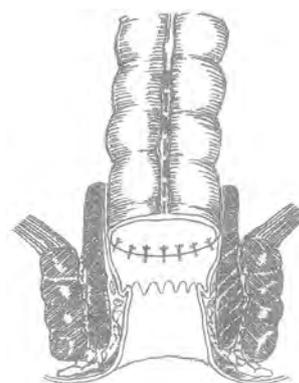
## MATERIALS AND METHODS

### Patients

From August 1993 to August 2012, we treated 1510 patients with rectal cancer surgically at the Department of General Surgery, General Hospital of Beijing Military Command, China. Of these patients, 576 (38.1%) underwent Miles' procedure and 420 (27.8%) underwent TCMA for rectal carcinoma less than 7 cm from the anal verge. There were 253 male and 167 female patients, with an average age of 55.7 years (range: 21-91 years). The distance between the lower margin of the tumor and the anal verge varied from 5 to 7 cm, and was 7 cm in 287 patients, 6 cm in 127, and 5 cm in 6 patients. The distance was measured with a rigid sigmoidoscope. All 420 patients were examined by rectal touch, colonoscopy, barium enema, magnetic resonance imaging (MRI) and endorectal ultrasonography. Primary malignant rectal neoplasms

**Table 1 Comparison of pathological stages of 56 patients who underwent preoperative neoadjuvant therapy (T stage)**

| Preoperative stage | Cases | Postoperative stage (n)          |
|--------------------|-------|----------------------------------|
| T3                 | 50    | T0 (4), T1 (24), T2 (22)         |
| T4                 | 6     | T0 (0), T1 (0), T2 (4), T3 (2)   |
| Total              | 56    | T0 (4), T1 (24), T2 (26), T3 (2) |



**Figure 1 Schematic layout of the anus-preserving procedure *via* trans-abdominal radical anterior resection and trans-anal telescopic colorectal mucosal anastomosis.**

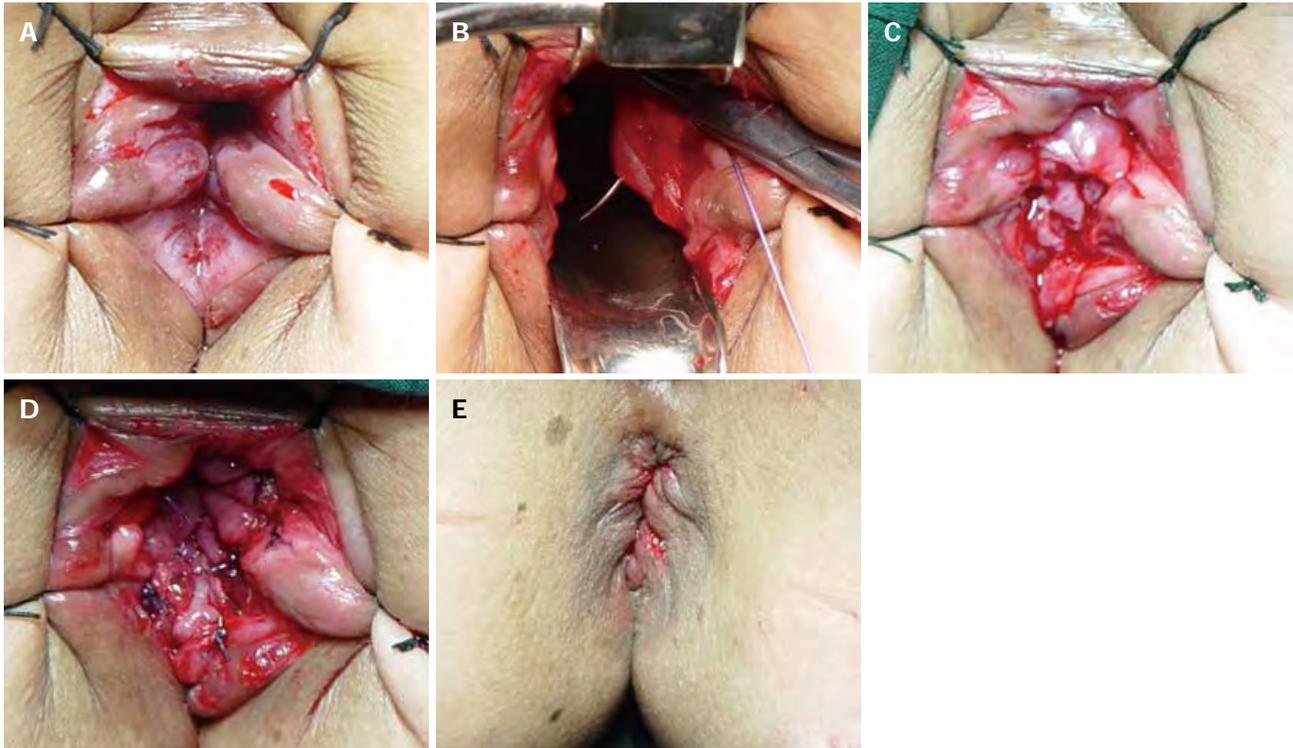
were confirmed by biopsy, and preoperative tumor-node-metastasis (TNM) staging of the patients was also carried out.

Preoperative TNM staging in the 420 patients was as follows: stage I,  $n = 136$ ; stage II,  $n = 252$  and stage III,  $n = 32$ . Of these patients, 56 above T3 received neoadjuvant chemoradiotherapy. They were given capecitabine 1500 mg twice a day orally for 1 mo. Radiotherapy was added during the chemotherapy cycle (45-50 Gy in 25-28 fractions to the pelvis). The tumor staging (assessed by MRI) after 6-8 wk of neoadjuvant therapy was as follows: T0,  $n = 4$ ; T1,  $n = 24$ ; T2,  $n = 26$ ; T3,  $n = 2$  (Table 1).

### Surgical procedures

Surgical procedures were performed according to the TME principles and the methods previously described by Li *et al*<sup>[11]</sup>. The schematic layout of the anus-preserving procedure *via* TCMA is shown in Figure 1. Under general anesthesia and continuous epidural anesthesia, the patient was placed in the lithotomy position. The procedures routinely involved high ligation of the inferior mesenteric artery and dissection to the levator ani under direct vision. The rectum was mobilized to the pelvic floor as low as possible to facilitate the perianal approach. If the lower edge of the tumor was reached, a clamp was applied below the tumor to close the rectum when possible.

Following the abdominal approach, sufficient relaxation of the anal sphincter was achieved by finger expansion under continuous epidural anesthesia. Wide exposure of the operative field above the dentate line was achieved using the "5-stitches-suspension" method (Figure 2A). To prevent bleeding, 2-3 mL of saline adrenaline solution (1:10000) was injected into the anal canal 1.0 cm



**Figure 2** Surgical procedures. A: The "5-stitches-suspension" method; B: Dissection was performed by mobilizing the rectum through the mucosal plane to 2-4 cm above the dentate line; C: Telescopic colorectal mucosal anastomosis (TCMA) of the sero-muscular layer and muscular sheath was performed at 2.0 cm above the dentate line; D: Four interrupted absorbable sutures in the distal end of the colon and the residual of the rectal mucosa were also placed at the 12, 3, 6 and 9 o'clock positions, followed by 4-8 additional sutures; E: After TCMA, the 5-suspension-stitches were removed, and the anastomotic stoma was repositioned.

above the dentate line, which resulted in swelling of the mucosa. A circumferential incision of the mucosa was made at 1.5-2.0 cm above the dentate line. Dissection was performed by mobilizing the rectum through the mucosal plane to approximately 2-4 cm (Figure 2B), and then the distal margin of the rectum was clamped and cut, with preservation of the entire muscular sheath of the rectum. Later, the distal end of the colon was pulled through the anus, and TCMA of the sero-muscular layer and muscular sheath was performed at 2.0 cm above the dentate line (Figure 2C). Four interrupted absorbable sutures were placed at the 12, 3, 6 and 9 o'clock positions in the lithotomy position, respectively, for fixation and relaxation. Similarly, 4 interrupted absorbable sutures in the distal end of the colon and the residual rectal mucosa were also placed at the 12, 3, 6 and 9 o'clock positions, followed by 4-8 additional sutures (Figure 2D). To remove the dermal sutures and reposition the anastomotic stoma back in the anal canal (Figure 2E), a pelvic drainage tube was placed before closure of the abdominal wall, and was removed 4-5 d after surgery.

#### **Postoperative adjuvant radiation and chemotherapy**

Patients with greater than T2 stage received 7-12 cycles of postoperative systemic chemotherapy with the mFOLF-FOX4 protocol (oxaliplatin, 5-fluorouracil and calcium folinate). Eighty-eight patients with T4 stage and 23 patients with positive circumferential margins after resection were given postoperative pelvis radiotherapy at a total dose of 10-20 Gy before adjuvant chemotherapy.

#### **Complications and follow-up**

Patients were seen within one month following resection for monitoring postoperative complications, such as bleeding, pelvic abscess and anastomotic leakage. Follow-up was performed every 3 mo for 2 years, every 6 mo for 3 years and then every 1 year thereafter. All patients underwent digital examination, laboratory studies (stool analysis including occult blood, serum carcinoembryonic antigen levels) and imaging examination (abdominal ultrasound, chest X-ray, and pelvic computed tomography/MRI). Colonoscopy was performed every 6 mo for 5 years after surgery. Anastomotic stenosis was confirmed by colonoscopy under direct vision within 1 year after operation. Local recurrence was defined as the first clinical, radiologic and/or pathologic evidence of tumor of the same histologic type within the pelvis 2-3 years after surgery. Distant recurrence was defined as clinical, radiologic, and/or pathologic evidence of systemic disease outside the pelvis, at sites including liver and lungs 5 years after surgery. Death of patients was recognized as the end of follow-up. All the clinical data were collected from the follow-up records at different time-points.

#### **Anal function evaluation**

A questionnaire on anal function was completed after surgery according to Kirwan staging criteria<sup>[17]</sup>.

#### **Statistical analysis**

Overall survival and disease-free survival were calculated by the Kaplan-Meier method. Statistical analysis was per-

**Table 2 Patient characteristics**

| Characteristics                        | Data                                |
|--|-------------------------------------|
| Patients (n)                           | 420                                 |
| Age (yr)                               | 55.7 (range: 21.0-91.0)             |
| Gender                                 |                                     |
| Male                                   | 253                                 |
| Female                                 | 167                                 |
| Distance of tumor from anal verge (cm) |                                     |
| 7                                      | 287                                 |
| 6                                      | 127                                 |
| 5                                      | 6                                   |
| Preoperative tumor stage               |                                     |
| I                                      | 136                                 |
| II                                     | 252                                 |
| III                                    | 32                                  |
| Postoperative tumor stage              |                                     |
| I                                      | 142                                 |
| II                                     | 250 (II a: 177, II b: 61, II c: 12) |
| III                                    | 28 (III a: 13, III b: 9, III c: 6)  |
| Differentiation of tumors              |                                     |
| Well differentiated                    | 148                                 |
| Moderately differentiated              | 249                                 |
| Poorly differentiated                  | 16                                  |
| Adenomatous canceration                | 7                                   |
| Temporary diverting stoma              |                                     |
| Yes                                    | 0                                   |
| No                                     | 420                                 |
| Surgical time (min)                    | 130 (range: 110-190)                |
| Intraoperative blood loss (mL)         | 360 (range: 150-1200)               |
| Hospital stay (d)                      | 13 (range: 7-31)                    |

formed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, United States).

## RESULTS

The postoperative follow-up rate in this series was 91.9% (386/420), with a median time of 6.4 years. All 420 patients underwent radical resection. The distance between the distal margins of the tumor ranged from 2 to 5 cm (mean 3.2 cm). Negative distal margins were confirmed pathologically in all 420 cases, while positive circumferential margins were observed in 23 cases (5.4%). A pathological diagnosis was made in 148 patients with well differentiated adenocarcinoma, in 249 patients with moderately differentiated adenocarcinoma, in 16 patients with poorly differentiated adenocarcinoma, and in 7 patients with adenomatous canceration.

According to the TNM staging principles of the 2010 National Comprehensive Cancer Network guidelines, postoperative pathological staging showed: 142 patients with stage I, 250 patients with stage II (IIa: 177 patients, II b: 61 patients, and II c: 12 patients) and 28 patients with stage III (IIIa: 13 patients, III b: 9 patients, and III c: 6 patients) (Table 2).

### Mortality and morbidity

No postoperative death occurred in this series. Anastomotic leakage occurred in 13 patients (3.1%), of whom 7 received conservative therapy (total parenteral nutrition and continuous drainage), and 5 underwent transverse colostomy (stoma apothesis after 3 mo). Anastomotic steno-

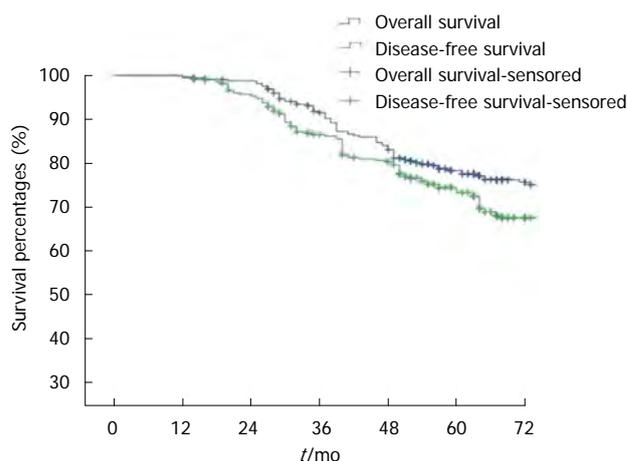


Figure 3 Kaplan-Meier method were used to calculate the 5-year survival rate and the 5-year disease-free survival rate.

sis occurred in 7 patients (1.6%), these patients recovered with continuous expansion of the anus for 1-3 mo. The postoperative local recurrence rate in the series was 6.2% (26 cases), recurrence was seen 2-3 years after surgery. The rate of metastasis to the liver and the lung was 13.2% (51 cases) and 2.3% (9 cases), respectively. The postoperative 5-year survival rate was 74% and the 5-year disease-free survival rate was 71% (Figure 3).

### Functional results

Enteral nutrition was administered to patients during the early postoperative period. These patients had poor continence, with approximately 6-9 bowel movements per day, which could be controlled to 3-6 times per day following oral intake of compound diphenoxylate 2 pills three times per day. Two to 4 mo after surgery, patients had better continence and recovered anal function 12 mo after surgery. Kirwan staging<sup>[17]</sup> was stage I in 369 patients (94.4%), stage II in 20 patients (5.1%), and stage III in 2 patients (0.5%).

## DISCUSSION

### Assessment of therapeutic results

Despite the improved clinical results of anterior resection *via* all types of anus-preserving procedures for treating low rectal cancer, several issues remain controversial such as the incidence of anastomotic leakage, the local recurrence rate and anal function outcome<sup>[11,18,19]</sup>. In 1993, Li *et al* developed the telescopic anastomosis technique for treating low rectal cancer, focusing on relaxation sutures, while strengthening the anastomotic stoma. The use of this method with wide exposure and a refined surgical technique effectively controlled anastomotic leakage. In our series, the rate of anastomotic leakage was 3.1%, which was significantly lower than the rate of 8.0%-18.0% reported elsewhere. Clinical data indicated that TCMA was a reliable, safe and superior surgical procedure<sup>[6,8,14]</sup>.

The mechanisms of defecation involve both sphincter contraction reflection<sup>[20]</sup> and complete physiological reflection of the rectal mucosa<sup>[21]</sup>. In anus-preserving

procedures, low anastomosis may impair the regions of these reflections. Thus, the intraoperative prevention of impairment in such regions could contribute to better anal function after surgery. In TCMA, colorectal anastomosis should be performed on the rectum plane 1.5-2.0 cm above the dentate line, with the preservation of anal sphincter function and enough residual rectum to protect complete physiological nervous reflection of defecation, which is completely different from that of anus-preserving procedures with resection of the internal sphincter<sup>[22-24]</sup>. Obviously, without impairment of the internal sphincter and normal anal construction, patients could have a recovery of 97.6%-99.5% Kirwan stages I and II defecation function 6-12 mo after operation, with improved quality of life.

Long-term clinical outcome depends on radical resection of the tumor and sufficient dissection of lymph nodes according to the TME principles<sup>[25]</sup>, which help to obtain both negative distal and circumferential margins for lowering local recurrence after surgery<sup>[26,27]</sup>. In our series, patients with T3-4 staging received preoperative neoadjuvant radiochemotherapy to downgrade tumor staging and to facilitate radical resection and anus-preserving procedures. Patients with stage II or above underwent postoperative systemic chemotherapy, and those with T4 staging or positive circumferential margins confirmed by pathological examination received postoperative pelvic radiotherapy. The data showed that the patients who received comprehensive treatment exhibited a local recurrence rate of 6.2% and a 5-year survival rate of 74%, which were not significantly different from those of Miles. With 20 years of use in clinical practice, TCMA has been proved to be a safe and feasible treatment for low rectal cancer and one of the effective standard anus-preserving procedures.

### Surgical skills and notes

Wide exposure of the operative field by the “5-stitches-suspension” method (Figure 3) and adequate muscle relaxation during anesthesia play a dominant role in TCMA<sup>[11]</sup>. Perianal anastomosis could be facilitated after satisfactory relaxation of the anal sphincter, and the injury of perianal architectures could be avoided and anal function restored after surgery<sup>[11,13]</sup>.

Perianal dissection through the rectal mucosa plane requires prevention of bleeding by the circumferential injection of saline adrenaline solution (1:10000) 1.0 cm above the dentate line and adequate exposure of the internal sphincteric plane to achieve complete excision of the distal mucosa, while avoiding injury of the internal sphincter<sup>[11,13]</sup>. In brief, a circumferential incision of the mucosa and internal anal sphincter is made at 1.5-2.0 cm above the dentate line. The dissection continues upward between the mucosa and the superficial layer of the internal sphincter for the resection of 2-4 cm of distal rectal mucosa (Figure 2B).

Relaxation and strengthening prevent anastomotic leakage, using a 4-stitches relaxation suture of the colonic sero-muscular layer and residual rectal muscular sheath<sup>[11,13]</sup> (Figure 2C). More importantly, penetrating the whole layer

of the bowel wall and/or the posterior wall of the vagina is avoided to prevent the occurrence of intestinal and vaginal fistula.

TCMA involves telescopic anastomosis between the whole layer of the colon and residual mucous and the submucous layer of the rectum. The distal margin of the colon should be modified by the resection of adipose tissue with well preserved blood supply to facilitate healing of the anastomotic stoma. Absorbable interrupted sutures are placed at the 6 and 12 o'clock positions, then at the 3 and 9 o'clock positions, followed by the addition of 4-8 sutures to avoid postoperative stenosis (Figure 2D).

After TCMA, the 5-suspension-stitches are removed, and the anastomotic stoma is repositioned. Vaseline gauze can be used for support and is removed 48-72 h after surgery (Figure 2E).

Anus-preserving operations should be performed under the conditions mentioned above in addition to sufficient mobilization of the rectum.

In conclusion, anus-preserving procedures *via* trans-abdominal radical anterior resection and trans-anal TCMA to treat patients with low rectal cancer can achieve satisfactory recovery of anal function with a decreased incidence of anastomotic leakage and a moderate local recurrence rate. In comparison with APR, this modified treatment can improve patient quality of life. TCMA might be one of the standard surgical options in treating low rectal cancer.

## COMMENTS

### Background

For many years, abdominoperineal resection (APR) was the treatment of choice for most patients with rectal cancer. Recent advances in surgical technique and other treatment modalities have led to a marked increase in the rate of sphincter-sparing operations, with a concomitant decrease in APR as permanent colostomy leads to inconvenience in terms of the social life of patients and mental health issues. In 1993, Li *et al* developed the telescopic colorectal mucosal anastomosis (TCMA) for treating low rectal cancer, focusing on relaxation sutures, while strengthening the anastomotic stoma. This modified technique effectively controlled anastomotic leakage.

### Research frontiers

With the modified surgical techniques used over the past 20 years in this study, the incidence of anastomotic leakage was decreased significantly and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence. Anus-preserving procedures *via* trans-abdominal radical anterior resection and trans-anal TCMA in treating patients with low rectal cancer could achieve a satisfactory recovery of anal function with a decreased incidence of anastomotic leakage and a moderate local recurrence rate. In comparison with APR, TCMA can greatly improve the quality of life of the patients, and could be one of the standard surgical options in treating low rectal cancer.

### Innovations and breakthroughs

Despite the improved clinical results of anterior resection in treating low rectal cancer by all kinds of anus-preserving procedures, several issues remain controversial such as the incidence of anastomotic leakage, the local recurrence rate and anal functional outcome. The telescopic anastomosis technique was developed to treat low rectal cancer while strengthening the anastomotic stoma. Using this method with wide exposure and refined surgical technique, the anastomotic leakage could be controlled effectively. In this series, the rate of anastomotic leakage was significantly lower than the reported rate elsewhere. Clinical data indicated that TCMA is a reliable, safe and superior surgical procedure for low rectal cancer.

### Applications

TCMA could be one of the standard surgical options in treating low rectal cancer. The transabdominal and transanal anterior resection for low rectal cancer *via* colorectal mucosal anastomosis leads to a better life quality with satisfac-

tory defecation function, while lowering the occurrence of anastomotic leakage. Telescopic anastomosis is one of the safe operative procedures in anus-preserving rectectomy for patients with low rectal cancer.

### Terminology

APR is considered to be a gold standard for the treatment of low rectal cancer less than 5 cm from the anal verge. It completely removes the distal colon, rectum, and anal sphincter complex using both anterior abdominal and perineal incisions, resulting in a permanent colostomy. The technique of TCMA was developed by Li *et al* for anus-preserving rectectomy in patients with low rectal cancer, which improved the anastomotic stoma and alleviated tension. With this modified technique, the incidence of anastomotic leakage was significantly decreased and the long-term outcome was satisfactory with good anal function and a lower rate of incontinence.

### Peer review

This study is innovative and is of interest for surgical community. Methods used are innovative and advanced. Detailed description is provided to allow other investigators to reproduce or validate authors' findings. Results provide sufficient evidence to draw firm scientific conclusions. Sample size and statistical data, especially graphic data, are adequate for a clinical study. However, some revisions should be made on the presentation and evaluation of the results.

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P- Reviewers Barauskas G, Kim YJ, Kopljar M  
S- Editor Gou SX L- Editor A E- Editor Xiong L



## Mitochondrial ATP 6 and 8 polymorphisms in irritable bowel syndrome with diarrhea

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Received: February 6, 2013 Revised: March 30, 2013

Accepted: April 9, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate mitochondrial ATP 6 and 8 polymorphisms in the colon and ileum of patients with irritable bowel syndrome with diarrhea (IBS-D).

**METHODS:** Twenty-eight patients fulfilling the Rome III criteria for IBS-D and 28 healthy subjects were investigated. All study participants underwent screening colonoscopy and mucosal biopsies were obtained from the colon and/or terminal ileum. Genomic DNA was extracted from specimens based on standard protocols. Mitochondrial ATP (MT-ATP) 6 and 8 genes in speci-

mens were polymerase chain reaction amplified and sequenced. Sequencing data were analyzed *via* Variant Reporter™ Software and compared with the reference sequence from Genbank (accession No. NC\_012920) to indicate possible polymorphisms. The protocol was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT01028898.

**RESULTS:** Twenty-five polymorphic sites of MT-ATP 6 and 8 genes were detected and 12 of them were missense mutations. A median of two polymorphic sites in MT-ATP genes was found in colon specimens of controls while a median of three polymorphic sites was noted in patients with IBS-D (Mann-Whitney test,  $P = 0.012$ ). The variants of the colon and ileum specimens from the same subjects were identical in all but one case. Symptom duration in IBS was not found to be a significant factor associated with the mtDNA polymorphism (Spearman correlation,  $P = 0.592$ ). The mitochondrial DNA change at 8860 was present in all cases of both groups. The frequency of the 8701 polymorphism was found to be the second most frequent; however, no statistical difference was noted between the groups ( $\chi^2$  test,  $P = 0.584$ ).

**CONCLUSION:** Patients with IBS-D have a higher incidence of MT-ATP 6 and 8 polymorphisms than healthy subjects, implying that the mtDNA polymorphism may play a role in IBS-D.

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**Key words:** Irritable bowel syndrome; Diarrhea; Mitochondrial ATP 6 gene; Mitochondrial ATP 8 gene; Polymorphism

**Core tip:** Mitochondrial DNA, the only source of extranuclear genome, was assessed by Camilleri's group in a study of patients with functional gastrointestinal disorders in 2009 which was the first study exploring the possible role of mitochondrial DNA in irritable bowel

syndrome (IBS). Up till now, few research efforts have focused on this topic and there is little knowledge about it. Present study revealed that mitochondrial ATP 6 and 8 genes were more frequently mutated in IBS with diarrhea than in healthy individuals. We believe this preliminary finding could help promote future research on mitochondrial DNA in IBS.

Wang WF, Li X, Guo MZ, Chen JD, Yang YS, Peng LH, Wang YH, Zhang CY, Li HH. Mitochondrial ATP 6 and 8 polymorphisms in irritable bowel syndrome with diarrhea. *World J Gastroenterol* 2013; 19(24): 3847-3853 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3847.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3847>

## INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most prevalent functional gastrointestinal disorders and affects 10%-20% of the population<sup>[1]</sup>. To date, the pathogenesis of IBS remains unclear.

Family<sup>[2,3]</sup> and twin<sup>[4]</sup> studies suggested that genetic changes may predispose individuals to IBS, although reported data are controversial<sup>[5]</sup>. Furthermore, some candidate nuclear genes are associated with IBS (serotonin transporter and receptor genes and inflammatory markers)<sup>[6]</sup>. Recently, mitochondrial DNA (mtDNA), the only source of extranuclear genes, was assessed by Camilleri *et al*<sup>[7]</sup> in a study of patients with functional gastrointestinal disorders. This initial study on the role of haplogroup H, 16519C>T and 3010G>A (located in the D-loop and the 16S rRNA gene) of mitochondrial DNA (mtDNA) indicated that the mitochondrial haplogroup and variations may be associated with gastrointestinal motor and sensory function in gastrointestinal disorders such as IBS. The human mitochondrial genome encodes 13 genes<sup>[8]</sup> for ATP subunits 6 and 8, and other polypeptides of respiratory complexes crucial for ATP production in mitochondria. ATP synthesis requires ATP synthase, of which subunits 6 and 8 are mtDNA encoded. Based upon this knowledge, we hypothesized that mitochondrial ATP (MT-ATP) 6 and 8 may be involved in the pathogenesis of IBS. In this study, we aimed to investigate the mitochondrial ATP (MT-ATP) 6 and 8 polymorphisms in the colon and ileum of IBS with diarrhea (IBS-D).

## MATERIALS AND METHODS

### Subjects

Twenty-eight patients who satisfied Rome III criteria<sup>[1]</sup> for IBS-D and 28 healthy controls were enrolled at the Chinese PLA General Hospital between June 2009 and May 2011. Inclusion criteria for IBS with diarrhea were: (1) male and female individuals aged 18-60 years; (2) recurrent abdominal pain or discomfort associated with a change in stool frequency or form over a 3-mo period; (3) loose or watery stool more than 25% of the time,

and hard stool less than 25% of the time; (4) symptom onset at least 6 mo prior to diagnosis; and (5) pain or discomfort at least 2 d a week during the screening period. Controls were healthy subjects undergoing routine health evaluation, with normal bowel movements (*i.e.*, fewer than 3/d and more than 3/wk) and no gastrointestinal complaints in the previous year. Patients were excluded in both IBS-D and control groups if they: (1) were pregnant or lactating; (2) were found to have abnormal colonoscopic findings; (3) had symptoms of a significant clinical illness; (4) had previous gastrointestinal surgery; and (5) had diabetes or other diseases affecting gastrointestinal function. This study was approved by the Ethics Committee of Chinese PLA General Hospital and written informed consent was obtained from each subject. The protocol was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) as NCT01028898. This study was conducted in compliance with the principles of the Declaration of Helsinki.

### Endoscopy procedure

All study participants underwent screening colonoscopy with a CF-H260AI or CF-Q260AI colonoscope (Olympus, Tokyo, Japan); terminal ileum intubation was attempted when applicable. During withdrawal of the scope, the colonic mucosa was carefully visualized and biopsies of the sigmoid colon were obtained (FB-55U-1 biopsy forceps; Olympus, Tokyo, Japan). Terminal ileal biopsies were obtained from 10 patients with IBS-D.

All colonoscopies were performed at the Chinese PLA General Hospital and samples were stored in covered storage tubes at -20 °C before analysis.

### Polymerase chain reaction and mtDNA sequencing

The MT-ATP 6 and 8 genes were polymerase chain reaction amplified and sequenced according to the mt-SEQr™ protocol (Applied Biosystems, Foster City, CA, United States).

**Polymerase chain reaction reaction:** Genomic DNA was extracted from specimens based on standard protocols<sup>[9]</sup>. Thermocycling conditions with the AB 9700 (Applied Biosystems, Foster City, CA, United States) were as follows: Heat activation at 96 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s; final extension at 72 °C for 10 min. Polymerase chain reaction (PCR) reaction clean-up was performed by adding 2 µL of ExoSAP-IT® (USB Corporation), incubating at 37 °C for 30 min and heat inactivation at 80 °C for 15 min.

**Sequencing reaction and electrophoresis:** A forward and reverse sequencing reaction mix was prepared from ready-to-use resequencing sets (Applied Biosystems, Foster City, CA, United States). The sequencing master mixes contained the M13 forward and reverse primers. Cycling conditions were: heat activation at 96 °C for 1 min, followed by 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. Sequencing reaction clean-up was performed. PCR products were electrophoresed using the

**Table 1** Demographic data and clinical profile of the participants

|                       | Control (n = 28) | IBS (n = 28) | P value             |
|-----------------------|------------------|--------------|---------------------|
| Age (yr)              | 43.6 ± 1.9       | 41.6 ± 2.2   | > 0.05 <sup>1</sup> |
| Gender (male:female)  | 22:6             | 18:10        | > 0.05 <sup>2</sup> |
| Disease duration (yr) | N/A              | 3.6 ± 0.6    | N/A                 |

<sup>1</sup>P values were obtained using *t* test ( $t = -0.682$ ,  $df = 54$ ,  $P = 0.498$ ); <sup>2</sup>Pearson's  $\chi^2$  test ( $\chi^2 = 1.400$ ,  $df = 1$ ,  $P = 0.237$ ) between the control and patients with irritable bowel syndrome (IBS). Values expressed as mean ± SE. N/A: Not available.

AB 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, United States).

#### Analysis of the sequences of MT-ATP 6 and 8 genes:

Sequencing data were analyzed *via* Variant Reporter™ Software version 1.0 (Applied Biosystems, Foster City, CA, United States) and compared with the reference sequence from Genbank (accession No. NC\_012920) to indicate possible polymorphisms. The corresponding changes of amino acid with missense polymorphisms were excerpted from MITOMAP ([www.mitomap.org](http://www.mitomap.org)). For polymorphism not recorded in MITOMAP, HmtDB (<http://www.hmtdb.uniba.it:8080/hmtdb/>) was applied.

#### Statistical analysis

The  $\chi^2$  and Student's *t* test were used to analyze demographic data between control and IBS-D patients, wherever applicable. Since MT-ATP 6 and 8 genes overlap in the mtDNA genome, these two genes were considered as a single gene during analysis. The non-parametric Mann-Whitney *U* test was performed to analyze polymorphism frequency between groups. The Spearman rank correlation coefficient was used to determine the association between the presence of the mtDNA polymorphism and IBS-D duration. A *P* value of less than 0.05 was considered statistically significant. Statistical analysis was performed using SPSS software (Chicago, IL, United States).

## RESULTS

#### Characteristics of study participants

The characteristics of study participants are summarized in Table 1. No significant difference was found in age or gender between patients with IBS-D and controls.

#### mtDNA polymorphisms in colon and terminal ileum

The nuclear positions and base changes of the mtDNA polymorphism, as compared with the Genbank mtDNA sequence (accession No. NC\_012920) are listed in Table 2. Twenty-five polymorphic sites in MT-ATP 6 and 8 genes were observed, of which 48% (12/25) were missense. No deletions or insertions were detected.

The median 2 polymorphic sites (range 1-3) in the MT-ATP 6 and 8 genes were found in control colon specimens (Table 3) while the median 3 polymorphic sites (range 1-4) were found in IBS-D patients (Table 4). Similarly, 1-4 polymorphic sites in MT-ATP 6 and 8

**Table 2** Summary of mitochondrial DNA mutations in the ATP 6 and 8 genes

| Nucleotide position | Base change (N>T) | mtDNA region  | Amino acid change         |
|---------------------|-------------------|---------------|---------------------------|
| 8392                | G>A               | ATP8          | syn                       |
| 8394                | C>T               | ATP8          | Missense                  |
| 8414                | C>T               | ATP8          | Missense                  |
| 8459                | A>G               | ATP8          | Missense                  |
| 8473                | T>C               | ATP8          | syn                       |
| 8563                | A>G               | ATP8 and ATP6 | ATP6: missense; ATP8: syn |
| 8584                | G>A               | ATP6          | Missense                  |
| 8684                | C>T               | ATP6          | Missense                  |
| 8697                | G>A               | ATP6          | syn                       |
| 8701                | A>G               | ATP6          | Missense                  |
| 8705                | T>C               | ATP6          | Missense                  |
| 8727                | C>T               | ATP6          | syn                       |
| 8772                | T>C               | ATP6          | syn                       |
| 8784                | A>G               | ATP6          | syn                       |
| 8793                | T>C               | ATP6          | syn                       |
| 8794                | C>T               | ATP6          | Missense                  |
| 8829                | C>T               | ATP6          | syn                       |
| 8856                | G>A               | ATP6          | syn                       |
| 8860                | A>G               | ATP6          | Missense                  |
| 8943                | C>T               | ATP6          | syn                       |
| 8994                | G>A               | ATP6          | syn                       |
| 9053                | G>A               | ATP6          | Missense                  |
| 9123                | G>A               | ATP6          | syn                       |
| 9128                | T>C               | ATP6          | Missense                  |
| 9180                | A>G               | ATP6          | syn                       |

mtDNA: Mitochondrial DNA; syn: Synonymous; N: Sequence obtained from National Center for Biotechnology Information genbank; T: Mutated sequence in the specimens; A: Adenine; T: Thymine; G: Guanine; C: Cytosine.

genes from the terminal ileum specimens were found in IBS-D patients (Table 5). When comparing with mtDNA polymorphisms in the colon and terminal ileum, all were identical except in one case (IBS22).

#### Association between clinical profiles and mtDNA polymorphisms

Polymorphisms in the MT-ATP 6 and 8 genes were increased in IBS patients as compared with controls (non-parametric Mann-Whitney test,  $U = 245$ ,  $P = 0.012$ ; Figure 1). In addition, the difference in percentage of missense polymorphisms between groups was statistically significant (non-parametric Mann-Whitney test,  $U = 265$ ,  $P = 0.016$ ). No correlation was found between the presence of mtDNA polymorphisms and disease duration in IBS-D patients (Spearman rho = 0.106,  $P = 0.592$ ).

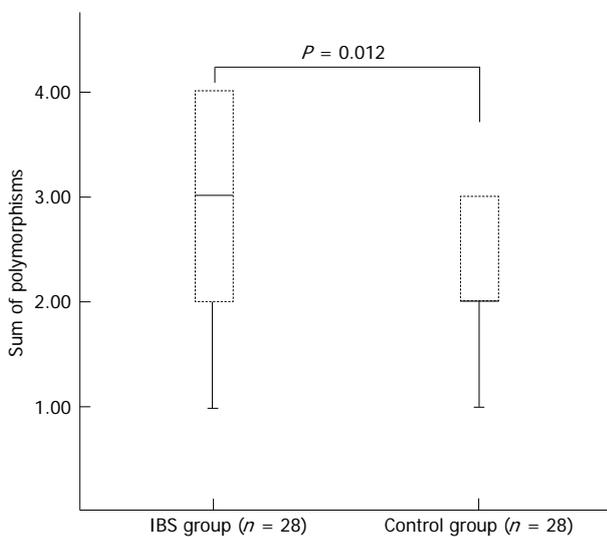
The mtDNA change at 8860 was present in all cases. The frequency of the 8701 polymorphism was 64% (18/28) in the IBS group and 57% (16/28) in controls, not statistically significant (Pearson  $\chi^2 = 0.299$ ,  $df = 1$ ,  $P = 0.584$ ). Several IBS-D patients (*e.g.*, IBS13, IBS14 and IBS18) had the same combination of polymorphisms but no similarities in clinical profile (age, gender, duration of disease) were observed.

## DISCUSSION

We found that polymorphisms in mitochondrial ATP 6

**Table 3 Mitochondrial DNA changes in the colon from controls**

| Case | Nucleotide position |      |      |      |      |      |      |      |      |      |      |      |      |      |          | Sum |   |
|------|---------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|----------|-----|---|
|      | 8392                | 8394 | 8414 | 8473 | 8563 | 8584 | 8701 | 8705 | 8727 | 8794 | 8860 | 8994 | 9053 | 9180 | Missense |     |   |
| 1    |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 2    |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | +        | 2   | 3 |
| 3    |                     |      |      |      |      |      |      |      |      |      |      |      | +    |      | 2        | 2   |   |
| 4    |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 5    |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |
| 6    |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 7    |                     |      |      |      |      |      |      | +    |      |      |      |      | +    |      | 3        | 3   |   |
| 8    |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |
| 9    |                     |      |      |      | +    |      |      |      |      | +    |      |      |      |      | 3        | 3   |   |
| 10   |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |
| 11   |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 12   |                     | +    |      |      |      |      |      | +    |      |      |      |      |      |      | 3        | 3   |   |
| 13   |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 14   |                     |      |      |      |      | +    |      | +    |      |      |      |      |      |      | 3        | 3   |   |
| 15   |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 16   |                     |      |      |      |      | +    |      |      | +    |      |      |      |      |      | 2        | 3   |   |
| 17   |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 2   |   |
| 18   |                     |      |      |      |      | +    |      | +    |      |      |      |      |      |      | 3        | 3   |   |
| 19   |                     |      |      | +    |      |      |      | +    |      |      |      |      |      |      | 2        | 3   |   |
| 20   |                     |      |      |      |      |      |      |      |      |      |      |      | +    |      | 2        | 2   |   |
| 21   |                     |      |      |      |      | +    |      | +    |      |      |      |      |      |      | 3        | 3   |   |
| 22   | +                   |      |      |      |      |      |      | +    |      |      |      |      |      |      | 2        | 3   |   |
| 23   |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |
| 24   |                     |      |      |      |      |      |      |      |      |      |      | +    |      |      | 1        | 2   |   |
| 25   |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |
| 26   |                     |      |      |      |      |      |      | +    |      |      |      |      |      | +    | 2        | 3   |   |
| 27   |                     |      | +    |      |      |      |      | +    |      |      |      |      |      |      | 3        | 3   |   |
| 28   |                     |      |      |      |      |      |      |      |      |      |      |      |      |      | 1        | 1   |   |



**Figure 1** Box plots showing the interquartile range (box) and median (solid line). An increase of mitochondrial DNA polymorphisms in irritable bowel syndrome (IBS) group was observed ( $P = 0.012$ ).

and 8 genes were more frequent in IBS with diarrhea, than in healthy individuals. Nearly half of the mutations studied were non-synonymous, the meaning of which from a pathophysiological point of view has not been previously clarified.

Blood samples were used typically when analyzing mtDNA. However, previous studies reported that mtDNA changes in the blood were not always the same

as those from organ tissues<sup>[10,11]</sup>. IBS is anatomically attributed to the lower gastrointestinal tract<sup>[12]</sup>; thus we have chosen colonic and ileal tissues rather than blood. We found that there was almost no difference in mtDNA variants between colon and ileum specimens from the same individual with IBS, except for one case. Both the small and large intestines, harboring the same mtDNA mutation, indicate that a similar pathogenesis may exist in both locations.

mtDNA is highly polymorphic<sup>[13,14]</sup>, and may occur across the entire mtDNA genome, with the MT-ATP gene as one of the hot spots. A high frequency of mtDNA polymorphisms has been implicated in a variety of diseases<sup>[15,16]</sup>, including mitochondrial diseases, degenerative diseases, aging, tumors and so on. Thus far, studies investigating the association between mtDNA and IBS are scarce. Camilleri's group reported that the mtDNA polymorphism in functional gastrointestinal disorders may be associated with digestive functions, such as satiation, gastric emptying, and pain<sup>[7]</sup>. Moreover, the mtDNA haplotype might affect the risk of developing IBS. Human mtDNA haplogroups are defined by a particular single nucleotide polymorphism (SNP). Haplotype H (7028C genotype) is predominant in the European population<sup>[17,18]</sup>; hence, Caucasians are often divided into Haplotype H and non-Haplotype H groups. Camilleri studied the difference between these groups, and found that constipation-predominant and mixed IBS were more likely to be associated with Haplotype H. Most Chinese people are Haplotype A, D and G, but not Haplotype

**Table 4 Mitochondrial DNA changes in the colon from irritable bowel syndrome group**

| Case  | Nucleotide position |      |      |      |      |      |      |      |      |      |      |      |      |      |      | Missense | Sum |      |      |      |      |      |   |   |
|-------|---------------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|----------|-----|------|------|------|------|------|---|---|
|       | 8392                | 8414 | 8459 | 8473 | 8563 | 8584 | 8684 | 8697 | 8701 | 8772 | 8784 | 8793 | 8794 | 8829 | 8856 |          |     | 8860 | 8943 | 9123 | 9128 | 9180 |   |   |
| IBS1  |                     |      |      |      |      |      |      |      |      |      |      |      |      |      |      | +        |     |      |      |      |      | 1    | 1 |   |
| IBS2  |                     | +    |      |      |      |      |      |      | +    |      |      |      |      |      |      |          | +   |      |      |      |      |      | 3 | 3 |
| IBS3  |                     |      |      |      |      |      |      | +    |      |      |      |      |      |      |      |          |     |      |      |      |      |      | 1 | 2 |
| IBS4  |                     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |          |     |      |      |      |      |      | 1 | 1 |
| IBS5  |                     |      |      |      | +    |      |      |      |      |      |      |      | +    |      |      |          |     |      |      |      |      |      | 3 | 3 |
| IBS6  |                     | +    |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 3 | 3 |
| IBS7  |                     |      |      |      |      | +    | +    |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 4 | 4 |
| IBS8  |                     |      |      |      |      |      |      |      |      | +    |      |      |      |      |      |          |     |      |      |      |      |      | 1 | 2 |
| IBS9  | +                   |      |      |      |      |      |      |      |      |      |      |      |      |      |      |          |     |      |      |      |      |      | 1 | 2 |
| IBS10 |                     |      |      |      |      | +    |      |      |      |      | +    |      |      | +    |      |          |     |      |      |      |      |      | 2 | 4 |
| IBS11 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      | +    |      | 2 | 3 |
| IBS12 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     | +    |      |      |      |      | 2 | 3 |
| IBS13 |                     | +    |      | +    |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 3 | 4 |
| IBS14 |                     | +    |      | +    |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 3 | 4 |
| IBS15 |                     |      |      |      |      |      |      |      | +    |      | +    |      |      |      | +    |          |     |      |      |      |      |      | 2 | 4 |
| IBS16 |                     |      |      |      |      |      |      |      | +    |      | +    |      |      |      | +    |          |     |      |      |      |      |      | 2 | 4 |
| IBS17 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 2 | 2 |
| IBS18 |                     | +    |      | +    |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 3 | 4 |
| IBS19 |                     |      | +    |      |      |      |      |      |      |      |      | +    |      |      |      |          |     |      |      |      |      |      | 3 | 3 |
| IBS20 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      | +    | 2 | 3 |
| IBS21 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 2 | 2 |
| IBS22 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 2 | 2 |
| IBS23 |                     |      |      | +    |      | +    |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 3 | 4 |
| IBS24 |                     |      |      |      |      | +    | +    |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 4 | 4 |
| IBS25 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      |      | 2 | 2 |
| IBS26 |                     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |          |     |      |      |      |      |      | 1 | 2 |
| IBS27 |                     |      |      |      |      |      |      |      |      |      |      |      |      |      |      |          |     |      |      |      | +    |      | 2 | 2 |
| IBS28 |                     |      |      |      |      |      |      |      | +    |      |      |      |      |      |      |          |     |      |      |      |      | +    | 3 | 4 |

IBS: Irritable bowel syndrome.

**Table 5 Mitochondrial DNA changes in terminal ileum from irritable bowel syndrome group**

| Case  | Nucleotide position |      |      |      |      |      |      |      |      |      |      | Sum |   |
|-------|---------------------|------|------|------|------|------|------|------|------|------|------|-----|---|
|       | 8414                | 8459 | 8473 | 8563 | 8584 | 8684 | 8697 | 8701 | 8772 | 8794 | 8860 |     |   |
| IBS1  |                     |      |      |      |      |      |      |      |      |      |      | +   | 1 |
| IBS2  | +                   |      |      |      |      |      |      | +    |      |      |      | +   | 3 |
| IBS3  |                     |      |      |      |      |      |      |      | +    |      |      | +   | 2 |
| IBS5  |                     |      |      |      | +    |      |      |      |      |      | +    | +   | 3 |
| IBS7  |                     |      |      |      | +    | +    |      |      |      |      |      | +   | 4 |
| IBS17 |                     |      |      |      |      |      |      |      | +    |      |      | +   | 2 |
| IBS18 | +                   |      |      | +    |      |      |      |      | +    |      |      | +   | 4 |
| IBS19 |                     | +    |      |      |      |      |      |      |      |      | +    | +   | 3 |
| IBS21 |                     |      |      |      |      |      |      |      | +    |      |      | +   | 2 |
| IBS22 |                     |      |      |      |      |      |      |      |      | +    |      | +   | 2 |

IBS: Irritable bowel syndrome.

H<sup>[19]</sup>, which is why we did not investigate the haplotype association with IBS in our study.

More than 3000 mtDNA polymorphisms are reported in various mtDNA databases<sup>[20]</sup>, and are often categorized as neutral or pathogenic. The role of pathogenic mtDNA mutations in mitochondrial diseases has been established<sup>[21,22]</sup>, while many polymorphisms in the mtDNA genome present in aging, tumorigenesis and other disease states are considered to be of little pathogenic significance<sup>[22,23]</sup>. Although efforts have been made to discover the underlying mechanism of these “neutral” polymorphisms, we are far from having a complete un-

derstanding of it. Recently, accumulation of mtDNA polymorphisms was proposed to underlie the pathogenesis of these diseases<sup>[24-26]</sup>. In our study, the polymorphisms detected were neutral, and cumulative polymorphisms in MT-ATP genes were higher in the IBS group. Accumulation of mtDNA polymorphism may play a role in IBS pathogenesis, however, data are limited on functional studies of neutral polymorphisms. In one study, neutral polymorphisms in the mtDNA control region were found to play a role in individual differences such as exercise tolerance<sup>[27]</sup>. Another study showed that 8701 and 10398 polymorphisms were associated with changes

in the mitochondrial matrix pH and intracellular calcium dynamics in the cell line<sup>[28]</sup>, which may help understand the functional significance of some neutral mtDNA polymorphisms. We also identified the 8701 polymorphism, but found no statistical difference in this polymorphism between IBS-D and controls. How these neutral polymorphisms are involved in functional diseases such as IBS-D remains unknown and further research is needed.

This study has several strengths and limitations. A major strength of this study lies in its direct analysis of small intestinal and colon samples, which are considered the areas of IBS symptomatology. As mentioned previously, blood mtDNA represents the status of circulating mitochondria, not specifically that of the bowel. However, due to the nature of intestinal biopsy, it is difficult to recruit a large number of subjects to participate. The relatively small number of study subjects limits our conclusions. Also, there is another concern that no functional studies focusing on these polymorphisms were involved, limiting the interpretation of the results. Further studies with larger sample sizes from multiple centers are required.

In conclusion, we found that cumulative polymorphisms in the mitochondrial ATP genes are associated with IBS-D. While it remains unknown how these polymorphisms are associated with ATP synthase function and cellular energy production in the intestine, our study has important implications for future research on the mtDNA genome in IBS.

## ACKNOWLEDGMENTS

We would like to thank Dr. Qing-Sen Liu, Dr. Wen Li and Dr. En-Qiang Linghu for their support. We are also grateful to Drs. Yuan-Zi Yu, and Min Min for their technical assistance. This work was presented in abstract form at UEG Week 2012 in Amsterdam.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) is one of the most prevalent functional gastrointestinal disorders and affects 10%-20% of the population. To date, the pathogenesis of IBS remains unclear. Family and twin studies suggested that genetic changes may predispose individuals to IBS.

### Research frontiers

A previous study indicated that the mitochondrial (MT) haplogroup and variations may be associated with gastrointestinal motor and sensory function in gastrointestinal disorders such as irritable bowel syndrome. Up till now, few research efforts have focused on this topic and little knowledge is available about it.

### Innovations and breakthroughs

The authors sequenced the coding region of mitochondrial DNA and found that polymorphisms in MT-ATP 6 and 8 genes were more frequent in IBS with diarrhea, than in healthy individuals.

### Applications

This study results implied that the MT-DNA polymorphism may play a role in irritable bowel syndrome with diarrhea, but further studies are needed.

### Terminology

MT-DNA: Human MT DNA is approximately 16.6 kbp long, which is inherited solely from the mother and encodes 13 genes for ATP subunits 6 and 8, and other polypeptides of respiratory complexes crucial for ATP production in mitochondria; IBS: According to the Rome III criteria, irritable bowel syndrome is

defined as a functional bowel disorder in which abdominal pain or discomfort is associated with defecation or a change in bowel habit, and with features of disordered defecation.

### Peer review

In the short paper, the authors analyzed the polymorphisms of MT-ATP 6 and 8 genes in the colon and ileum of IBS with diarrhea (IBS-D). Based on clinical samples, they concluded that patients with IBS-D have a higher incidence of MT-ATP 6 and 8 polymorphisms, compared with healthy controls. This finding is interesting and will be helpful for clinicians to emphasize the importance of the mtDNA polymorphism of IBS-D patients.

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**P- Reviewers** Clave P, Igor SL, Ren XF **S- Editor** Song XX  
**L- Editor** Ma JY **E- Editor** Li JY



## Laparoscopic splenectomy for treatment of splenic marginal zone lymphoma

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Received: January 10, 2013 Revised: April 28, 2013

Accepted: May 17, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the short-term and long-term efficacy and safety of laparoscopic splenectomy (LS) for treatment of splenic marginal zone lymphoma (SMZL).

**METHODS:** A total of 18 continuous patients who were diagnosed with SMZL and underwent LS in our department from 2008 to 2012 were reviewed. The perioperative variables and long-term follow-up were evaluated. To evaluate the efficacy and safety of this procedure better, we also included 34 patients with liver cirrhosis who underwent LS, 49 patients with immune thrombocytopenia (ITP) who underwent LS, and 20 patients with SMZL who underwent open splenectomy (OS). The results observed in the different groups were compared.

**RESULTS:** No differences were found in the sex and Child-Pugh class of the patients in SMZL-LS, SMZL-OS, ITP, and liver cirrhosis groups. The splenic length of the patients in the SMZL-LS group was similar to that in the SMZL-OS and liver cirrhosis groups but significantly longer than in the ITP group. The SMZL-LS group had a significantly longer operating time compared with the SMZL-OS, ITP, and liver cirrhosis groups, and the SMZL-LS group exhibited significantly less blood loss compared with the SMZL-OS group. No difference was found in the length of the postoperative hospital stay between the SMZL-LS, SMZL-OS, ITP, and liver cirrhosis-LS groups. After surgery, 6 (33.3%) SMZL-LS patients suffered slight complications. During mean follow-up periods of 13.6 and 12.8 mo, one patient from the SMZL-LS group and two from the SMZL-OS group died as a result of metastasis after surgery. None of the ITP and liver cirrhosis patients died.

**CONCLUSION:** LS should be considered a feasible and safe procedure for treatment of SMZL in an effort to improve the treatment options and survival of patients.

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**Key words:** Splenic marginal zone lymphoma; Laparoscopic splenectomy; Open splenectomy; Liver cirrhosis; Immune thrombocytopenia

**Core tip:** Laparoscopic splenectomy (LS) achieves excellent results for treatment of benign hematological diseases. The role of LS in treatment of splenic marginal zone lymphoma (SMZL) is difficult to define due to the associated splenomegaly, which may influence long-term outcomes. We investigated the perioperative variables and long-term follow-up of 18 SMZL patients who underwent LS and compared them with SMZL patients who underwent open splenectomy, immune thrombocytopenia patients who underwent LS, and liver cirrhosis patients who underwent LS. LS should be considered an appropriate treatment strategy for SMZL

patients in an effort to improve the treatment options and survival of these patients.

Wu Z, Zhou J, Wang X, Li YB, Niu T, Peng B. Laparoscopic splenectomy for treatment of splenic marginal zone lymphoma. *World J Gastroenterol* 2013; 19(24): 3854-3860 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3854.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3854>

## INTRODUCTION

Due to their low incidence rate, it is difficult and often ambiguous to determine the appropriate strategy for the treatment/management of splenic masses, which are considered uncommon diseases<sup>[1]</sup>. The most common splenic malignancy is lymphoma<sup>[1]</sup>. Splenic marginal zone lymphoma (SMZL) with or without villous lymphocytes is a disorder that was recently recognized as a distinct pathological entity in the World Health Organization classification<sup>[2]</sup>. This disease mainly affects elderly and middle-aged patients with a median age of 65 years<sup>[3]</sup>. At diagnosis, SMZL presents as an indolent and disseminated disease that is originally recognized after histopathological examination of surgically removed spleens as SMZL itself, or by means of morphological and immunophenotypic characterization of circulating neoplastic lymphocytes as splenic lymphoma with villous lymphocytes<sup>[4-6]</sup>. Cytopenia and lymphocytosis are frequently observed<sup>[7]</sup>. To date, there is no definitive standard treatment for SMZL. Approximately 2/3 of the patients are asymptomatic at diagnosis, and as many as one third of the patients will never require therapy. The diagnosis of this disease in patients who do not undergo splenectomy involves the morphological and immunophenotypic analysis of the peripheral blood and bone marrow<sup>[8]</sup>.

When splenectomy is indicated, laparoscopic splenectomy (LS) is the favored approach for treatment of benign hematological disorders. The role of LS in the treatment of a variety of hematological diseases, such as immune thrombocytopenia (ITP) and thrombotic thrombocytopenia, for which all other medical therapies have been exhausted has been elaborately documented<sup>[8]</sup>. The technical success, minimal morbidity, reduced disability, and high patient acceptance have resulted in the classification of LS as the gold standard for treatment of ITP<sup>[9,10]</sup>. Although splenomegaly was once considered a contraindication for laparoscopy, an increasing number of studies have proven the efficacy and safety of LS in both the short-term and the long-term treatment of splenomegaly and hypersplenism<sup>[11,12]</sup>.

The role of LS in patients with hematological malignancies remains ambiguous due to the skepticism regarding the use of minimally invasive techniques for the management of malignant or potentially malignant splenic diseases<sup>[12]</sup>. However, the increased incidence of patients with non-Hodgkin's lymphoma, particularly elderly pa-

tients, and the relative increase in the number of splenectomies performed in the treatment of hematological malignancies makes this issue particularly germane<sup>[13]</sup>. To date, there are only a few case studies that have analyzed the use of LS in the treatment of SMZL<sup>[1,4,8]</sup>. The present study aimed to reveal whether the surgical outcomes of LS are beneficial, safe, and/or secure for the treatment of SMZL to determine whether this procedure should be considered a standard protocol in the management of SMZL. To achieve the most meaningful comparison between patients with similar disease mechanisms, we analyzed 20 patients with SMZL that underwent open splenectomy (OS), 49 with ITP, and 34 with splenomegaly due to liver cirrhosis and portal hypertension who were treated with LS.

## MATERIALS AND METHODS

### Patients

Our retrospective comparative study was designed to determine the efficacy and surgical outcomes of SMZL patients who underwent LS (SMZL-LS group) and to compare the outcomes with those observed in SMZL patients who underwent OS (SMZL-OS group) and in ITP and liver cirrhosis patients who underwent LS in West China Hospital at Sichuan University in 2008-2012. We include our published report on the use of LS in the management of ITP and liver cirrhosis and compared these results with the outcomes obtained for LS in the management of SMZL.

The chief diagnostic indicator of SMZL was histological confirmation. The diagnosis of ITP was based on bone marrow aspirate that documented a sufficient number of megakaryocytes. All of the patients with liver cirrhosis underwent LS and subsequent liver biopsy. All of the patients were characterized by the principal indicators for splenectomy: diagnostic and therapeutic. The major anticipated therapeutic benefits were the relief of the local symptoms of splenomegaly and the correction of cytopenia.

The patients included in this study underwent a detailed demographic, clinical, and biochemical assessment. The hematological response and liver function were assessed before and 7 d after surgery using peripheral blood count (leukocytes, hemoglobin, and platelets) and total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and albumin assays. At the time of preoperative evaluation for splenectomy, all of the patients underwent a color Doppler ultrasonography (US) scan and computed tomography (CT) to calculate the length of the spleen and to determine the presence of any portal or splenic vein thrombosis (PSVT). Seven days after the operation, all of the patients underwent careful screening for thrombosis. The patients who showed evidence of splenic vein thrombosis by US underwent CT to confirm the extent of thrombosis.

### Operative technique of LS

The operative techniques of OS, LS, and hand-assisted

**Table 1** Demographic characteristics

| Variable           | SMZL        |             | Liver cirrhosis | ITP         | P value <sup>1</sup> | P value <sup>2</sup> | P value <sup>3</sup> |
|--------------------|-------------|-------------|-----------------|-------------|----------------------|----------------------|----------------------|
|                    | LS          | OS          |                 |             |                      |                      |                      |
| Cases              | 18          | 22          | 34              | 49          |                      |                      |                      |
| Age, yr            | 56.4 ± 10.5 | 52.0 ± 10.8 | 47.7 ± 12.2     | 36.2 ± 15.9 | 0.191                | 0.013                | 0.000                |
| Sex (M/F)          | 8/10        | 10/12       | 16/18           | 10/39       | 0.949                | 0.857                | 0.049                |
| Child-Pugh class   |             |             |                 |             | 0.336                | 0.522                | 0.282                |
| A                  | 16 (88.9)   | 17 (77.3)   | 27 (79.4)       | 47 (95.9)   |                      |                      |                      |
| B                  | 2 (11.1)    | 5 (22.7)    | 5 (14.7)        | 2 (4.1)     |                      |                      |                      |
| C                  | 0           | 0           | 2 (5.9)         | 0 (0)       |                      |                      |                      |
| Comorbidity        |             |             |                 |             |                      |                      |                      |
| ITP                | 1           | 2           |                 |             |                      |                      |                      |
| SLE                | 1           | 0           |                 |             |                      |                      |                      |
| Pulmonary effusion | 1           | 2           |                 |             |                      |                      |                      |
| Herpes zoster      | 1           | 1           |                 |             |                      |                      |                      |

Data are presented as mean ± SD or *n* (%). <sup>1</sup>SMZL-LS vs SMZL-OS groups; <sup>2</sup>SMZL-LS vs liver cirrhosis group; <sup>3</sup>SMZL-LS vs ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; M: Male; F: Female; SLE: Systemic lupus erythematosus.

laparoscopic splenectomy (HALS) have been described previously by our group<sup>[11,12]</sup>. In addition, we removed and biopsied a 1 cm × 1 cm piece of hepatic tissue from the left lobe of the liver of patients with liver cirrhosis.

### Follow-up

The mean follow-up time of SMZL patients who underwent LS was 13.6 mo. US and CT studies were performed at 1-mo intervals for 6 mo and at 3-mo intervals thereafter to determine whether the patients relapsed or developed PSVT. Upon the detection of PSVT by CT, we initiated anticoagulation therapy, which consisted of heparin (10000 U/d, intravenously), followed by warfarin. The dose of warfarin was adjusted to achieve an international normalized ratio (INR) of 1.5-2.0. Warfarin was administered until the disappearance of PSVT was confirmed by CT. All of the tests and examinations were repeated depending on the clinical condition of the patient.

### Statistical analysis

The continuous variables are expressed as the mean ± SD. The statistical analyses were performed using the SPSS for Windows version 16.0 (SPSS, Chicago, IL, United States). The differences between the variables were compared using Student's *t* test and the  $\chi^2$  test. Differences with *P* < 0.05 were considered statistically significant.

## RESULTS

### Demographic characteristics

No differences were found between the demographic characteristics of the SMZL-LS and SMZL-OS groups. The SMZL-LS patients were significantly older than the liver cirrhosis and ITP patients. In addition, women tend to suffer from ITP, and thus there were significant sex differences between the ITP group and both the SMZL-LS and the liver cirrhosis groups (Table 1). There were no differences in the Child-Pugh class between the SMZL-LS group and the SMZL-OS and the liver cir-

rhosis groups, whereas the ITP patients had normal liver function. The comorbidity of the SMZL patients in both groups is shown in Table 1.

### Perioperative outcomes

No patients in the ITP group exhibited conversion, but one patient from the liver cirrhosis group underwent conversion due to bleeding during the operation. In addition, one SMZL-LS patient underwent conversion because the harmonic was unable to stop the bleeding from the VASA during the operation. The SMZL-LS group had a significantly longer operation time compared with the SMZL-OS, ITP and liver cirrhosis groups. The EBL of the SMZL-OS group exhibited the most established blood loss, whereas the estimated blood loss (EBL) of the SMZL-LS and liver cirrhosis groups was not significantly different from that of the ITP group (Table 2). The SMZL-OS group exhibited a higher transfusion rate compared with the SMZL-LS group, whereas the transfusion rates of the other three types of patients were not significantly different. The spleen length of the SMZL-LS group was similar to that of the SMZL-OS and liver cirrhosis groups and longer than that of the ITP patients. The spleens of SMZL and liver cirrhosis patients usually exhibit splenomegaly or massive splenomegaly. The operation methods for the treatment of SMZL were LS (*n* = 8) or HALS (*n* = 10), whereas LS was used for the treatment of patients with liver cirrhosis and ITP.

### Postoperative results

No difference was found in the length of postoperative hospital stay between the SMZL-LS, liver cirrhosis and ITP groups, whereas the SMZL-OS group experienced a significantly longer stay (Table 3). Six SMZL-LS, 10 SMZL-OS, 5 liver cirrhosis, and three ITP patients suffered complications. Patients with pulmonary effusion, pancreatic leakage, and abdominal cavity effusion were all cured through conservative treatment, such as somatostatin and drainage. One liver cirrhosis patient experienced

**Table 2 Comparison of intraoperative details**

| Variable             | SMZL          |               | Liver cirrhosis | ITP          | P value <sup>1</sup> | P value <sup>2</sup> | P value <sup>3</sup> |
|----------------------|---------------|---------------|-----------------|--------------|----------------------|----------------------|----------------------|
|                      | LS            | OS            |                 |              |                      |                      |                      |
| Conversion           | 1             | -             | 1               | 0            |                      |                      |                      |
| Operation time (min) | 238.4 ± 37.9  | 185.9 ± 54.9  | 210.1 ± 48.5    | 163.9 ± 67.2 | 0.001                | 0.037                | 0.000                |
| EBL                  | 171.9 ± 228.4 | 310.0 ± 192.0 | 150.0 ± 146.1   | 65.7 ± 114.0 | 0.045                | 0.675                | 0.014                |
| Transfusion          | 4/18 (22.2)   | 9/13 (69.0)   | 3/34 (8.8)      | 8/49 (16.3)  |                      | 0.178                | 0.577                |
| Spleen length (cm)   | 22.8 ± 5.5    | 23.7 ± 5.6    | 23.9 ± 3.9      | 12.1 ± 3.2   | 0.624                | 0.408                | 0.000                |
| Additional operation |               |               |                 |              |                      |                      |                      |
| Liver biopsy         | 0             | 0             | 34              | 0            |                      |                      |                      |
| Lymph node biopsy    | 3             | 5             |                 | 0            |                      |                      |                      |
| LC                   | 1             | -             | 2               | 5            |                      |                      |                      |
| Operation Method     |               |               |                 |              |                      |                      |                      |
| LS                   | 8             | -             | 34              | 49           |                      |                      |                      |
| HALS                 | 10            | -             | 0               | 0            |                      |                      |                      |

Data are presented as mean ± SD or *n* (%). <sup>1</sup>SMZL-LS vs SMZL-OS groups; <sup>2</sup>SMZL-LS vs liver cirrhosis groups; <sup>3</sup>SMZL-LS vs ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; HALS: Hand-assisted laparoscopic splenectomy; EBL: Estimated blood loss; LC: Laparoscopic cholecystectomy.

**Table 3 Comparison of postoperative details**

| Variable                       | SMZL       |            | Liver cirrhosis | ITP       | P value <sup>1</sup> | P value <sup>2</sup> | P value <sup>3</sup> |
|--------------------------------|------------|------------|-----------------|-----------|----------------------|----------------------|----------------------|
|                                | LS         | OS         |                 |           |                      |                      |                      |
| PHS (d)                        | 8.17 ± 3.7 | 10.8 ± 4.2 | 7.5 ± 2.0       | 7.6 ± 2.1 | 0.044                | 0.378                | 0.389                |
| Complication                   |            |            |                 |           |                      |                      |                      |
| Pulmonary effusion             | 3          | 5          | 1               | 0         |                      |                      |                      |
| Pancreatic leakage             | 1          | 2          | 1               | 1         |                      |                      |                      |
| Abdominal cavity effusion      | 1          | 1          | 0               | 0         |                      |                      |                      |
| Postoperative bleeding         | 0          | 0          | 1               | 2         |                      |                      |                      |
| Portal/splenic vein thrombosis | 1          | 2          | 2               | 0         |                      |                      |                      |
| Total                          | 6 (33.3)   | 10 (45)    | 5 (14.7)        | 3 (6.1)   |                      |                      |                      |

Data are presented as mean ± SD or *n* (%). <sup>1</sup>SMZL-LS vs SMZL-OS groups; <sup>2</sup>SMZL-LS vs liver cirrhosis groups; <sup>3</sup>SMZL-LS vs ITP groups. SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; PHS: Postoperative hospital stay.

postoperative bleeding. As a result, an emergency laparotomy and blood transfusion were performed, and the patient was discharged 14 d after LS. Two ITP patients suffered postoperative bleeding and received blood transfusion and conservative medical treatment. Both of these patients recovered 10 d after surgery. Patients were diagnosed with portal splenic vein thrombosis by postoperative dynamic CT. These patients received anticoagulation therapy consisting of heparin (10000 U/d *iv*) followed by warfarin. The dose of warfarin was adjusted to achieve an INR of 2. The administration of warfarin was continued every 3 mo until thrombosis disappeared.

After surgery and during follow-up, almost no significant differences in the hematological parameters and liver function outcomes were observed between the SMZL-LS and SMZL-OS groups. Total bilirubin of the liver cirrhosis group was much higher than that of the SMZL-LS and ITP groups because liver cirrhosis usually causes liver damage. The same result was observed in the analysis of the ALT and AST of the three groups of patients. The SMZL-LS and liver cirrhosis patients had a low white blood cell count (WBC) compared with the ITP patients ( $P = 0.000$ ), and the WBCs of the SMZL-LS and liver cirrhosis groups were the same. The platelet counts of

the three types of patients were all different. The platelet count of the SMZL-LS group was higher than that of the liver cirrhosis group ( $P = 0.000$ ), and the platelet count of the liver cirrhosis group was higher than that of the ITP group ( $P = 0.000$ ). Postoperative comparison revealed that the liver cirrhosis patients had a higher level of total bilirubin and albumin than the SMZL patients. The ALT and AST levels in these patients were equal. The WBC of the ITP group was higher than that of the lymphoma and liver cirrhosis patients, but the WBC of the lymphoma and liver cirrhosis patients did not differ significantly. The platelet count of the three types of patients exhibited no significant differences (Table 4).

#### Follow-up outcomes

The SMZL-LS and SMZL-OS groups had a mean follow-up of 13.6 and 12.8 mo, respectively. At these times, none of the patients became septic or experienced wound complications following LS. One SMZL-LS and 2 SMZL-OS patients died as a result of metastasis following surgery. The other 17 patients experienced disease-free survival. None of the patients in the ITP and liver cirrhosis groups died.

**Table 4 Comparison of the preoperative and postoperative hematological parameters and liver function variables**

| Variable                   | SMZL          |               | Liver cirrhosis | ITP           | P value <sup>1</sup> | P value <sup>2</sup> | P value <sup>3</sup> |
|----------------------------|---------------|---------------|-----------------|---------------|----------------------|----------------------|----------------------|
|                            | LS            | OS            |                 |               |                      |                      |                      |
| Preoperation               |               |               |                 |               |                      |                      |                      |
| TBIL (mmol/L)              | 15.7 ± 8.5    | 23.3 ± 11.2   | 28.3 ± 17.2     | 13.3 ± 6.5    | 0.023                | 0.005                | 0.231                |
| ALT (U/L)                  | 25.1 ± 17.6   | 30.6 ± 11.1   | 54.7 ± 44.0     | 36.2 ± 33.6   | 0.237                | 0.009                | 0.187                |
| AST (U/L)                  | 25.7 ± 16.9   | 33.9 ± 16.9   | 59.3 ± 40.1     | 25.9 ± 21.9   | 0.133                | 0.001                | 0.956                |
| Albumin (g/L)              | 36.8 ± 6.6    | 35.1 ± 8.4    | 37.6 ± 5.7      | 40.7 ± 5.3    | 0.478                | 0.653                | 0.015                |
| HGB (g/L)                  | 102.7 ± 26.1  | 106.3 ± 30.3  | 112.2 ± 22.5    | 123.7 ± 23.1  | 0.687                | 0.175                | 0.002                |
| WBC (× 10 <sup>9</sup> /L) | 4.2 ± 3.3     | 4.0 ± 3.2     | 3.2 ± 2.6       | 11.3 ± 6.7    | 0.867                | 0.255                | 0.000                |
| PLT (× 10 <sup>9</sup> /L) | 65.8 ± 35.6   | 56.1 ± 30.5   | 38.1 ± 15.7     | 20.6 ± 20.2   | 0.359                | 0.000                | 0.000                |
| Postoperation              |               |               |                 |               |                      |                      |                      |
| TBIL (mmol/L)              | 11.9 ± 6.7    | 16.2 ± 7.8    | 19.4 ± 11.3     |               | 0.078                | 0.014                |                      |
| ALT (U/L)                  | 23.4 ± 12.9   | 28.1 ± 13.9   | 32.0 ± 25.9     |               | 0.279                | 0.192                |                      |
| AST (U/L)                  | 27.4 ± 17.7   | 29.5 ± 14.7   | 28.6 ± 9.7      |               | 0.682                | 0.753                |                      |
| Albumin (g/L)              | 31.2 ± 5.5    | 33.7 ± 3.9    | 34.5 ± 3.9      |               | 0.114                | 0.017                |                      |
| HGB (g/L)                  | 101.1 ± 15.1  | 99.2 ± 15.1   | 138.6 ± 172.1   | 117.3 ± 20.4  | 0.699                | 0.362                | 0.003                |
| WBC (× 10 <sup>9</sup> /L) | 9.9 ± 6.7     | 11.3 ± 6.5    | 7.9 ± 1.8       | 14.4 ± 4.9    | 0.481                | 0.122                | 0.003                |
| PLT (× 10 <sup>9</sup> /L) | 298.8 ± 304.1 | 318.1 ± 211.1 | 237.2 ± 165.0   | 287.8 ± 140.1 | 0.814                | 0.346                | 0.840                |

Data are presented as mean ± SD. <sup>1</sup>SMZL-LS vs SMZL-OS groups; <sup>2</sup>SMZL-LS vs liver cirrhosis groups; <sup>3</sup>SMZL-LS vs ITP groups. HGB: hemoglobin; PLT: platelet count; TBIL: Total bilirubin; WBC: White blood cell count; SMZL: Splenic marginal zone lymphoma; OS: Open splenectomy; LS: Laparoscopic splenectomy; ITP: Immune thrombocytopenia; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

## DISCUSSION

SMZL is globally deemed a low-grade lymphoma with an indolent clinical course. Numerous cases exhibit a protracted straightforward progression, an excellent response to splenectomy or chemotherapy treatment, and sometimes an unmodified clinical picture in the absence of any treatment. The 5-year survival rate ranges from 65% to 78%<sup>[14,15]</sup>. Retrospective studies have shown that patients who underwent splenectomy exhibited a significantly improved survival rate compared with those patients who underwent chemotherapy<sup>[14]</sup>. Splenectomy is the generally preferred treatment for SMZL. Although this process is not preventive, splenectomy offers superior swift relief of symptoms and often completely modifies any affiliated cytopenia. Additionally, this surgical procedure offers excellent disease management, which usually makes it possible for individuals to avoid systemic therapy<sup>[14]</sup>. Although the advantages of LS, such as shorter hospital stay, less scarring, earlier return to activity, and less inflammatory responses<sup>[16]</sup>, have been documented previously, the residual tumor and tumor recurrence should be taken into account in the consideration of LS as an appropriate procedure for the treatment of a potentially malignant lesion.

Extensive experience with LS at many centers has led to its use in the treatment of a wide variety of benign hematological diseases. Furthermore, our previous results demonstrated that LS is an efficient and safe strategy for the treatment of hypersplenism secondary to liver cirrhosis<sup>[11]</sup>. Our current data suggest that the results of LS for treatment of SMZL are comparable with the results for treatment of ITP and liver cirrhosis, which confirms the safety of this procedure for these diseases. Although the SMZL group included a significantly older patient population compared with the ITP group and exhibited

a spleen length comparable to that of the liver cirrhosis patients, the SMZL patients underwent successful operations with low morbidity and no mortality. The significantly longer operating time and the significantly higher blood loss in the SMZL patients compared with the ITP and liver cirrhosis groups were expected but did not correlate with adverse outcomes<sup>[9]</sup>.

The ability to achieve a satisfactory outcome in this difficult patient group is probably related to the technical expertise of the surgeon<sup>[9]</sup>. It has been shown that splenic size is an independent predictor of postoperative complications<sup>[14]</sup>. Yano *et al*<sup>[17]</sup> reported their experience with HALS for the treatment of splenic tumors in 10 patients. They have recommended the HALS approach because it allows easier mobilization of the spleen (particularly with splenomegaly) and easier resection of the adjacent organs or tissue if necessary. However, Makrin *et al*<sup>[18]</sup> concluded that most splenic tumors can be treated using a completely laparoscopic approach. This total laparoscopic approach may be unsuitable when the tumor is associated with massive splenomegaly; in these cases HALS may be considered. In our study, eight patients underwent total LS, whereas 10 patients underwent HALS. We performed LS on patients with splenic length > 20 cm. To ensure sufficient space throughout the surgical procedure, additional movements of the spleen were required, which escalated the blood loss and the chance of perisplenic organ injury. In contrast, the majority of our patients with splenomegaly underwent LS effectively<sup>[19]</sup>. In this particular analysis, we attempted to appraise the intraoperative and postoperative consequences with respect to substantial splenomegaly, utilizing LS and HALS for the treatment of SMZL. Of the 81 patients studied by Thieblemont *et al*<sup>[20]</sup>, 44 exhibited spleen lymphoma and anemia, and 13 of these had Coombs-positive hemolytic anemia. Of our 18 patients, 38.9% exhibited Coombs-

positive hemolytic anemia. A study of 309 patients revealed the 50% of the patients remained anemic<sup>[21]</sup>. However, our comparative study is unique because it analyzed the effectiveness of LS in the treatment of an assortment of diseases, particularly SMZL. Our outcomes demonstrate that, regardless of the numerous strategies for the treatment of SMZL, LS might prove advantageous for a number of reasons, including its significantly shorter hospital stay and low postoperative stress; these findings have been confirmed by several other investigators. Splenectomy frequently contributes to somatic compensation of patients, which results in local relapse in the spleen, prevents continuing dissemination of the primary tumor site, and mostly corrects cytopenia, thereby creating better conditions for chemotherapy<sup>[22]</sup>. One of the patients enrolled in our study died as a result of metastasis several weeks after surgery; the patient's death was therefore unrelated to our treatment approach.

The sex of the different groups differed significantly, mainly because of the characteristics and epidemiology of SMZL and ITP. The splenic size was an important indicator of the conversion rate, the operation time, and the blood loss. The SMZL and the liver cirrhosis patients had significantly longer spleens. The operation time of the SMZL group was significantly longer than that of the liver cirrhosis and ITP groups, which implies that surgery for lymphoma is more difficult than for liver cirrhosis and ITP. We found that the spleen of the lymphoma patients usually adhered to the greater omentum or intestine. It therefore requires a longer time to separate these tissues and organs. LS is the gold standard for the treatment of ITP. Compared with LS for the treatment of ITP, LS for liver cirrhosis may be more difficult because the blood vessels are thick and varicose. The EBL of the SMZL and the liver cirrhosis groups was higher than that of the ITP group, whereas there was no significant difference between the EBL of the SMZL group and that of the liver cirrhosis group. This finding indicates that LS exhibits similar outcomes in the treatment of both types of patients.

Fine-needle aspiration (FNA) was used in the diagnosis of the splenic mass with a high positive rate of approximately 80%-88.9%<sup>[23,24]</sup>. Previous studies reported a low morbidity rate and no biopsy-site seeding of the tumor. However, the incidentally discovered lesions comprised the minority of the lesions (20%-27%)<sup>[1]</sup>. Furthermore, this technique may be associated with bleeding complications and the risk of tumor dissemination<sup>[21]</sup>. Tessier *et al*<sup>[1]</sup> demonstrated that FNA biopsy is unnecessary unless the patient cannot tolerate splenectomy, that is, in the setting of a solitary splenic mass with no history of malignancy. Based on the results of Tessier *et al*<sup>[1]</sup>, the SMZL patients in our study did not undergo FNA.

In conclusion, we evaluated the safety and efficacy of LS for the treatment of SMZL and compared these results with the outcome of LS for treatment of ITP and liver cirrhosis, and from the use of OS for the treatment of SMZL. Our findings show that LS is usually safe and effective for the treatment of SMZL. Although the

SMZL patients who underwent LS required a significantly longer operation time than those with ITP and liver cirrhosis, no significant differences were observed in the transfusion requirements, postoperative complications, or length of postoperative hospital stay. LS might be a favored procedure for the treatment of SMZL. However, further research is required to determine more definitely its effectiveness in the treatment of SMZL. Furthermore, the role of HALS as a first-choice approach or an alternative approach for the treatment of massive splenomegaly needs to be investigated.

## COMMENTS

### Background

Laparoscopic splenectomy (LS) is the favored operative approach for the treatment of benign hematological disorders that require splenectomy. Although splenomegaly was once considered a contraindication for laparoscopy, an increasing number of studies have proven the efficacy and safety of the use of LS for both the short-term and long-term treatment of splenomegaly. However, the role of LS in the treatment of patients with hematological malignancies remains ambiguous due to skepticism regarding the use of minimally invasive techniques for the treatment of malignant or potentially malignant splenic diseases.

### Research frontiers

To date, there is no definitive standard for the treatment of splenic marginal zone lymphoma (SMZL). Approximately two-thirds of patients are asymptomatic at the time of diagnosis, and as many as one-third of the patients will never require therapy. However, the incidence of patients with SMZL is increasing, especially in the elderly population. The use of LS for the treatment of hematological malignancy has gradually improved. In this study, the authors demonstrated that LS might be a feasible and safe treatment option for SMZL.

### Innovations and breakthroughs

To date, there are only a few case studies that have analyzed the use of LS for the treatment of SMZL. In addition, only a few studies have compared LS and open splenectomy (OS) for the treatment of SMZL. Furthermore, no study has shown differences in the perioperative and long-term outcomes between SMZL, immune thrombocytopenia (ITP), and splenomegaly. This study demonstrated that LS is a feasible and safe procedure for the treatment of SMZL.

### Applications

To achieve the most meaningful comparison between patients with similar disease mechanisms, the authors included patients with SMZL who underwent OS, patients with ITP, and patients with splenomegaly due to liver cirrhosis and portal hypertension who were treated with LS. The study revealed that LS is safe for the treatment of SMZL and should be considered in its management.

### Terminology

SMZL with or without villous lymphocytes is a disorder that was recently recognized as a distinct pathological entity in the World Health Organization classification. SMZL was originally recognized either after histopathological examination of surgically removed spleens as SMZL itself, or by means of morphological and immunophenotypic characterization of circulating neoplastic lymphocytes as splenic lymphoma with villous lymphocytes.

### Peer review

This was an interesting study in which the authors analyzed the perioperative and long-term variables in the use of LS for the treatment of lymphoma. This study shows that the morbidity associated with treatment of SMZL is no more than expected compared with the outcomes obtained for LS treatment of other diseases. The results are instructive and suggest that LS is a feasible and safe procedure for the treatment of SMZL.

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## Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations

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Supported by Health Bureau of Sichuan Province, China, No. 070283 and 100175

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Received: February 6, 2013 Revised: April 12, 2013

Accepted: May 7, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To investigate the relationship between hepatitis B virus (HBV) genotype with spontaneous YMDD mutations and hepatocellular carcinoma (HCC) in HBV-related cirrhosis.

**METHODS:** We investigated 264 liver cirrhosis patients who were not treated with antiviral drugs, including 81 patients with HCC. YMDD mutations were detected by fluorescent hybridization bioprobe polymerase chain reaction (PCR) and melting curve assay using the Diagnosis Kit for HBV YMDD Mutation. Serum HBV genotypes were detected by real-time PCR using genotype-specific TaqMan probes. Statistical analysis was performed according to data type using the *t* test,  $\chi^2$  test and unconditional logistic regression analysis.

**RESULTS:** In the HCC group, genotype C strains, spontaneous YMDD mutations, and genotype C strains with YMDD mutations were detected in 33 (40.74%), 13 (16.05%) and 11 (13.58%) patients, respectively. In the liver cirrhosis (LC) group, HBV genotype C strains,

spontaneous YMDD mutations, and genotype C strains with YMDD mutations were detected in 33 (18.03%), 7 (3.83%) and 2 (1.09%) patients, respectively. The differences in genotype C strains, spontaneous YMDD mutations, and genotype C strains with YMDD mutations between the two groups were statistically significant ( $\chi^2 = 15.441, P = 0.000; \chi^2 = 11.983, P = 0.001; P = 0.000$ ). In the HCC and LC groups, there were seven patients infected by genotype B strains with YMDD mutations and 13 by genotype C strains with YMDD mutations. Further research revealed that HCC occurred in 2 patients infected by genotype B strains with YMDD mutations and 11 infected by genotype C strains with YMDD mutations. The difference was statistically significant ( $P = 0.000$ ). Unconditional logistic regression analysis revealed that patients infected by genotype C strains with spontaneous YMDD mutations had a 7.775-fold higher risk for the development of HBV-related HCC than patients infected by other type HBV strains ( $P = 0.013, 95\%CI: 1.540-39.264$ ).

**CONCLUSION:** Genotype C strains with spontaneous YMDD mutations are an independent risk factor for HCC in LC patients and are important for early warning of HCC.

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**Key words:** Hepatitis B virus; Liver cirrhosis; Primary hepatocellular carcinoma; Hepatitis B virus genotype; YMDD mutation

**Core tip:** YMDD mutation is a research hotspot globally. Until recently, most research about YMDD mutation focused on the occurrence of lamivudine-related YMDD mutation and its impact on antiviral treatment. In our research, 264 hepatitis B virus (HBV)-related liver cirrhosis patients not treated with antiviral drugs, including 81 with primary hepatocellular carcinoma (HCC), were investigated for the association between infection by different HBV genotype strains with spontaneous

YMDD mutations and occurrence of primary HCC in cirrhosis patients. Infection by genotype C strains with spontaneous YMDD mutations is an independent risk factor for the development of HCC in cirrhosis patients.

Yang JH, Zhang H, Chen XB, Chen G, Wang X. Relationship between hepatocellular carcinoma and hepatitis B virus genotype with spontaneous YMDD mutations. *World J Gastroenterol* 2013; 19(24): 3861-3865 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v19/i24/3861.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3861>

## INTRODUCTION

According to the estimation of the World Health Organization, about 350 million people worldwide are chronically infected by hepatitis B virus (HBV)<sup>[1-3]</sup>. HBV infection is endemic in China. The seropositive rate of hepatitis B surface antigen is 7.18% and about 93 million people are chronically infected by HBV<sup>[4-6]</sup>. Chronic infection by HBV is the major cause of hepatocellular carcinoma (HCC)<sup>[7,8]</sup>, and in addition to HCC, leads to a series of HBV-related liver diseases, including asymptomatic carrier, chronic hepatitis, and liver cirrhosis (LC)<sup>[9-11]</sup>.

HBV replicates actively in host liver cells and the reverse transcriptase domain of HBV polymerases lacks a proofreading function. Thus, the mutation rate of HBV is relatively high. YMDD motif is a highly conserved sequence in domain C of HBV reverse transcriptase. YMDD mutation, also known as M204V/I mutation, is the substitution of methionine by valine or isoleucine and designated as YVDD or YIDD variant<sup>[12,13]</sup>. Until recently, most research about YMDD mutations has focused on the occurrence of lamivudine-related YMDD mutations and their effect on antiviral treatment<sup>[14-16]</sup>. During recent years, spontaneous YMDD mutations have been detected in patients with chronic HBV infection not previously treated with antiviral drugs. The relationship between spontaneous YMDD mutation and HBV-related HCC has rarely been reported. Different HBV genotype strains are formatted by accumulation of point mutations in the viral genome. Previous research has revealed that infection by genotype C strains is associated with HCC. However, the relationship between infection by different HBV genotype strains with spontaneous YMDD mutations and the occurrence of HCC in HBV-related LC patients has not been reported before.

In order to investigate the association between infection by different genotype strains with spontaneous YMDD mutation and the occurrence of HBV-related HCC, we investigated 264 cirrhosis patients not previously treated with antiviral drugs, including 81 with HCC.

## MATERIALS AND METHODS

### Patients

To ensure that HBV genotype and YMDD mutations

could be detected by our kit, 264 HBV-related LC patients with serum HBV DNA load  $> 5 \times 10^3$  copies/mL, diagnosed and treated at the Department of Infectious Diseases in our hospital from May 2010 to August 2012, were selected for further research. According to the criteria "Chinese Standard for the Diagnoses and Treatment of Primary Hepatocellular Cancer in 2011" and "Prevention and Treatment Standard of Chinese Viral Hepatitis in 2000", 81 LC patients with HCC and 183 without HCC were selected and assigned to the HCC group and LC group, respectively. In the HCC group, there were 65 male patients (80.25%) and 16 female patients (19.75%). Their ages ranged from 31 to 78 years, with a mean of  $53.86 \pm 11.05$  years. In the LC group, 129 patients (70.49%) were male and 54 patients (29.51%) were female. Their ages ranged from 22 to 79 years, with a mean of  $52.66 \pm 11.42$  years. None of the patients had been treated previously with antiviral drugs and were not affected by other liver injury factors, such as co-infection with hepatitis A virus, hepatitis C virus, hepatitis D virus or hepatitis E virus, alcoholic hepatitis, autoimmune hepatitis, and fatty liver.

### Sample collection

Fasting venous blood was collected from these patients. The serum was separated immediately and stored at  $-70^\circ\text{C}$ .

### Detection methods

Serum HBV DNA was quantified by real-time polymerase chain reaction (PCR) (Qiagen, Shenzhen, Guangdong Province, China). YMDD mutant types were determined by fluorescence hybridization bioprobe PCR and melting curve assay with the use of the care HBV mutation PCR assay (Qiagen, Shenzhen, China) and distinguished by melting temperature value. HBV genotype was detected by real-time PCR using genotype-specific TaqMan probe (Fuxing, Shanghai, China). Serum HBV markers were tested by enzyme-linked immunosorbent assay (Huamei, Shanghai, China).

### Statistical analysis

Statistical analysis including Student's *t* test,  $\chi^2$  test and unconditional logistic regression analysis were performed using SPSS 17.0 software. A difference with  $P < 0.05$  was considered statistically significant.

## RESULTS

### Patient characteristics

Patient characteristics are shown in Table 1. Age, sex distribution, hepatitis B e antigen (HBeAg)-positive rate, and serum HBV load did not differ significantly between the HCC and LC groups. Thirteen and seven spontaneous YMDD mutations were detected in the HCC and LC groups, respectively. In the HCC group, 33 (40.74%) patients were infected by genotype C strains and 47 (58.02%) were infected by genotype B strains. In the LC group, 33 (18.03%) patients were infected by genotype C strains

**Table 1 Patient characteristics in the hepatocellular carcinoma and liver cirrhosis groups**

|   | HCC group         | LC group          | <i>t</i> or $\chi^2$ value | <i>P</i> value |
|---|-------------------|-------------------|----------------------------|----------------|
| No. of patients                                   | 81                | 183               |                            |                |
| Age (yr; mean $\pm$ SD)                           | 53.86 $\pm$ 11.05 | 52.66 $\pm$ 11.42 | -0.797                     | 0.426          |
| Sex (male)  | 65                | 129               | 2.742                      | 0.098          |
| HBeAg positive                                    | 18                | 37                | 0.137                      | 0.712          |
| Serum HBV DNA loads (log <sub>10</sub> copies/mL) | 5.43 $\pm$ 1.16   | 5.25 $\pm$ 1.28   | -1.077                     | 0.283          |
| Spontaneous YMDD mutation                         | 13                | 7                 | 11.983                     | 0.001          |
| Genotype C virus                                  | 33                | 33                | 15.441                     | 0.000          |
| Genotype B virus                                  | 47                | 146               | 13.518                     | 0.000          |
| Co-infected by genotype B and C viruses           | 1                 | 4                 | -                          | -              |

HCC: Hepatocellular carcinoma; LC: Liver cirrhosis; HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus.

and 146 (79.78%) were infected by genotype B strains. The ratio of patients infected by genotype C virus and spontaneous YMDD mutation rate in the HCC group was higher than in the LC group, and these differences were significant.

#### **Ratio of patients infected by genotype B or C strains with spontaneous YMDD mutation**

There were 2 (1.09%) and 11 (13.58%) patients infected by genotype C strains with YMDD mutation in the LC ( $n = 183$ ) and HCC ( $n = 81$ ) groups, respectively. The ratio of patients infected by genotype C strains with spontaneous YMDD mutation was higher in the HCC than in the LC group, and the difference was significant ( $P = 0.000$ ). The constituent ratio difference of patients infected by genotype B strains with spontaneous YMDD mutations between the HCC and LC groups was not significant [2 (2.47%) *vs* 5 (2.73%),  $P = 1.000$ ].

#### **HCC in LC patients infected by genotype B or C strains with spontaneous YMDD mutation**

Thirteen patients were infected by genotype C strains with YMDD mutations; 11 in the HCC group and two in the LC group. Seven patients were infected by genotype B strains with YMDD mutations; two in the HCC group and five in the LC group. The occurrence of HCC was significantly higher in patients infected by genotype C strains with YMDD mutation than in patients infected by genotype B strains with YMDD mutation ( $P = 0.022$ ).

#### **Identification of risk factors for HBV-related HCC**

Unconditional logistic regression analysis was performed with the exclusion criterion  $P \geq 0.05$ . Sex, HBV genotype, and genotype C strains with spontaneous YMDD mutation were included in the regression model. As shown in Table 2, the occurrence of HCC in the male patients was 2.114 times higher than in the female patients. Patients infected by genotype C virus were 2.469 times more susceptible to HCC than those infected by genotype B virus. Risk of HCC in patients infected by genotype C strains with spontaneous YMDD mutation was 7.775-fold higher than in other patients. Other factors, including age, HBeAg status, serum HBV DNA load, spontaneous YMDD mutation, and genotype B strains with YMDD mutation, were excluded from the regression model.

## **DISCUSSION**

Prior research has suggested that lamivudine is the major cause of YMDD mutation in HBV P-ORF. However, the mechanism remains unclear. Further research has revealed that strains with YMDD mutation also exist in patients with chronic HBV infection not previously treated with lamivudine<sup>[17-20]</sup>. Hosaka *et al*<sup>[21]</sup> have suggested that lamivudine-related YMDD mutation is an independent risk factor for HCC. Our results showed that spontaneous YMDD mutations were detected in LC and HCC patients, and spontaneous YMDD mutation rate in HCC patients was significantly higher than in LC patients. This suggests that spontaneous YMDD mutations are associated with the occurrence of HCC. Unlike the study of Hosaka *et al*<sup>[21]</sup>, our unconditional logistic regression analysis showed that spontaneous YMDD mutation was not an independent risk factor for HBV-related HCC. One possible reason for this difference is that we studied the effect of spontaneous YMDD mutations, and the carcinogenicity of HBV strains with spontaneous YMDD mutations and lamivudine-related YMDD mutations may be different. This needs to be studied further.

Previous research has revealed that HBV-related LC is a leading cause of HCC<sup>[22-24]</sup>. Infection with genotype C strains can induce continuous gangrenous inflammation in the host liver and increase the risk of LC and HCC<sup>[25]</sup>. Our results showed that the infection rate of genotype C strains in the HCC group was higher than in the LC group. This suggests that infection by genotype C strains is associated with HCC. Consistent with previous reports, our unconditional logistic regression showed that the risk of HCC in patients infected by genotype C strains was 2.469 times higher than in those infected by genotype B strains, and infection by genotype C strains was an independent risk factor for HCC. This may have been caused by different genotype strains having different biological properties, pathogenicity and carcinogenicity.

Our research revealed that the ratio of patients infected by genotype C strains with spontaneous YMDD mutation was higher in the HCC than LC group. Unconditional logistic regression analysis showed that patients infected by genotype C strains with spontaneous YMDD mutations were 7.775-fold more susceptible to HCC than patients infected by genotype B strains with or without spontaneous YMDD mutations and genotype C strains

**Table 2** Multivariate regression analysis for occurrence of hepatitis B virus-related hepatocellular carcinoma

| Related factors                                | B     | SE    | Wald  | P value | Exp (B) | 95%CI        |
|--|-------|-------|-------|---------|---------|--------------|
| Sex (male)                                     | 0.749 | 0.353 | 4.500 | 0.034   | 2.114   | 1.059-4.224  |
| HBV genotype C strain                          | 0.904 | 0.335 | 7.257 | 0.007   | 2.469   | 1.279-4.764  |
| Spontaneous YMDD mutation in genotype C strain | 2.051 | 0.826 | 6.161 | 0.013   | 7.775   | 1.540-39.264 |

HBV: Hepatitis B virus.

without spontaneous YMDD mutations. This suggests that infection by genotype C strains with spontaneous YMDD mutations is an independent risk factor for HCC. Spontaneous YMDD mutation and formation of different genotype strains are the result of mutation in the HBV genome. Mutations in the reverse transcriptase domain and different HBV genotypes may result in changes in amino acid sequence and protein configuration in HBV polymerase. These may change HBV biological properties, influence the process of HBV-related diseases, and increase HBV carcinogenicity. Previous research has shown that mutated HBV may be easier to integrate into host hepatocytes. The integration may lead to chromosome mutation in host cells and increase host chromosomal instability. As a result, chromosome repeat, inversion, deletion and translocation can be detected in many HCC cells. Virus integration may activate many proto-oncogenes and cause mutations in anti-oncogenes. The activation of proto-oncogenes and repression of anti-oncogenes causes loss of control of cell proliferation and differentiation and results in the formation of cell clusters with accelerated division and malignant transformation<sup>[26,27]</sup>.

To the best of our knowledge, this is the first study to show that infection by genotype C strains with spontaneous YMDD mutation is an independent risk factor for the development of HCC in patients with HBV-related LC. Our results pave the way for exploring the molecular biological mechanism of HCC and have important clinical value for early warning of HBV-related HCC.

## COMMENTS

### Background

YMDD motif is a highly conserved sequence in the C zone of the reverse-transcriptase domain of hepatitis B virus (HBV) polymerase. The YMDD motif is the binding site for lamivudine to interfere with the replication of HBV. YMDD mutation, also known as M204V/I mutation, is the substitution of methionine by valine or isoleucine and designated as YVDD or YIDD variant. YMDD mutation, caused by amino acid substitution, leads to a change in protein configuration, abolishes its binding affinity with lamivudine, and weakens the antiviral activity of lamivudine. The emergence of YMDD mutation can induce a series of symptoms including elevation of serum alanine aminotransferase level, positive conversion of serum HBV DNA, and lamivudine resistance. Resistance to lamivudine increases in parallel with the duration of treatment and affects clinical application of the drug. These findings have made YMDD mutation a research hotspot globally.

### Research frontiers

In order to investigate the association between infection by different genotype strains with spontaneous YMDD mutation and occurrence of HBV-related hepatocellular carcinoma (HCC), 264 cirrhosis patients not previously treated with antiviral drugs, including 81 with HCC, were included in our research.

### Innovations and breakthroughs

Until recently, most research about YMDD mutations have focused on the oc-

currence of lamivudine-related mutations and their effect on antiviral treatment. The relationship between spontaneous YMDD mutation and HBV-related HCC and the association between infection by different HBV genotype strains with spontaneous YMDD mutations and the occurrence of HCC in HBV-related liver cirrhosis patients have rarely been reported.

### Applications

It is believed that our study is the first to identify infection by genotype C strains with spontaneous YMDD mutation as an independent risk factor for the development of HCC in HBV-related liver cirrhosis. These results pave the way for exploring the molecular biological mechanism of HCC and have important clinical value for early warning of HBV-related HCC.

### Terminology

Spontaneous YMDD mutations: Tyrosine (Y)-methionine (M)-aspartic acid (D)-aspartic acid (D) (YMDD) mutation occurs in the absence of known mutagens, such as antiviral drugs. YMDD motif mutations can naturally occur in chronic HBV patients without antiviral treatment.

### Peer review

This is a case control study. The case and control group should at least have similar characteristics in age, HBV viral load, and hepatitis status (HBV carrier, chronic hepatitis B, liver cirrhosis), otherwise the statistical analysis would be biased.

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P- Reviewer Lee CZ S- Editor Song XX L- Editor A  
E- Editor Li JY



## Clinical prognostic factors for disabling Crohn's disease: A systematic review and meta-analysis

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Received: December 1, 2012 Revised: December 20, 2012

Accepted: February 7, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To identify demographic and clinical factors associated with disabling Crohn's disease (CD).

**METHODS:** A systematic review and meta-analysis

of observational studies, focusing on the factors that can predict the prognosis of different outcomes of CD was undertaken. PubMed, ISI Web of Knowledge and Scopus were searched to identify studies investigating the above mentioned factors in adult patients with CD. Studies were eligible for inclusion if they describe prognostic factors in CD, with inclusion and exclusion criteria defined as follows. Studies with adult patients and CD, written in English and studying association between clinical factors and at least one prognosis outcome were included. Meta-analysis of effects was undertaken for the disabling disease outcome, using odds ratio (OR) to assess the effect of the different factors in the outcome. The statistical method used was Mantel-Haenszel for fixed effects. The 16-item quality assessment tool (QATSDD) was used to assess the quality of the studies (range: 0-42).

**RESULTS:** Of the 913 papers initially selected, sixty studies were reviewed and three were included in the systematic review and meta-analysis. The global QATSDD scores of papers were 18, 21 and 22. Of a total of 1961 patients enrolled, 1332 (78%) were classified with disabling disease five years after diagnosis. In two studies, age at diagnosis was a factor associated with disabling disease five years after diagnosis. Individuals under 40 years old had a higher risk of developing disabling disease. In two studies, patients who were treated with corticosteroids on the first flare developed disabling disease five years after diagnosis. Further, perianal disease was found to be relevant in all of the studies at two and five years after diagnosis. Finally, one study showed localization as a factor associated with disabling disease five years after diagnosis, with L3 being a higher risk factor. This meta-analysis showed a significantly higher risk of developing disabling disease at five years after initial diagnosis among patients younger than 40 years of age (OR = 2.47, 95%CI: 1.74-3.51), with initial steroid treatment for first flare (OR = 2.42, 95%CI: 1.87-3.11) and with perianal disease (OR = 2.00, 95%CI: 1.41-2.85).

**CONCLUSION:** Age at diagnosis, perianal disease, initial use of steroids and localization seem to be independent prognostic factors of disabling disease.

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**Key words:** Crohn's disease; Disabling disease; Prognostic factors; Outcome; Systematic review; Meta-analysis

Dias CC, Rodrigues PP, da Costa-Pereira A, Magro F. Clinical prognostic factors for disabling Crohn's disease: A systematic review and meta-analysis. *World J Gastroenterol* 2013; 19(24): 3866-3871 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3866.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3866>

## INTRODUCTION

Crohn's disease (CD) occurs in equal proportion in both genders and its incidence has been growing worldwide in the last decades<sup>[1]</sup>. CD is a disabling disease affecting psychological, familial, and social dimensions of life<sup>[2]</sup>. Therefore, the need to develop a specific instrument able to evaluate disabilities and identify specific factors as predictors is paramount. This is particularly true since in the last decades the medical treatment options have been dramatically changed. Other strategies are now approaching, namely accelerate step-up and top-down treatment<sup>[3]</sup>. The top-down strategy is based on the very early use of intensive therapy (immunosuppressants and/or biologics) to maintain a good quality of life from the first flare-up of the disease and prevent any irreversible consequences<sup>[3]</sup>. Therefore, it is now crucial to identify simple clinical criteria at diagnosis to predict CD outcome. This work aims to systematically review the evidence with respect to predictive clinical prognostic factors for CD.

## MATERIALS AND METHODS

A systematic review and meta-analysis of observational studies focusing on the factors that can predict the prognosis of different outcomes of CD was undertaken. The methodology included the definition of eligibility criteria, search strategies, study selection and characteristics, outcome measures, quantitative data synthesis and sensitivity analysis, methodological quality of studies, and statistical data analysis.

### Eligibility criteria

Studies that described prognostic factors in CD were eligible for inclusion. The criteria for inclusion were studies with adult patients and CD written in English and studying association between clinical factors and disabling disease. Studies not in English, without available abstract, with genetic or serologic factors, biomarker studies, or those addressing diagnosis or quality of life were excluded.

### Search strategy

The main method to search for the eligible articles was a broad literature search using PubMed with the following keywords and MeSH terms: "crohn disease"[MeSH Terms] OR "crohn"[All Fields] AND predictor [All Fields] OR predictors [All Fields] OR predict [All Fields] OR "prognostic factor" [All Fields] OR "prognostic factors" [All Fields]. Literature searches were also undertaken in Scopus database and ISI Web of Knowledge using the same search keywords: *crohn disease AND (predictors OR predict OR prognostic factors)*.

### Study selection

The studies were screened and selected by two reviewers. First, all titles and abstracts were read and the inclusion and exclusion criteria were applied. Second, the reviewers read the full text of all papers considered for inclusion after abstract selection, again applying the inclusion and exclusion criteria.

### Study characteristics

The following properties of each study were recorded: total number of patients, prognostic variables, and percentage of patients with disabling disease.

### Outcome measures

The aim of the study is to assess prognostic factors to predict disabling CD.

### Methodological quality of included studies

The 16-item quality assessment tool (QATSD), developed by Higgins *et al*<sup>[4]</sup>, was used to assess the quality of the included studies. This tool includes 16 items, scored between 0 and 3, and can be applied to different types of studies using different approaches. However, two of the items were not evaluated as they only address qualitative studies, hence we only considered a maximum score of 42.

### Statistical analysis

Statistical evidence of effects is presented as described in the original studies. Meta-analysis of effects was undertaken for the disabling disease outcome using odds ratio (OR) to assess the effect of the different factors in the outcome. The statistical method used was Mantel-Haenszel for fixed effects. All included estimates are recomputed from original articles descriptions, potentially resulting in slightly different values. All reported *P*-values are 2-sided with a significance level of 5%. Statistical heterogeneity was assessed with the *I*<sup>2</sup> statistic; values higher than 50% indicate a substantial level of heterogeneity<sup>[5]</sup>. RevMan v5.1 (The Nordic Cochrane Center, The Cochrane Collaboration, 2011) was used to calculate OR and 95%CI for disabling disease and to derived forest plots showing the results of individual studies and pooled analysis.

## RESULTS

### Search and study selection

A total of 913 articles were identified using the search

**Table 1 Characteristics of the studies**

| Ref.                                  | Country | Sample size (n) | Type of study | Disabling | Follow-up (yr) | Factor  | QATSDD |
|---------------------------------------|---------|-----------------|---------------|-----------|----------------|---|--------|
| Beaugerie <i>et al</i> <sup>[6]</sup> | France  | 1123            | Retrospective | 85.2%     | 5              | Age (under 40 yr)<br>Steroids for 1 <sup>st</sup> flare | 22     |
| Loly <i>et al</i> <sup>[7]</sup>      | Belgium | 361             | Retrospective | 57.9%     | 5              | Perianal disease<br>Steroids for 1 <sup>st</sup> flare  | 18     |
| Yang <i>et al</i> <sup>[8]</sup>      | China   | 207             | Retrospective | 71.0%     | 2              | Perianal disease<br>L3                                  | 21     |
|                                       |         |                 |               | 80.2%     | 5              | Age (under 40 yr)                                       |        |

QATSDD: Sixteen-item quality assessment tool.

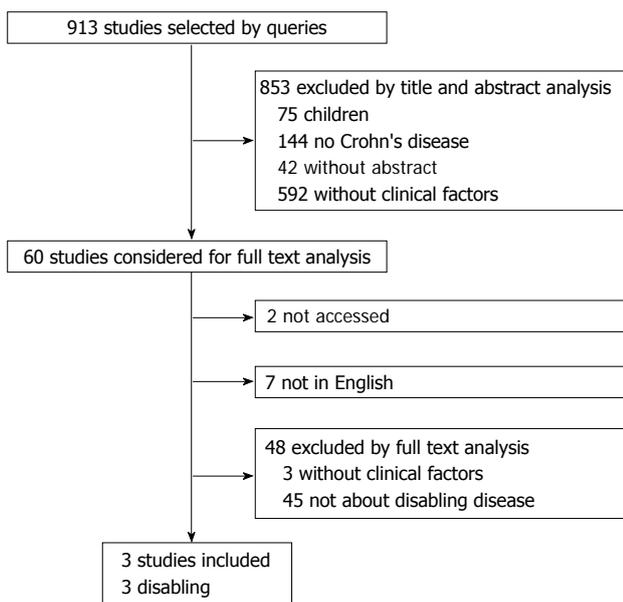


Figure 1 Flowchart of the selection process for this meta-analysis.

strategy. After reading all titles and abstracts, 853 articles were excluded (Figure 1). Sixty studies were reviewed in detail and three articles were included in the study. A new search of the literature focused on the outcome was made in order to find other papers that could have been missed by the generic search. The global QATSDD scores ranged between 18 and 22. The main characteristics of the studies are summarized in Table 1.

**Predicting factors of disabling disease**

Beaugerie *et al*<sup>[6]</sup> and Loly *et al*<sup>[7]</sup> define disabling disease by the presence of at least one of the following criteria: two steroid courses required and/or steroid dependency; further hospitalization after diagnosis for flare up or complications of the disease; chronic symptoms (diarrhea with nocturnal and/or urgent stools, intensive abdominal pain due to intestinal obstruction, fever, fatigue attributable to the disease); joint pain; painful uveitis or pyoderma gangrenous for 12 mo within the five year study; immunosuppressive therapy and intestinal resection or surgical operation for perianal disease. Yang *et al*<sup>[8]</sup> defined CD as disabling if patients satisfy at least one of the follow-

ing criteria: require two or more steroids courses and/or steroid dependency; need immunosuppressive therapy; intestinal resection or surgical operation for perianal disease and hospitalization after diagnosis for the treatment of acute exacerbation, or complication of the disease.

According to Beaugerie *et al*<sup>[6]</sup>, 957 of 1123 patients (85.2%) were classified with disabling disease. With a sample of 361 patients, Loly *et al*<sup>[7]</sup> found 209 patients (57.9%) with disabling disease, while Yang *et al*<sup>[8]</sup> found 80.2% of 207 patients with disabling disease five years after diagnosis, and 71% of patients already had disabling disease two years after diagnosis.

Different factors were found that could predict disabling disease, namely age at diagnosis, use of steroids, perianal disease, and localization.

**Age at diagnosis**

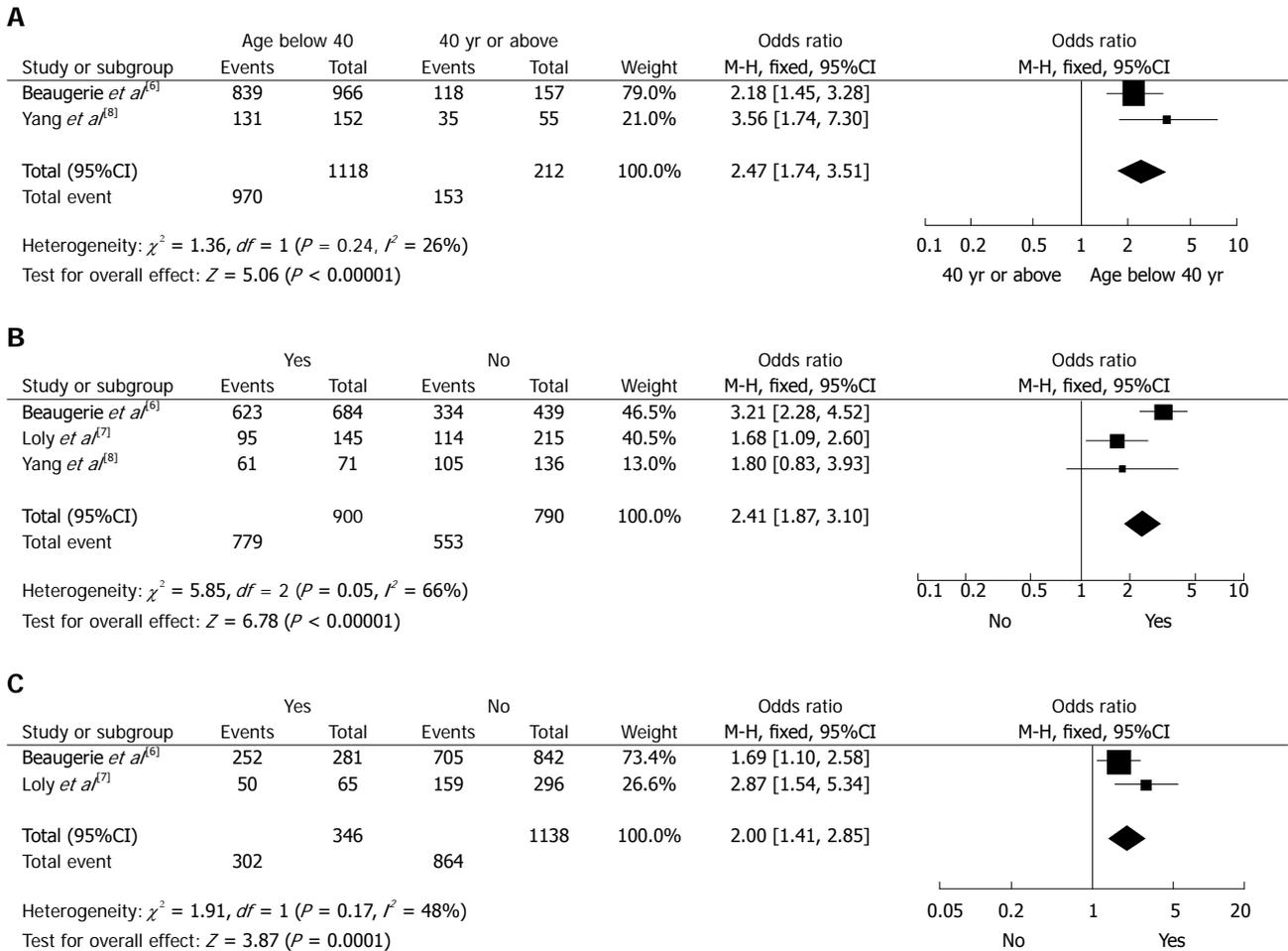
Beaugerie *et al*<sup>[6]</sup> found age at diagnosis as a factor associated with disabling disease. Patients less than 40 years old had a higher risk of developing disabling disease than older patients (OR = 2.1, 95%CI: 1.3-3.6) five years after the diagnosis. Yang *et al*<sup>[8]</sup> also showed that patients under 40 had a higher risk of developing disabling disease (OR = 3.56, 95%CI: 1.74-7.30).

Results of studies comparing younger patients (under 40) with older patients (over 40) are shown in Figure 2A. A fixed effects model shows that younger patients had a higher risk of disabling disease five years after diagnosis (OR = 2.47, 95%CI: 1.74-3.51). There was no evidence of statistical heterogeneity among the studies ( $I^2 = 26\%$ ).

**Steroids for treatment of first flare**

Both Beaugerie *et al*<sup>[6]</sup> and Loly *et al*<sup>[7]</sup> show patients who had initial requirement of steroids for treating the first flare had a higher risk of developing disabling disease five years after diagnosis when compared to those who did not require treatment (OR = 3.1, 95%CI: 2.2-4.4 and OR = 1.7, 95%CI: 1.02-2.7, respectively). Yang *et al*<sup>[8]</sup> found similar results two years after diagnosis (OR = 2.142, 95%CI: 1.068-4.298).

Results of these different studies comparing patients with and without steroid requirement treatment are presented in Figure 2B. A fixed effects model shows that patients with steroid treatment had a higher risk of disabling



**Figure 2 Predictor of disabling disease.** A: Age at diagnosis as a predictor of disabling disease; B: The use of steroids for treatment of the first flare as a predictor of disabling disease; C: Perianal disease as a predictor of disabling disease.

disease five years after diagnosis (OR = 2.41, 95%CI: 1.87-3.10). Significant heterogeneity was found among the studies ( $I^2 = 66\%$ ). Nevertheless, all studies found a higher risk of disabling disease for patients on steroids.

**Perianal disease**

In all three studies, patients with perianal disease had a higher risk of developing disabling disease five years after diagnosis when compared to patients without perianal disease: Beaugerie *et al*<sup>[6]</sup> (OR = 1.8, 95%CI: 1.2-2.8), Loly *et al*<sup>[7]</sup> (OR = 2.6, 95%CI: 1.4-5.1), Yang *et al*<sup>[8]</sup> (two years after diagnosis) (OR = 5.433, 95%CI: 1.585-18.620).

The comparison between patients with and without perianal disease is shown in Figure 2C. A fixed effects model shows the presence of perianal disease as a high risk of disabling disease five years after diagnosis (OR = 2.00, 95%CI: 1.41-3.85). There was no evidence of statistical heterogeneity among the studies ( $I^2 = 48\%$ ).

**Localization**

One study associated disabling disease to the localization of the disease. In this study, patients with L3 localization had a higher risk of developing disabling disease five years after the diagnosis (OR = 1.74, 95%CI: 1.06-2.8)<sup>[7]</sup>.

**DISCUSSION**

CD is a chronic disease with no known medical or surgical cure, requiring several appointments and hospitalizations for those afflicted. There are several reasons stressing the importance of prognostic factors: (1) Recent available drugs, namely anti-tumour necrosis factor (TNF), having the potential of inducing mucosal healing and prolonged clinical remission; (2) Mucosal healing has been considered a therapeutic goal; and (3) Early therapeutic interventions are followed by a better outcome. Therefore, it is imperative that therapeutic options are optimized.

The present systematic review and meta-analysis presented some of the factors that could help clinicians identify risk groups for disabling CD. Age, perianal disease, the use of steroids and localization were all associated with disabling disease. Although other markers can help clinicians to predict disease course of CD, namely genetic, serologic and endoscopic findings, we limited this meta-analysis to demographic and clinical characteristics due to feasibility to apply at diagnosis at the bedside.

Three studies address disabling disease and used similar definitions, although in Yang *et al*<sup>[8]</sup> the presence of chronic symptoms like diarrhea, fever, fatigue, was not

considered. All studies were retrospective and the number of patients in each study ranged from 207 to 1123<sup>[6,8]</sup>. It was clear that a large number of patients had disabling disease (range: 59%-81%), which gives an indication of the severity of the disease. Moreover, the study with the highest percentage of disabling disease included the least amount of defining characteristics<sup>[8]</sup>. Our meta-analysis showed that patients under 40 years old and patients with an initial requirement of steroids, or patients with perianal disease had a higher risk of having disabling disease five years following initial diagnosis. These results are in line with the three studies used in the meta-analysis<sup>[6-8]</sup>. Although the definition of disabling disease in Yang *et al*<sup>[8]</sup> is slightly different, this study had a lower weight in the final result of the meta-analysis, hence limiting the possible bias. Even though the effect of age at diagnosis was clear in the meta-analysis; further studies are necessary to better assess relative CD risk, including an evaluation of more patients diagnosed after the age threshold. We call into question some of the recent points included in disability definition, namely steroids following the first flare, the need for immunosuppressants, and surgery. The percentage of patients treated with steroids in the first flare (65%) in Beaugerie *et al*<sup>[6]</sup> was very similar to the percentage of patients who received steroids within in the first year of disease in the North-European population-based study, however this only reflects the step-up strategy, and the population on immunosuppressants was very low<sup>[9]</sup>. Markowitz *et al*<sup>[10]</sup> showed children requiring steroids for the treatment of the first flare-up that a very early use of 6-Mercaptopurine was associated with steroid sparing and a more favorable clinical outcome in the 18-mo period following diagnosis. Similar results were observed in those treated with anti-TNF in the first two years of disease<sup>[11]</sup>. Finally, the role of early surgery in limiting ileal disease with regard to CD prognosis is also debatable. In conclusion, the risk factors analyzed in this meta-analysis should be considered when new scores or approaches are taken concerning risk factors in CD outcome, particularly when more early therapeutic approaches are imminent.

The QATSDD scale, developed by Sirriyeh *et al*<sup>[5]</sup>, allows the comparison of the quality of the included papers even when their designs are different. The included papers consistently presented low quality scores, especially considering the representativeness of the sample and the absence of a critical discussion of strengths and limitations.

The results of this study may need further confirmation due to the small number of reviewed studies and their low quality (maximum QATSDD score of 22 out of 42). Nevertheless, this work presents a step-forward in the definition of clinical predictors for disabling CD, exposing their relevance and impact in disease prognosis.

In summary, this review and meta-analysis showed that age, perianal disease and the use of steroids are associated with disabling disease. The use of these factors in building predictive models for CD prognosis could enhance the initial clinical approach, and therefore improve the clinical

outcome of patients with severe disease. However, more elaborate and precise definitions of disabling and severe disease are needed.

## COMMENTS

### Background

Crohn's disease (CD) occurs in equal proportion in both genders and its incidence has been growing worldwide in the last decades. CD is a disabling disease affecting psychological, familial, and social dimensions of life. Therefore, the need to develop a specific instrument able to evaluate disabilities and identify specific factors as predictors is paramount.

### Research frontiers

The top-down strategy is based on the very early use of intensive therapy (immunosuppressants and/or biologics) to maintain a good quality of life from the first flare-up of the disease and prevent any irreversible consequences. Therefore, it is now crucial to identify simple clinical criteria at diagnosis to predict CD outcome.

### Innovations and breakthroughs

This work aims to systematically review the evidence with respect to predictive clinical prognostic factors for CD.

### Applications

This review and meta-analysis showed that age, perianal disease and the use of steroids are associated with disabling disease. The use of these factors in building predictive models for CD prognosis could enhance the initial clinical approach, and therefore improve the clinical outcome of patients with severe disease. However, more elaborate and precise definitions of disabling and severe disease are needed.

### Peer review

It is one of the first work searching the role of CD in disability of the patient. The authors performed an extensive review of multiple manuscript related with the topic. The manuscript is very well prepared and written and can be accepted for publication.

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**P- Reviewers** Perakath B, Rodriguez DC

**S- Editor** Gou SX **L- Editor** A **E- Editor** Xiong L



## Meta-analysis of radiofrequency ablation in combination with transarterial chemoembolization for hepatocellular carcinoma

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Received: December 11, 2012 Revised: March 12, 2013

Accepted: April 3, 2013

Published online: June 28, 2013

### Abstract

**AIM:** To compare radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE) with RFA monotherapy in hepatocellular carcinoma (HCC).

**METHODS:** We searched PubMed, Medline, Embase and Chinese databases (CBMdisc and Wanfang data) for randomized controlled trails comparing RFA plus TACE and RFA alone for treatment of HCC from January 2000 to December 2012. The overall survival rate, recurrence-free survival rate, tumor progression rate, and safety were analyzed and compared. The analysis was conducted on dichotomous outcomes and the standard meta-analytical techniques were used. Pooled odds ratios (ORs) with 95% CIs were calculated using either the fixed-effects or random-effects model. For each meta-analysis, the  $\chi^2$  and  $I^2$  tests were first calculated to assess the heterogeneity of the included trials. For  $P < 0.05$  and  $I^2 > 50\%$ , the assumption of homogeneity was deemed invalid, and the random-effects model was

used; otherwise, data were assessed using the fixed-effects model. All statistical analysis was conducted using Review Manager (version 4.2.2.) from the Cochrane collaboration.

**RESULTS:** Eight randomized controlled trials were identified as eligible for inclusion in this analysis and included 598 patients with 306 treated with RFA plus TACE and 292 with RFA alone. Our data analysis indicated that RFA plus TACE was associated a significantly higher overall survival rate (OR<sub>1-year</sub> = 2.96, 95%CI: 1.84-7.74,  $P < 0.001$ ; OR<sub>2-year</sub> = 3.72, 95%CI: 1.24-11.16,  $P = 0.02$ ; OR<sub>3-year</sub> = 2.65, 95%CI: 1.81-3.86,  $P < 0.001$ ) and recurrence-free survival rate (OR<sub>3-year</sub> = 3.00, 95%CI: 1.75-5.13,  $P < 0.001$ ; OR<sub>5-year</sub> = 2.26, 95%CI: 1.43-3.57,  $P = 0.0004$ ) vs that of RFA alone. The tumor progression rate in patients treated with RFA alone was higher than that of RFA plus TACE (OR = 0.60, 95%CI: 0.42-0.88,  $P = 0.008$ ) and there was no significant difference on major complications between two different kinds of treatment (OR = 1.20, 95%CI: 0.31-4.62,  $P = 0.79$ ). Additionally, the meta-analysis data of subgroups revealed that the survival rate was significantly higher in patients with intermediate- and large-size HCC underwent RFA plus TACE than in those underwent RFA monotherapy; however, there was no significant difference between RFA plus TACE and RFA on survival rate for small HCC.

**CONCLUSION:** The combination of RFA with TACE has advantages in improving overall survival rate, and provides better prognosis for patients with intermediate- and large-size HCC.

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**Key words:** Radiofrequency ablation; Transcatheter arterial chemoembolization; Hepatocellular carcinoma; Meta-analysis

**Core tip:** This study aimed to compare the effectiveness and prognosis of combination of transcatheter

arterial chemoembolization (TACE) and radiofrequency ablation (RFA) with that of RFA alone in hepatocellular carcinoma (HCC). To the best of our knowledge, there has been no comprehensive comparison on these two treatments in terms of small-, intermediate- and large-size HCC. Our analysis demonstrated that effectiveness of TACE combined with RFA was better than that of RFA for treatment of intermediate- and large-size HCC. We provide important evidence that TACE-RFA for intermediate- and large-size HCC may be performed more widely in clinical practice.

Ni JY, Liu SS, Xu LF, Sun HL, Chen YT. Meta-analysis of radiofrequency ablation in combination with transarterial chemoembolization for hepatocellular carcinoma. *World J Gastroenterol* 2013; 19(24): 3872-3882 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3872.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3872>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies. HCC ranks fifth for men and eighth for women and accounts for > 660000 new cases annually worldwide<sup>[1-3]</sup>. Due to poor baseline liver function, over tumor burden, or hepatic vessel invasion of HCC patients, it is barely possible to perform surgical resection. Transcatheter arterial chemoembolization (TACE) as a palliative therapy has become one of the most widely performed treatments for unresectable HCC<sup>[4,5]</sup>. However, the complete necrosis rate of tumors after TACE have only reached 10%-20%, and the 1-, 3- and 5-year overall survival rates range from 49%-71.9% to 23%-62.5% and 9%-17% in most studies<sup>[6-11]</sup>. Radiofrequency ablation (RFA) as a thermal *in situ* destruction technique has been proved to be a safe and effective treatment. RFA has been accepted as one of the best treatment options for small HCC<sup>[12,13]</sup>. However, it is difficult for RFA to achieve complete ablation in the treatment of relatively large HCC. Therefore, novel approaches to treating HCC patients have been extensively pursued and may offer opportunities for longer survival of patients with HCC. In recent years, the combination of interventional therapies has been widely performed for treatment of HCC. One such combined strategy is the combination of RFA and TACE.

Previous studies have reported that combination of RFA and TACE is more effective for induction of a significantly higher complete tumor necrosis rate than RFA monotherapy is, and improves overall survival rate in patients with HCC<sup>[14-16]</sup>. However, other studies assessing the clinical efficacy of RFA plus TACE and RFA alone for treatment of HCC have reported conflicting outcomes<sup>[17-19]</sup>. Hence, whether RFA combined with TACE or RFA monotherapy is the better treatment choice for HCC has long been debated. Meta-analysis is a suitable method to resolve this conflict. Several randomized

controlled trials have been published in an attempt to answer the above question. A meta-analysis of these trials to analyze and compare comprehensively the clinical efficacy and safety of RFA combined with TACE and RFA monotherapy will provide clinicians with an unbiased opinion and valuable information about the efficacy of these treatment options. Comparison of these two treatments could help stratify the benefits of treatment choices for patients with HCC. Hence, this meta-analysis was designed to compare comprehensively the efficacy and safety of combination of RFA and TACE with RFA monotherapy for treatment of patients with HCC.

## MATERIALS AND METHODS

### Study selection

A search of the literature was conducted in PubMed, Medline, Embase and Chinese databases (CBMdisc and Wanfang data) from January 2000 to December 2012, using the following MeSH search headings: “hepatocellular carcinoma”, “radiofrequency ablation” and “transcatheter arterial chemoembolization”. A limit was set on the randomized controlled trials, which was conducted to identify studies comparing the effectiveness and safety of the combination of RFA and TACE with that of RFA monotherapy for HCC. No language restriction was imposed in this search.

### Criteria for inclusion and exclusion

To be eligible for the present meta-analysis, studies were required to have an integrated baseline of patients and outcomes: (1) study design: randomized controlled trials on RFA plus TACE *vs* RFA monotherapy in the treatment of HCC; (2) baseline of population: randomization of no fewer than 30 formally diagnosed HCC patients with average age, percentage male, Child-Pugh class, tumor size, and tumor stage; and (3) results: studies were required to have good descriptions of the results for overall survival rate, recurrence-free survival rate, tumor progression rate, and major complications. Abstracts, letters, reviews without original data, expert opinions, editorials, case reports and studies lacking control groups were excluded from the analysis.

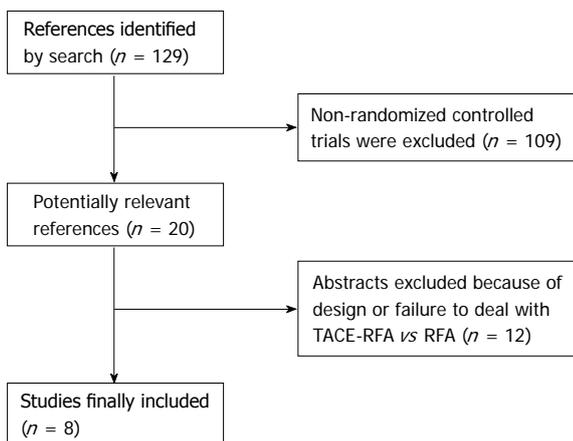
### Statistical analysis

All analysis was conducted on dichotomous outcomes and the standard meta-analytical techniques were used. Pooled odds ratios (ORs) with 95% CIs were calculated using either the fixed-effects or random-effects model. For each meta-analysis, the  $\chi^2$  and  $I^2$  tests were first calculated to assess the heterogeneity of the included trials.  $P < 0.05$  and  $I^2 > 50\%$  was considered significant. For  $P < 0.05$  and  $I^2 > 50\%$ , the assumption of homogeneity was deemed invalid, and the random-effects model was used; otherwise, data were assessed using the fixed-effects model. The risk of the publication bias of the included trials was assessed using the symmetry of the funnel plot. Statistical analysis was performed using the

**Table 1** Baseline characteristics of the trials included in the meta-analysis (mean  $\pm$  SD)

| Ref.                                  | Country | Design | Treatment  | No. of patients | Age (yr)               | Sex (male/female) | Tumor size (cm)                | Child-Pugh class (A/B/C) |
|---------------------------------------|---------|--------|------------|-----------------|------------------------|-------------------|--------------------------------|--------------------------|
| Peng <i>et al</i> <sup>[14]</sup>     | China   | RCT    | TACE + RFA | 69              | 57.5 $\pm$ 10.0        | 60/9              | $\leq$ 5.01                    | 60/9/0                   |
| Cheng <i>et al</i> <sup>[15]</sup>    | China   | RCT    | TACE + RFA | 70              | 55.1 $\pm$ 9.5         | 55/15             | -                              | 59/11/0                  |
|                                       |         |        | RFA        | 100             | $\leq$ 75 <sup>1</sup> | NA                | 3 < TS $\leq$ 7.5 <sup>1</sup> | NA                       |
| Yang <i>et al</i> <sup>[16]</sup>     | China   | RCT    | TACE + RFA | 24              | 59.1 $\pm$ 11.4        | 18/6              | 6.6 $\pm$ 0.6                  | NA                       |
|                                       |         |        | RFA        | 12              | 61.0 $\pm$ 10.4        | 8/4               | 5.2 $\pm$ 0.4                  | NA                       |
| Shibata <i>et al</i> <sup>[18]</sup>  | Japan   | RCT    | TACE + RFA | 46              | 67.2 $\pm$ 8.9         | 31/15             | 1.7 $\pm$ 0.6                  | 32/14/0                  |
|                                       |         |        | RFA        | 43              | 69.8 $\pm$ 8.0         | 33/10             | 1.6 $\pm$ 0.5                  | 33/10/0                  |
| Morimoto <i>et al</i> <sup>[20]</sup> | Japan   | RCT    | TACE + RFA | 19              | 70 (57-78)             | 15/4              | 3.6 $\pm$ 0.7                  | 12/7/0                   |
|                                       |         |        | RFA        | 18              | 73 (48-84)             | 12/6              | 3.7 $\pm$ 0.6                  | 16/2/0                   |
| Kang <i>et al</i> <sup>[22]</sup>     | China   | RCT    | TACE + RFA | 19              | 52.2                   | 14/5              | 6.7 $\pm$ 1.1                  | 12/7/0                   |
|                                       |         |        | RFA        | 18              | 50.7                   | 14/4              | 6.2 $\pm$ 1.2                  | 12/6/0                   |
| Shen <i>et al</i> <sup>[23]</sup>     | China   | RCT    | TACE + RFA | 18              | 52.7 (20-72)           | 5/13              | 5.6 (2.2-15.8)                 | 4/14/0                   |
|                                       |         |        | RFA        | 16              | 56.1 (36-75)           | 3/13              | 5.0 (2.3-12.3)                 | 6/10/0                   |
| Zhang <i>et al</i> <sup>[24]</sup>    | China   | RCT    | TACE + RFA | 15              | 57.8 (39-72)           | 12/3              | 4.6 (2.3-7.1)                  | NA                       |
|                                       |         |        | RFA        | 15              | 61.8 (38-78)           | 13/2              | 4.1 (2.4-6.0)                  | NA                       |

<sup>1</sup>Total data of relative study. RCT: Randomized controlled trial; NA: Not applicable; TS: Tumor size; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.



**Figure 1** Flow chart of searching strategy for study inclusion. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

software programs Review manager (version 4.2.2.) from the Cochrane collaboration.  $P < 0.05$  was considered significant.

## RESULTS

### Selection of trials

This meta-analysis yielded a total of 129 studies. Based on the inclusion and exclusion criteria, we included eight randomized controlled trials<sup>[14,15,18,20-24]</sup> (Figure 1). There was a total of 598 patients with 306 treated with RFA plus TACE and 292 treated with RFA alone (Table 1). Among these studies, there were eight, three, six and two studies that reported comparative data for overall survival rate at 1, 2, 3 and 5 years, respectively; five, three and two studies reported comparative data for recurrence-free survival rate at 1, 3 and 5 years, respectively (Tables 2 and 3). In the small HCC (tumor size  $\leq$  3 cm) subgroup, there were two studies with comparative data on survival rate at

1 and 3 years, respectively. In the intermediate-size HCC (3 cm < tumor size  $\leq$  5 cm) subgroup, there were four, three and two studies with comparative data on survival rate at 1, 3 and 5 years, respectively. In the large-size HCC (tumor size > 5 cm) subgroup, there were four and three studies with comparative data on survival rate at 1 and 3 years, respectively (Tables 2 and 3). There were six and three studies with comparative data on tumor progression rate and major complications, respectively (Tables 2 and 3). The quality of the included studies was detected using Review manager (version 4.2.2.) programs, and was judged to be high quality (Figure 2A-D).

### Meta-analysis results

**Heterogeneity assessment:** In the analysis of the effects of 1-, 2- and 3-year overall survival rates ( $P_{1\text{-year}} = 0.77$ ,  $I^2 = 0\%$ ;  $P_{2\text{-year}} = 0.56$ ,  $I^2 = 0\%$ ;  $P_{3\text{-year}} = 0.33$ ,  $I^2 = 13.1\%$ ); 1-, 3- and 5-year recurrence-free survival rates ( $P_{1\text{-year}} = 0.39$ ,  $I^2 = 3.5\%$ ;  $P_{3\text{-year}} = 0.60$ ,  $I^2 = 0\%$ ;  $P_{5\text{-year}} = 0.37$ ,  $I^2 = 0\%$ ); tumor progression rates ( $P = 0.18$ ,  $I^2 = 34.8\%$ ); major complications ( $P = 0.91$ ,  $I^2 = 0\%$ ), the meta-analysis data indicated that there was no significant heterogeneity among the studies, thus the fixed-effects model was used to pool the results. However, in the analysis of the effect of 5-year overall survival rates, there was significant heterogeneity among the studies ( $P_{5\text{-year}} = 0.03$ ,  $I^2 = 79.3\%$ ), thus the random-effects model was used to pool the results (Figures 2 and 3).

**Overall survival rate:** There was a significant difference in 1-, 2- and 3-year overall survival rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA plus TACE was associated with a significantly higher 1-, 2- and 3-year overall survival rate than RFA monotherapy was (OR<sub>1-year</sub> = 2.96, 95%CI: 1.84-7.74,  $P < 0.001$ ; OR<sub>2-year</sub> = 3.72, 95%CI: 1.24-11.16,  $P = 0.02$ ; OR<sub>3-year</sub> = 2.65, 95%CI: 1.81-3.86,  $P < 0.001$ ).

**Table 2** Prognosis of patients reported in the trials included in the meta-analysis

| Ref.                                  | Treatment | No. of patients | Recurrence-free survival rate |        |        | Overall survival rate |         |        |        |
|---------------------------------------|-----------|-----------------|-------------------------------|--------|--------|-----------------------|---------|--------|--------|
|                                       |           |                 | 1 yr                          | 3 yr   | 5 yr   | 1 yr                  | 2 yr    | 3 yr   | 5 yr   |
| Peng <i>et al</i> <sup>[14]</sup>     | TACE+RFA  | 69              | 80.00%                        | 45.00% | 40.00% | 94.00%                | NA      | 69.00% | 46.00% |
|                                       | RFA       | 70              | 64.00%                        | 18.00% | 18.00% | 82.00%                | NA      | 47.00% | 36.00% |
| Cheng <i>et al</i> <sup>[15]</sup>    | TACE+RFA  | 96              | NA                            | NA     | 58.00% | 83.00%                | NA      | 55.00% | 31.00% |
|                                       | RFA       | 100             | NA                            | NA     | 42.00% | 67.00%                | NA      | 32.00% | 8.00%  |
| Yang <i>et al</i> <sup>[16]</sup>     | TACE+RFA  | 24              | 29.00%                        | NA     | NA     | 68.00%                | NA      | NA     | NA     |
|                                       | RFA       | 12              | 34.70%                        | NA     | NA     | 57.00%                | NA      | NA     | NA     |
| Shibata <i>et al</i> <sup>[18]</sup>  | TACE+RFA  | 46              | 71.30%                        | 48.80% | NA     | 100.00%               | 100.00% | 84.80% | NA     |
|                                       | RFA       | 43              | 74.30%                        | 29.70% | NA     | 100.00%               | 88.80%  | 84.50% | NA     |
| Morimoto <i>et al</i> <sup>[20]</sup> | TACE+RFA  | 19              | 67.00%                        | NA     | NA     | 100.00%               | 93.00%  | 93.00% | NA     |
|                                       | RFA       | 18              | 56.00%                        | 28.00% | NA     | 89.00%                | 89.00%  | 80.00% | NA     |
| Kang <i>et al</i> <sup>[22]</sup>     | TACE+RFA  | 19              | NA                            | NA     | NA     | 84.20%                | 42.10%  | 36.80% | NA     |
|                                       | RFA       | 18              | NA                            | NA     | NA     | 66.10%                | 22.20%  | 16.70% | NA     |
| Shen <i>et al</i> <sup>[23]</sup>     | TACE+RFA  | 18              | 63.90%                        | 50.00% | NA     | 87.50%                | NA      | 73.30% | NA     |
|                                       | RFA       | 16              | 30.00%                        | 18.70% | NA     | 52.20%                | NA      | 20.40% | NA     |
| Zhang <i>et al</i> <sup>[24]</sup>    | TACE+RFA  | 15              | NA                            | NA     | NA     | 100.00%               | NA      | NA     | NA     |
|                                       | RFA       | 15              | NA                            | NA     | NA     | 80.00%                | NA      | NA     | NA     |

NA: Not applicable; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

**Table 3** Efficacy and major complications of radiofrequency ablation-transcatheter arterial chemoembolization *vs* radiofrequency ablation for treatment of hepatocellular carcinoma

| Variables                        | No. of studies furnishing data | Results  |        | OR (95%CI)         | P value | I <sup>2</sup> |
|----------------------------------|--------------------------------|----------|--------|--------------------|---------|----------------|
|                                  |                                | RFA-TACE | RFA    |                    |         |                |
| Efficacy overall survival rate   |                                |          |        |                    |         |                |
| 1 yr                             | 8                              | 89.20%   | 76.00% | 2.96 (1.84- 7.47)  | < 0.001 | 0.00%          |
| 2 yr                             | 3                              | 85.70%   | 73.40% | 3.72 (1.24-11.16)  | 0.02    | 0.00%          |
| 3 yr                             | 6                              | 66.70%   | 45.70% | 2.65 (1.81- 3.86)  | < 0.001 | 13.10%         |
| 5 yr                             | 2                              | 37.50%   | 19.40% | 2.78 (0.85-9.12)   | 0.09    | 79.30%         |
| Recurrence-free survival rate    |                                |          |        |                    |         |                |
| 1 yr                             | 5                              | 67.60%   | 60.30% | 1.59 (0.99-2.55)   | 0.05    | 3.50%          |
| 3 yr                             | 3                              | 46.60%   | 22.40% | 3.00 (1.75-5.13)   | < 0.001 | 0.00%          |
| 5 yr                             | 2                              | 50.90%   | 32.30% | 2.26 (1.43-3.57)   | 0.0004  | 0.00%          |
| Survival rate (TS ≤ 3 cm)        |                                |          |        |                    |         |                |
| 1 yr                             | 2                              | 98.90%   | 91.00% | 8.42 (1.01- 70.56) | 0.05    | NA             |
| 3 yr                             | 2                              | 78.10%   | 71.90% | 1.35 (0.67-2.74)   | 0.4     | 0.00%          |
| Survival rate (3 cm < TS ≤ 5 cm) |                                |          |        |                    |         |                |
| 1 yr                             | 4                              | 95.30%   | 84.60% | 3.46 (1.29-9.28)   | 0.01    | 0.00%          |
| 3 yr                             | 3                              | 78.20%   | 53.90% | 3.58 (1.79-7.15)   | 0.0003  | 28.60%         |
| 5 yr                             | 2                              | 49.30%   | 15.40% | 5.34 (2.42-11.75)  | < 0.001 | 0.00%          |
| Survival rate (TS < 5 cm)        |                                |          |        |                    |         |                |
| 1 yr                             | 4                              | 75.90%   | 52.50% | 2.91 (1.60-5.29)   | 0.0004  | 0.00%          |
| 3 yr                             | 3                              | 43.10%   | 10.30% | 6.96 (3.01-16.07)  | 0.00001 | 0.00%          |
| Tumor progression rate           | 6                              | 31.60%   | 42.80% | 0.60 (0.42-0.88)   | 0.008   | 34.80%         |
| Major complications              | 3                              | 3.70%    | 3.10%  | 1.20 (0.31-4.62)   | 0.79    | 0.00%          |

NA: Not applicable; TS: Tumor size; RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization.

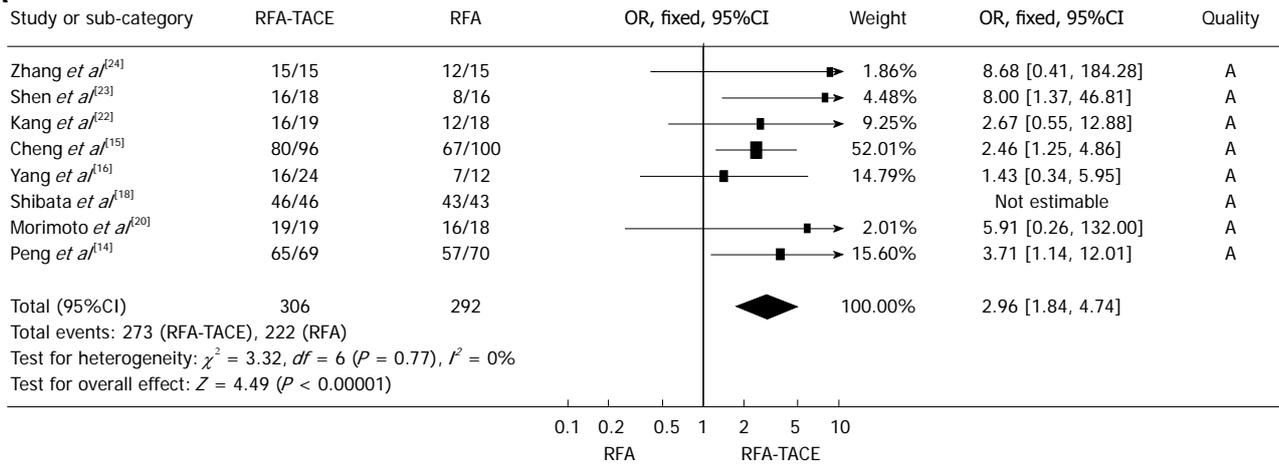
However, there was no significant difference in overall survival rate between these two treatments for 5-year overall survival rate (OR<sub>5-year</sub> = 2.78, 95%CI: 0.85-9.12, *P* = 0.09) (Figure 2A-D).

**Recurrence-free survival rate:** There was a significant difference in 3- and 5-year recurrence-free survival rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA plus TACE was associated with a significantly higher 3- and 5-year recurrence-free survival rate than RFA monotherapy was (OR<sub>3-year</sub> = 3.00, 95%CI: 1.75-5.13, *P* < 0.001;

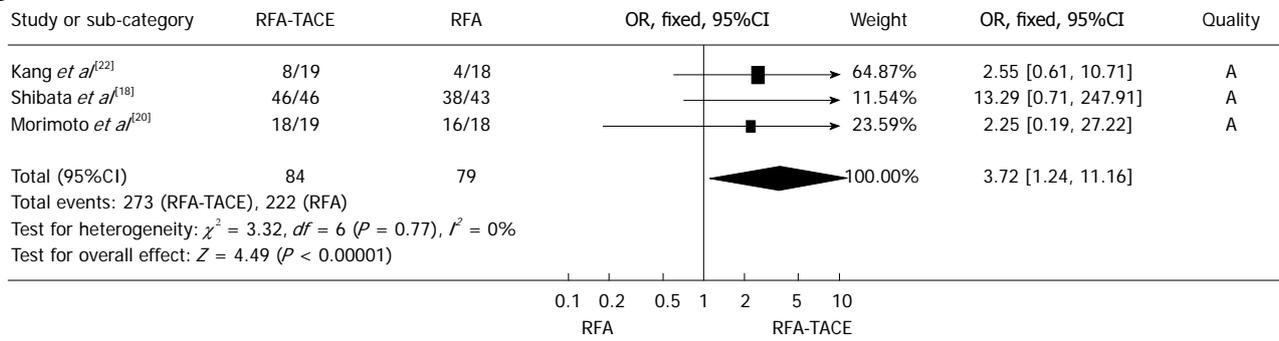
OR<sub>5-year</sub> = 2.26, 95%CI: 1.43-3.57, *P* = 0.0004). However, there was no significant difference in recurrence-free survival rate between the two treatments at 1 year (OR<sub>1-year</sub> = 1.59, 95%CI: 0.99-2.55, *P* = 0.05) (Figure 2E-G).

**Tumor progression rate:** There was a significant difference in tumor progression rate between treatment with RFA plus TACE and RFA alone, and the meta-analysis data suggested that RFA monotherapy was associated with a significantly higher tumor progression rate than RFA plus TACE treatment was (OR = 0.60, 95%CI: 0.42-0.88, *P* = 0.008) (Figure 3A).

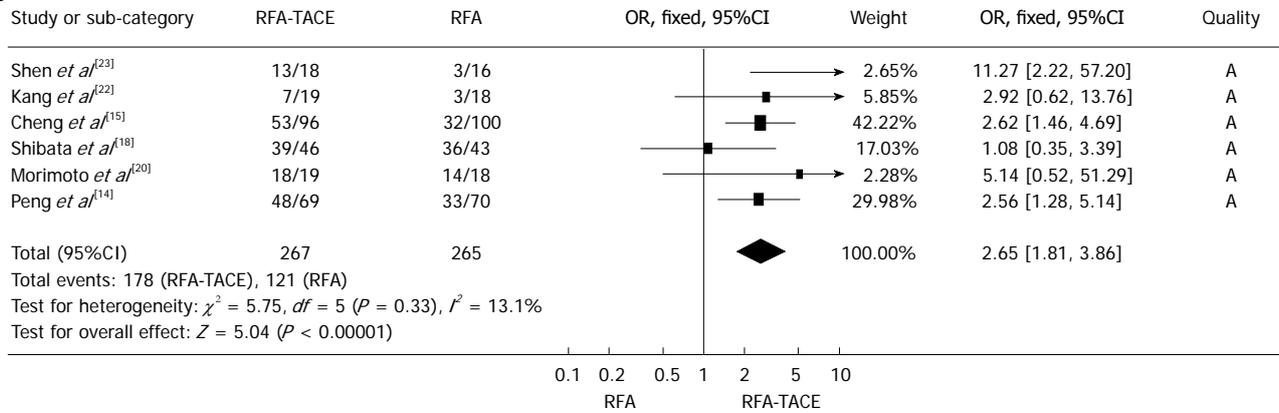
**A**



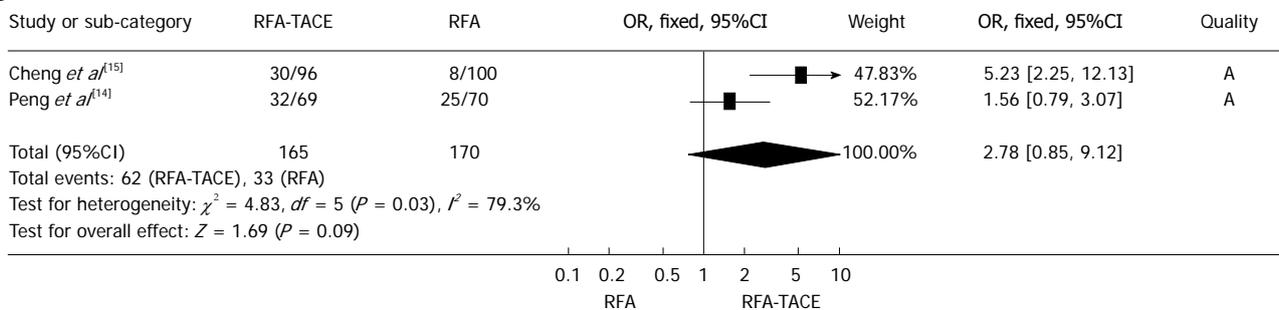
**B**



**C**



**D**



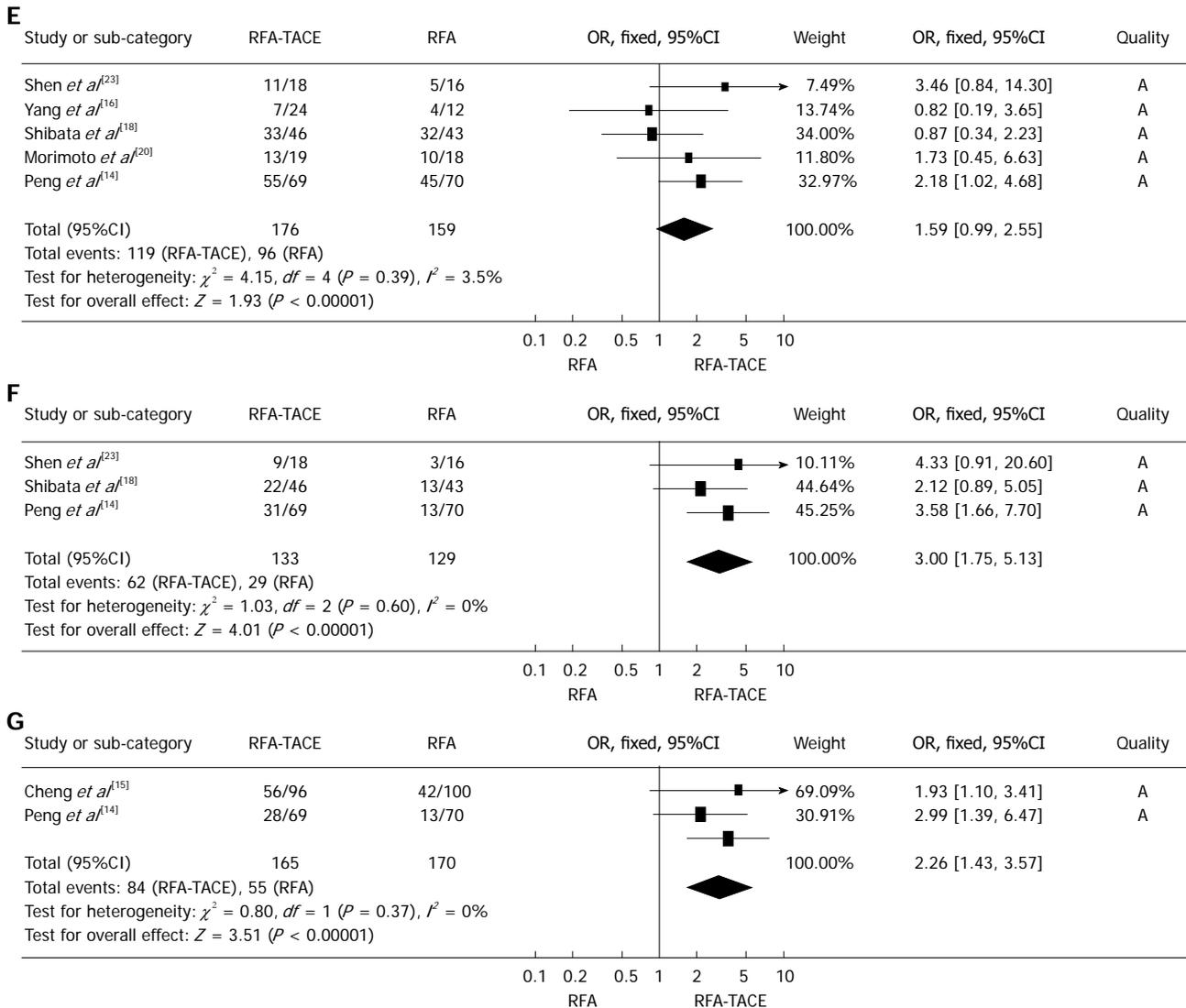


Figure 2 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for hepatocellular carcinoma in terms of overall survival rates (A-D) and recurrence-free survival rates (E-G). A, E: Meta-analysis of 1-year results; B: Meta-analysis of 2-year results; C, F: Meta-analysis of 3-year results; D, G: Meta-analysis of 5-year results. RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; OR: Odds ratio.

**Safety**

Meta-analysis data showed that there was no significant difference in major complications between treatment with RFA plus TACE and RFA monotherapy (OR = 1.20, 95%CI: 0.31-4.62,  $P = 0.79$ ) (Figure 3B).

**Comparison of survival rate in small HCCs (tumor size  $\leq 3$  cm)**

The meta-analysis data revealed that there was no significant difference in survival rate between the two treatments at 1 year (OR: 8.42, 95%CI: 1.01-70.56,  $P = 0.05$ ) and 3 years (OR: 1.35, 95%CI: 0.67-2.74,  $P = 0.40$ ) (Figure 4A and B).

**Comparison of survival rate in intermediate-size HCCs (3cm < tumor size  $\leq 5$ cm)**

There was a significant difference in survival rate between

the two treatments at 1, 3 and 5 years, and the meta-analysis data indicated that RFA plus TACE was associated with a significantly higher 1-, 3- and 5-year survival rate than RFA monotherapy was (OR<sub>1-year</sub> = 3.46, 95%CI: 1.29-9.28,  $P = 0.01$ ; OR<sub>3-year</sub> = 3.58, 95%CI: 1.79-7.15,  $P = 0.0003$ ; OR<sub>5-year</sub> = 5.34, 95%CI: 2.42-11.75,  $P < 0.001$ ) (Figure 4C-E).

**Comparison of survival rate in large-size HCCs (tumor size < 5 cm)**

There was a significant difference in survival rate between the two treatments at 1 and 3 years, and the meta-analysis data revealed that RFA plus TACE was associated with a significantly higher 1- and 3-year survival rate than RFA monotherapy was (OR<sub>1-year</sub> = 2.91, 95%CI: 1.60-5.29,  $P = 0.0004$ ; OR<sub>3-year</sub> = 6.69, 95%CI: 3.01-16.07,  $P < 0.001$ ) (Figure 4F and G).

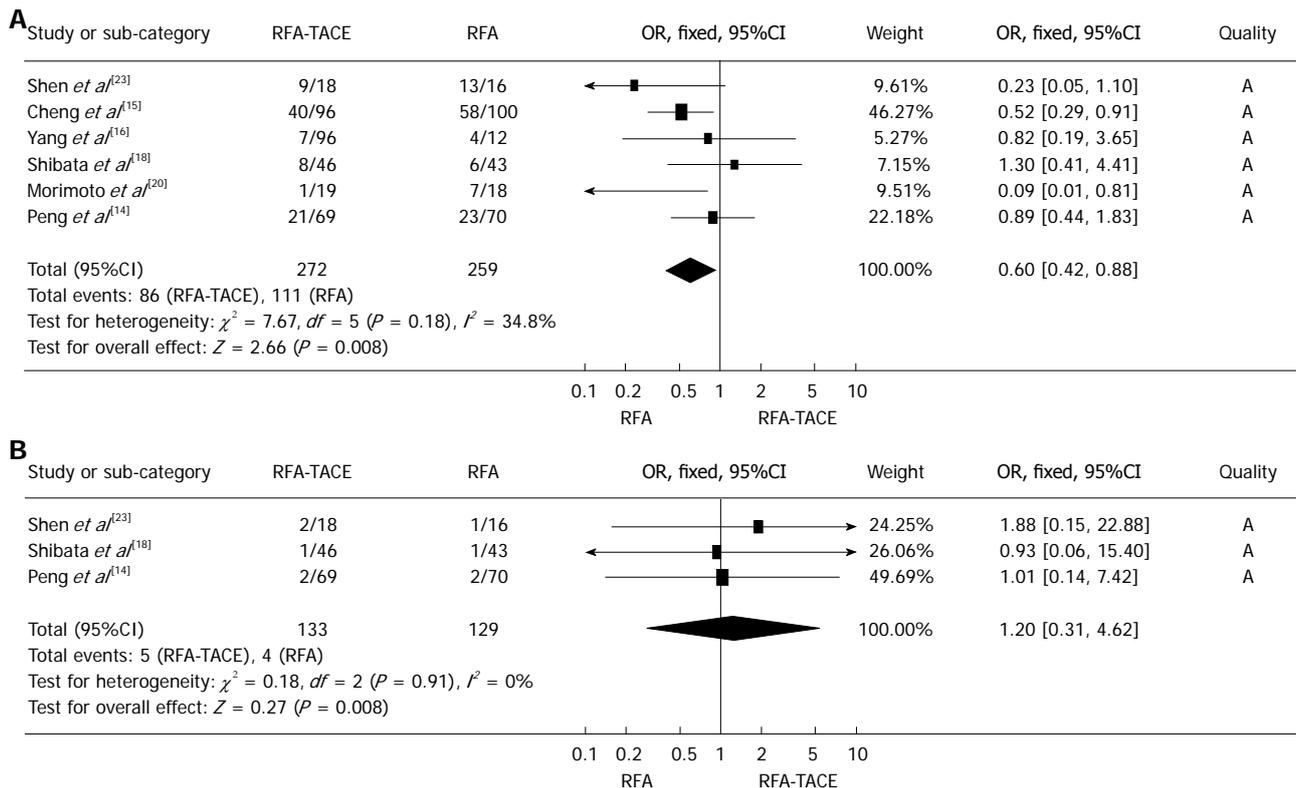


Figure 3 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for hepatocellular carcinoma in terms of tumor progression rate (A) and major complications (B). RFA: Radiofrequency ablation; TACE: Transcatheter arterial chemoembolization; OR: Odds ratio.

### Assessment of publication bias

The publication bias in this study was detected using the funnel plot of the meta-analysis results. All of the included studies reported comparative data on 1-year overall survival rate. In the analysis of the effect of 1-year overall survival rate, the symmetry of the funnel plot shape suggested that there was no obvious publication bias in this meta-analysis (Figure 5).

## DISCUSSION

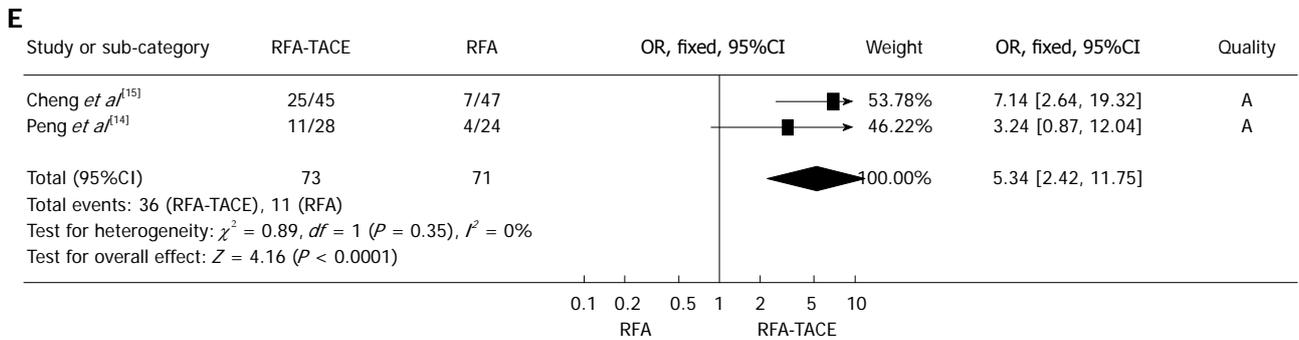
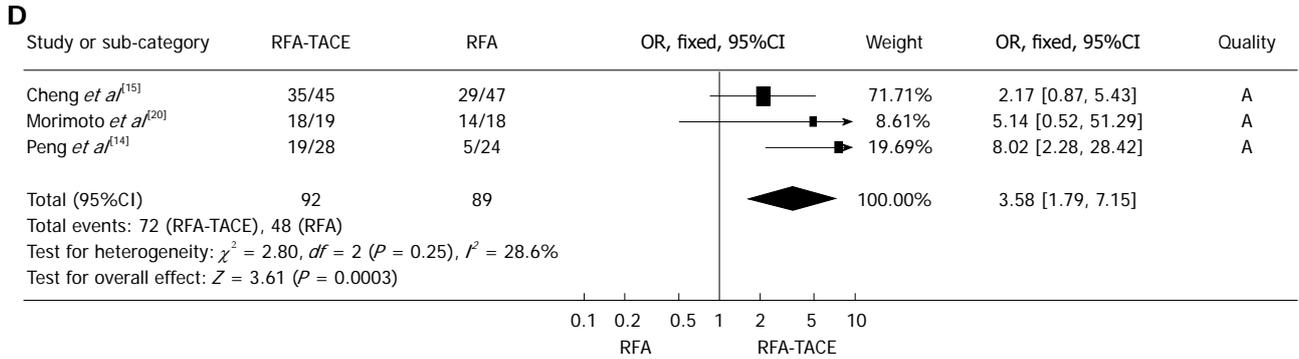
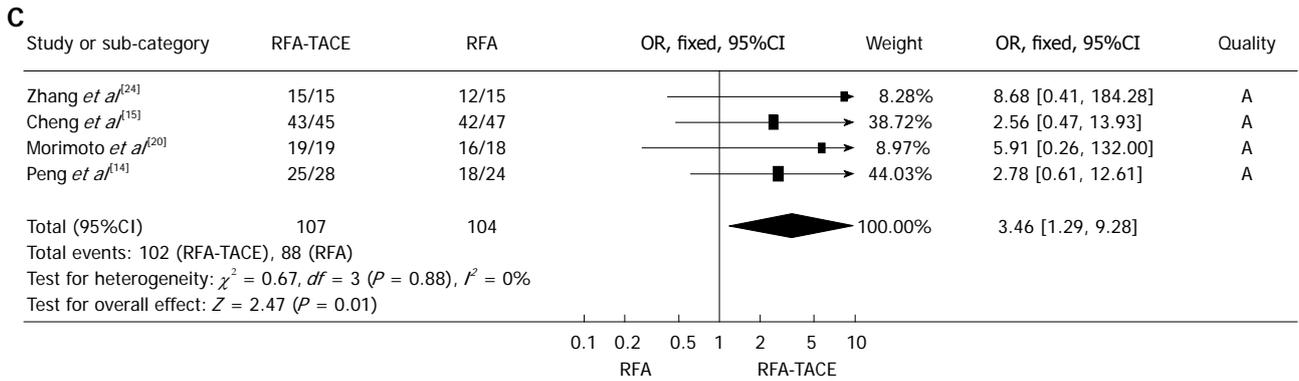
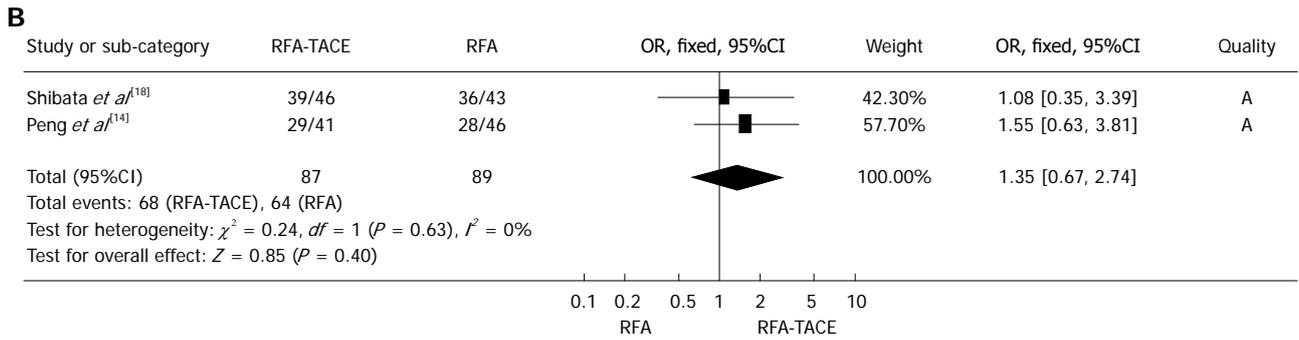
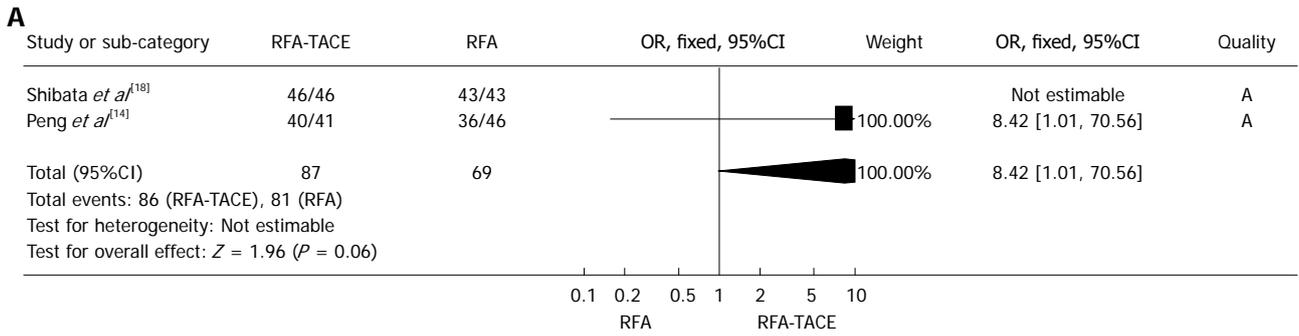
According to our data analysis, we found that the combination of TACE and RFA was associated with higher overall and recurrence-free survival rates than RFA monotherapy was in the treatment of HCC. Additionally, the subgroups analysis indicated that although there were no significant differences between TACE plus RFA and RFA alone in the treatment of small-size HCC, combination of TACE and RFA was associated with a higher survival rate than was RFA monotherapy for patients with intermediate- and large-size HCC. Thus, our analysis suggested that TACE combined with RFA was more effective than RFA monotherapy for treatment for intermediate- and large-size HCC.

Previous studies have reported that RFA combined with TACE is more effective than RFA monotherapy for treatment of HCC<sup>[14-16]</sup>. However, some other studies have reported conflicting results<sup>[17-19]</sup>. Meta-analysis is a method that combines data from all eligible studies, and

has the advantage of reducing random error, thus obtaining more precise estimates and defining the effect of clinical interventions more precisely<sup>[25,26]</sup>.

Tumor recurrence and progression are the major risk factors that affect the prognosis of HCC patients. A high rate of intrahepatic recurrence and progression after RFA plus TACE and/or RFA is the main cause of late death of patients with HCC. In the current analysis, the recurrence-free survival and progression rates were compared and analyzed. Our meta-analysis indicated that the 1-, 3- and 5-year recurrence-free survival rate was higher after RFA plus TACE than after RFA alone. However, RFA monotherapy was found to be associated with a higher tumor progression rate than RFA plus TACE was. The residual tumor tissue after RFA was the main cause of tumor recurrence and progression, which may be attributable to insufficient ablation of the primary tumor after RFA monotherapy. RFA combined with TACE has advantages in improving tumor necrosis rate. TACE is expected to reduce the cooling effect of hepatic blood flow in RFA by decreasing hepatic arterial flow, and plays a primary role in inducing tumor destruction<sup>[14,20,21]</sup>. Additionally, RFA cannot be a suitable treatment for tumors with multiple nodules, and TACE after RFA can effectively induce necrosis of multiple nodules and improve the tumor necrosis rate. This may explain the better results following RFA plus TACE in the treatment of HCC.

As regards the subgroups in our analysis, we found



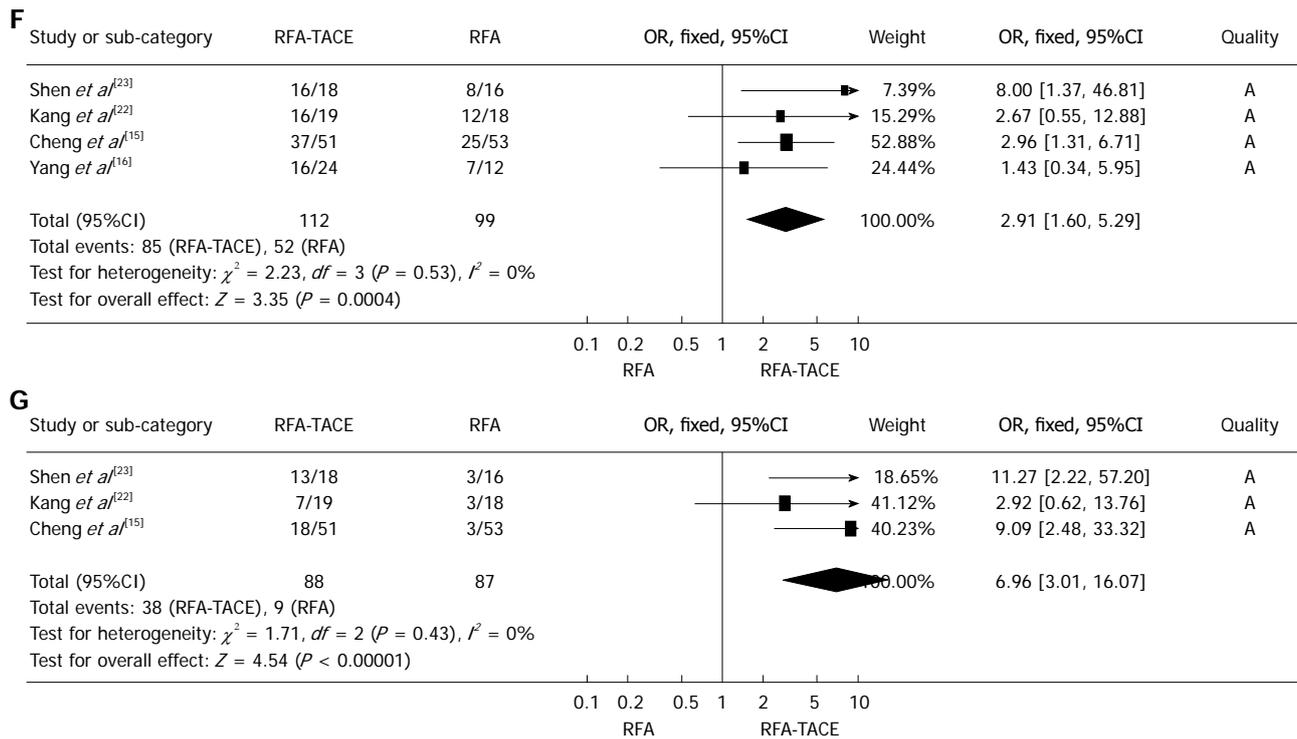


Figure 4 Comparison of combination of radiofrequency ablation and transcatheter arterial chemoembolization with radiofrequency ablation alone for small hepatocellular carcinoma in terms of survival rates. A, B: Tumor size  $\leq 3$  cm; C-F:  $3 \text{ cm} < \text{tumor size} \leq 5$  cm; G, H: Tumor size  $> 5$  cm; A, C, F: Meta-analysis of 1-year results; B, D, G: Meta-analysis of 3-year results; E: Meta-analysis of 5-year results.

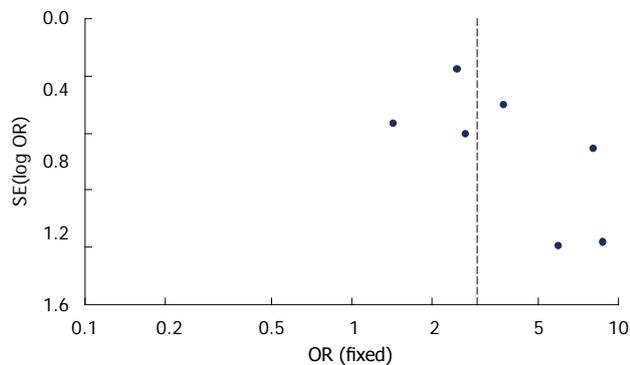


Figure 5 Funnel plot in the analysis of the effect of 1-year overall survival rate. OR: Odds ratio.

that there was no significant difference in survival rate between the two treatments for small HCC. However, there was a significant difference in survival rate between RFA plus TACE and RFA alone for treatment of intermediate- and large-size HCC, and our meta-analysis data showed that RFA plus TACE was associated with a significantly higher survival rate than RFA monotherapy was. RFA as a thermal *in situ* destruction technique was proved to be a safe and effective treatment. It is possible for RFA to achieve complete necrosis for treatment of small HCC. In recent years, RFA has been accepted as one of the best treatment options for small HCC. However, due to the limitation of the ablation area, it is barely possible for RFA to achieve complete necrosis during treatment of a relatively large HCC<sup>[27,28]</sup>. RFA combined with TACE can

resolve this problem effectively. According to the clinical studies and experience, we found that there were some synergistic effects between RFA and TACE in combined treatment: (1) TACE can reduce the cooling effect of hepatic blood flow by decreasing hepatic arterial flow, and improve the effect of percutaneous RFA thermal therapy, which plays a critic role in inducing tumor necrosis<sup>[14,20,21]</sup>; (2) edematous change in the tumors induced by ischemia and inflammation after TACE is expected to enlarge the area of tumor necrosis during RFA treatment; and (3) RFA combined with TACE can prevent HCC with hepatic artery portal fistula from invading the portal vein and provides a better prognosis for HCC patients<sup>[20,29]</sup>.

The risk of publication bias in this meta-analysis was assessed by the symmetry of the funnel plot<sup>[30,31]</sup>. All the studies included in the current meta-analysis had comparative data for 1-year overall survival rate. In the analysis of the effect of 1-year overall survival rate, we found that the level of the symmetry of the funnel plot was judged to be high, which indicated that there was no significant publication bias in this meta-analysis. Eight randomized controlled trials were included in this study. The overall quality of the studies was detected using Review Manager from the Cochrane Collaboration and was judged to be high. This suggests that the studies included in the study had strong evidence to support the results of our meta-analysis.

To the best of our knowledge, no meta-analysis has been performed to compare the efficacy and safety of RFA combined with TACE and RFA monotherapy in

terms of overall survival rate, recurrence-free survival rate, tumor progression, and major complications. Our meta-analysis is believed to be the first report to assess comprehensively combination of TACE and RFA compared with RFA alone for treatment of HCC. Additionally, we also analyzed and compared the effectiveness of RFA plus TACE and RFA monotherapy for treatment of small-, intermediate- and large-size HCC. The analysis of these studies is important and useful for precise and objective statistical assessment of the clinical efficacy of RFA plus TACE and RFA alone for treatment of HCC.

Our study had several limitations. First, the heterogeneity of the inclusion criteria (Child-Pugh class, number of tumors, tumor size, tumor stage, and treatment design) might have affected the consistency of the results and caused between-study heterogeneity, which could have affected the overall quality of our study. Second, the etiological factors of HCC such as viral hepatitis, alcoholic liver disease, and autoimmune liver disease, were not considered in the trials. Whether HCC patients with different etiological factors could obtain similar outcomes from treatment with RFA plus TACE needs further research.

In conclusion, this meta-analysis suggests that combination of RFA with TACE is more effective than RFA monotherapy in the treatment of patients with intermediate- and large-size HCC, with significantly higher survival rates achieved with the combined methods. The combination of interventional therapies may be applied more widely in the treatment of HCC.

## COMMENTS

### Background

Radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE) have been widely accepted as the established non-surgical treatment options for hepatocellular carcinoma (HCC). However, which is the superior treatment choice is still uncertain. Previous studies have suggested that the effectiveness of combination of TACE and RFA is much better than that of RFA monotherapy in HCC. However, other studies assessing the effectiveness of combination of TACE and PRFA compared with RFA alone have reported conflicting results. Hence, it is necessary to design a study to compare comprehensively the effectiveness and safety of TACE plus RFA and RFA monotherapy for treatment of patients with HCC.

### Research frontiers

In the current study, a meta-analysis was designed to compare comprehensively the overall survival rates, recurrence-free survival rates, and tumor progression rates for RFA plus TACE with those of RFA monotherapy. Additionally, based on the tumor size, the survival rates of patients with small-, intermediate- and large-size HCC underwent RFA plus TACE compared with those who underwent RFA alone.

### Innovations and breakthroughs

The current analysis comprehensively compared the effectiveness and safety of RFA combined with TACE with that of RFA monotherapy in HCC. This analysis indicated that the overall and recurrence-free survival rates of patients treated with RFA plus TACE were significantly higher than those with RFA alone. The authors also found that although there were no significant differences between RFA plus TACE and RFA monotherapy for small HCC, the efficacy of RFA plus TACE was obviously better than that of RFA alone for treatment of intermediate- and large-size HCC.

### Applications

The analysis showed that the effectiveness of RFA combined with TACE was much better than that of RFA monotherapy for treatment of intermediate- and large-size HCC. Comparison of these two treatments could provide the theoretic

cal basis for the use of RFA plus TACE for treatment of HCC and help stratify the benefits of treatment choices for patients with HCC.

### Terminology

TACE is one of the most widely performed treatments for unresectable HCC, which is a type of interventional radiology. RFA is a thermal *in situ* destruction technique, and it has been proved to be a safe and effective treatment. RFA has been accepted as one of the best treatment options for small HCC.

### Peer review

This was an excellent study that addresses an important topic, particularly the part comparing the efficacy and safety of combination of RFA and TACE with RFA monotherapy for treatment of patients with HCC. The report is concise and informative.

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P- Reviewers Shi HY, Sun ZH S- Editor Zhai HH  
L- Editor Kerr C E- Editor Xiong L



## Meta-analysis comparison of endoscopic papillary balloon dilatation and endoscopic sphincter papillotomy

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Received: January 3, 2013 Revised: March 20, 2013

Accepted: March 28, 2013

Published online: June 28, 2013

group were younger (OR = -1.16, 95%CI: -1.49 to 0.84,  $P < 0.01$ ). There were no significant differences in gender proportion, average size of stones, number of gallstones, previous cholecystectomy, the incidence of duodenal diverticulum, CBD diameter or the total follow-up time between EST and EPBD groups. Compared with EST, the total stone clearance in the EPBD group decreased (OR = 0.64, 95%CI: 0.42 to 0.96,  $P = 0.03$ ), the use of stone extraction baskets significantly increased (OR = 1.91, 95%CI: 1.41 to 2.59,  $P < 0.01$ ), and the incidence of pancreatitis significantly increased (OR = 2.79, 95%CI: 1.74 to 4.45,  $P < 0.0001$ ). The incidence of bleeding (OR = 0.12, 95%CI: 0.04 to 0.34,  $P < 0.01$ ) and cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84,  $P = 0.02$ ) significantly decreased. The stone recurrence rate also was significantly reduced in EPBD (OR = 0.48, 95%CI: 0.26 to 0.90,  $P = 0.02$ ). There were no significant differences between the two groups with the incidence of stone removal at first attempt, hours of operation, total short-term complications and infection, perforation, or acute cholangitis.

**CONCLUSION:** Although the incidence of pancreatitis was higher, the overall stone clearance rate and risk of bleeding was lower with EPBD compared to EST.

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**Key words:** Common bile duct stone; Endoscopic papillary balloon dilatation; Endoscopic sphincter papillotomy; Meta-analysis

**Core tip:** A meta-analysis was performed to evaluate the outcomes of endoscopic sphincter papillotomy (EST) and endoscopic papillary balloon dilatation (EPBD) from previously published reports. Fourteen randomized trials involving 1975 patients were analyzed. Of those, 980 were treated with EPBD and 995 were treated with EST. Differences were observed between the treatments in total stone clearance, short-term complications, and long-term complications. Compared to

### Abstract

**AIM:** To assess endoscopic papillary balloon dilatation (EPBD) and endoscopic sphincter papillotomy (EST) for common bile duct (CBD) stone removal using a meta-analysis.

**METHODS:** Randomized controlled trials published from 1990 to 2012 comparing EPBD with EST for CBD stone removal were evaluated. This meta-analysis was performed to estimate short-term and long-term complications of these two treatments. The fixed random effect model or random effect model was established to analysis the data. Results were obtained by analyzing the relative risk, odds ratio, and 95%CI for a given comparison using RevMan 5.1. Statistical significance was defined as  $P < 0.05$ . Risk of bias was evaluated using a funnel plot.

**RESULTS:** Of the 1975 patients analyzed, 980 of them were treated with EPBD and 995 were treated with EST. Of the patient population, patients in the EPBD

EST, the overall stone clearance rate was lower, and the incidence of pancreatitis was higher with EPBD. Thus, EPBD may decrease the incidence of long-term complications and be more suitable for patients who have a high risk of bleeding.

Zhao HC, He L, Zhou DC, Geng XP, Pan FM. Meta-analysis comparison of endoscopic papillary balloon dilatation and endoscopic sphincterotomy. *World J Gastroenterol* 2013; 19(24): 3883-3891 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3883.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3883>

## INTRODUCTION

Along with the advance of endoscope technology and related instruments, endoscopic sphincterotomy (EST) had become the first choice for common duct stones. But it was associated with some short-term and long-term complications such as pancreatitis, bleeding, cholangitis, perforation of the bile duct, narrow nipple, and sphincter loss of function. Endoscopic papillary balloon dilatation (EPBD), as a replacement for EST, was first reported by Staritz *et al*<sup>[1]</sup>. Compared with EST, EPBD is a less complicated procedure, and the incidence of short-term complications, such as bleeding and perforation, are decreased, while the function of the Oddi sphincter (OS) is protected. In a multicenter random control study, 117 patients who were definitively diagnosed with bile duct stones were treated by EPBD; after treatment, the incidence of pancreatitis among those patients reached 15.4% and two patients died from post-treatment complications<sup>[2]</sup>. Thus, a heated dispute still remains in the medical community as to the safety and application of EPBD. In the present study, a meta-analysis was performed to evaluate the curative effect and safety of EPBD relative to EST by systematically reviewing published randomized trials.

## MATERIALS AND METHODS

### Inclusion criteria

Only randomized controlled trials were considered for review, and no limitations were placed on the article language. In the included published studies, all cases were diagnosed with common duct stones and observational targets included at least one of the following criteria: (1) overall incidence of stone clearance, (2) incidence of a singular stone clearance, (3) incidence of short-term complications, or (4) incidence of long-term complications. EPBD was used as the experimental group while EST was used as the control group.

### Search strategy

Studies were identified by searching MEDLINE, EMBASE, Cochrane library, Current Contents and the Science Citation Index in July 2012. Key search terms were “com-

mon bile duct stone”, “endoscopic retrograde cholangiopancreatography”, “endoscopic sphincterotomy”, “endoscopic papillary balloon dilatation”, “endoscopic balloon dilatation”, “EST”, “ERCP”, “EPBD” and “EBD”. Additionally, articles were searched using Google in order to prevent any oversight.

### Statistical analysis

Articles were retrieved when they met the inclusion criteria. Two reviewers (He L and Zhou DC) applied the inclusion criteria independently to the retrieved articles as defined in the protocol. Any differences were resolved by discussion. Each included study was critically evaluated for its study quality, based on the criteria used by the Cochrane Collaboration, and level of evidence according to the standards of the Hierarchy of Evidence. One reviewer (He L) extracted information on data extraction sheets and a second reviewer (Zhou DC) checked them.

All data were calculated using RevMan5.1. Relative risks (for dichotomous outcome measures), odds ratio (OR) and weighted mean differences (for continuous outcome measures) with 95%CI were calculated. Statistical significance was defined as  $P < 0.05$ . If the outcome could not be calculated or the incidence was too small, a qualitative evaluation was given by a detailed explanation. A heterogeneity test was conducted according to the value of  $I^2$ ,  $\chi^2$  and  $P$ . If heterogeneity was positive, then a sensitivity analysis was carried out. Any article that showed positive heterogeneity was excluded. Risk of bias was evaluated using a funnel plot.

## RESULTS

After the initial screening, 178 relevant articles were considered for review. Of these, 163 articles were not in accordance with the defined inclusion criteria. Among the remaining 15 articles, one was excluded based on positive heterogeneity<sup>[3]</sup>. A total of 14 articles were included in this meta-analysis with available data from 1975 patients summarized in Table 1<sup>[4-16]</sup>.

Based on results from the analysis of these randomized controlled trials, significant differences were only found in the comparison of mean patient age between the two groups. The average age in the EPBD group was significantly younger than in the EST group (OR = -1.16, 95%CI: -1.49 to -0.84,  $P < 0.01$ ). There were no significant differences in other general characteristics, such as gender ratio (female ratio, OR = 1.10, 95%CI: 0.91 to 1.31,  $P = 0.33$ ; male ratio, OR = 0.91, 95%CI: 0.76 to 1.09,  $P = 0.91$ ), mean diameter of stones (OR = 0.04, 95%CI: -0.22 to 0.30,  $P = 0.77$ ), number of stones (OR = 0.22, 95%CI: -0.11 to 0.46,  $P = 0.06$ ), complicated cholelithiasis (OR = 1.13, 95%CI: 0.83 to 1.54,  $P = 0.43$ ), prior-cholecystectomy (OR = 1.03, 95%CI: 0.81 to 1.31,  $P = 0.80$ ), mean diameter of common bile duct (CBD) stones (OR = -0.21, 95%CI: -0.69 to 0.26,  $P = 0.38$ ) or total follow-up time (OR = -0.05, 95%CI: -0.51 to 0.41,  $P = 0.83$ ).

In addition, the analysis showed that there was no

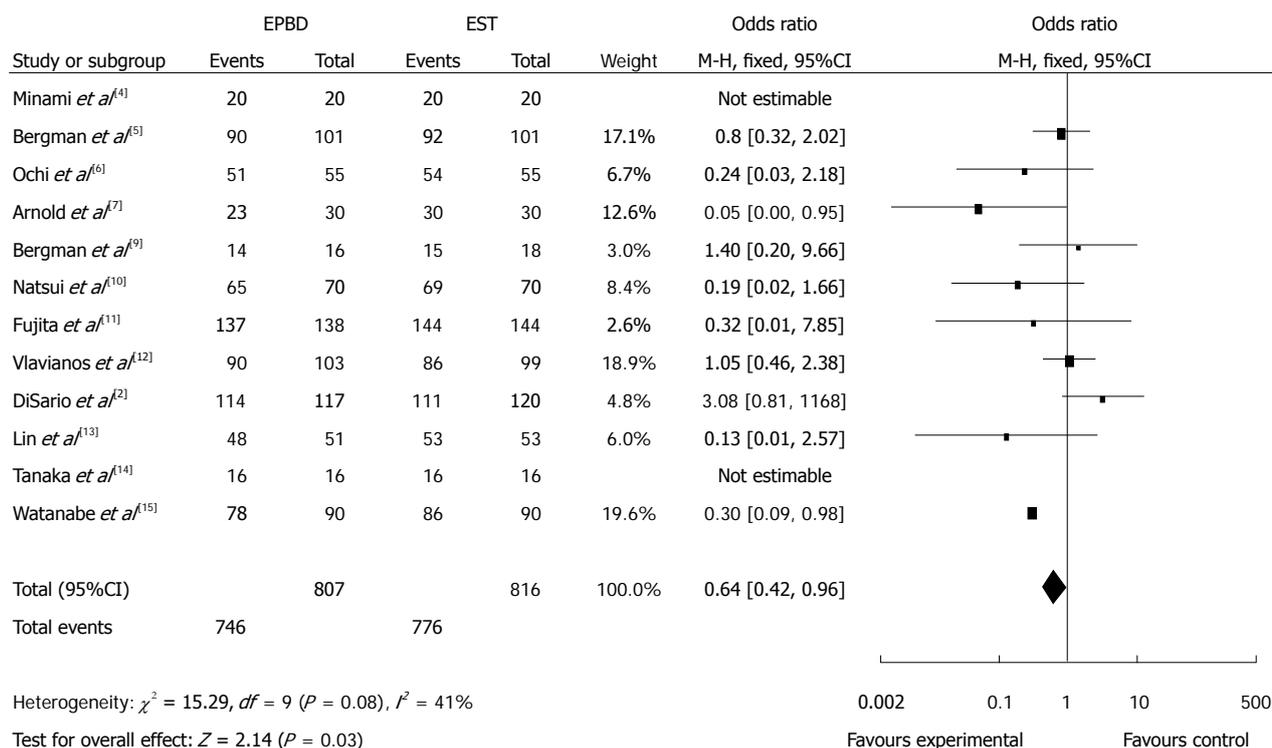


Figure 1 Comparison of total clearance of common bile duct stones between endoscopic papillary balloon dilatation (experimental group) and endoscopic sphincteropyllotomy (control group). EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincteropyllotomy.

significant difference in the incidence of stone clearance on the first attempt between the survey groups (OR = 0.82, 95%CI: 0.63 to 1.07,  $P = 0.15$ ). Relative to EST, the EPBD procedure was associated with a higher incidence of total clearance of CBD stones (Figure 1; OR = 0.64, 95%CI: 0.42 to 0.96,  $P = 0.03$ ) and with more frequent stone extraction basket use (OR = 1.91, 95%CI: 1.41 to 2.59,  $P < 0.01$ ). There were no significant differences in operation times between the two groups (OR = -0.62, 95%CI: -1.73 to 0.48,  $P = 0.27$ ).

**Incidence of short-term complications**

Our results show that there was no significant difference in the overall incidence of early complications (OR = 1.33, 95%CI: 0.96 to 1.85,  $P = 0.09$ ). Among the included randomized controlled trials, 11 studies reported the incidence of pancreatitis. Analysis of the data indicated that EPBD increased the incidence of pancreatitis when compared to EST in Western patients (OR = 4.05, 95%CI: 2.06 to 7.94,  $P < 0.0001$ ), but was not significantly different in Asian patients ( $P = 0.08$ ). In a combined analysis of Western and Asian studies, the incidence of pancreatitis was significantly increased in patients who received EPBD treatment (Figure 2A; OR = 2.79, 95%CI: 1.74 to 4.45,  $P < 0.0001$ ).

There were nine studies that reported incidences of bleeding. The analysis showed that the incidence of bleeding in the EPBD group was lower than that of the EST group (Figure 2B; OR = 0.12, 95%CI: 0.04 to 0.34,  $P < 0.01$ ). There was no significant difference in incidence of infection between EPBD and EST (OR = 0.85, 95%CI:

0.46 to 1.56,  $P = 0.59$ ). Likewise, there was no significant difference in the incidence of perforation between EPBD and EST (OR = 0.77, 95%CI: 0.17 to 3.50,  $P = 0.73$ ).

**Incidence of long-term complications**

Six of the included studies reported incidences of long-term complications. Overall, the results of our analysis indicated that this incidence was significantly lower if patients were treated by EPBD rather than by EST (Figure 3A; OR = 0.53, 95%CI: 0.36 to 0.77,  $P = 0.0008$ ). Compared to EST, EPBD markedly decreased the incidence of acute cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84,  $P = 0.02$ ), although there was no significant difference between EPBD and EST in the incidence of acute cholangitis (OR = 0.60, 95%CI: 0.18 to 1.95,  $P = 0.39$ ). Among the included studies, only seven reported recurrence of CBD stones, and our analysis showed that there was no significant difference between EPBD and EST (Figure 3B; OR = 0.66, 95%CI: 0.42 to 1.05,  $P = 0.08$ ). Further analysis of the studies with a follow-up of more than one year indicated that the stone recurrence rate decreased significantly in the EPBD group (Figure 4; OR = 0.48, 95%CI: 0.26 to 0.90,  $P = 0.02$ ).

**Study assessment**

A funnel plot analysis (Figure 5) was symmetrical on the whole, demonstrating that there was no significant publication bias. In addition, the included studies were of high quality and generally had a low risk of bias for selection, performance, attrition, and reporting as seen in Figure 6.

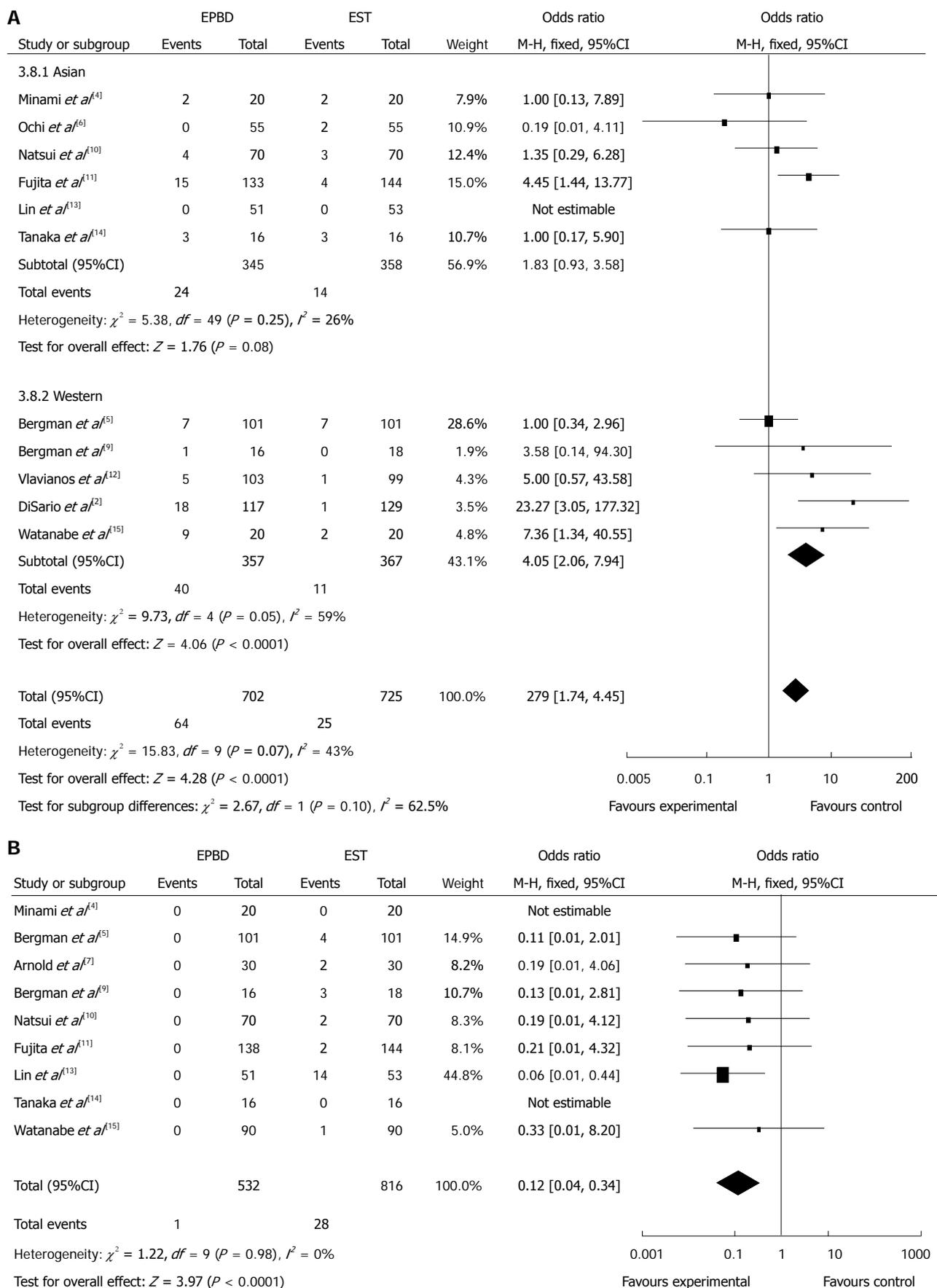


Figure 2 Incidence of pancreatitis (A) and bleeding (B) between endoscopic papillary balloon dilatation and endoscopic sphincter papillotomy. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincter papillotomy.

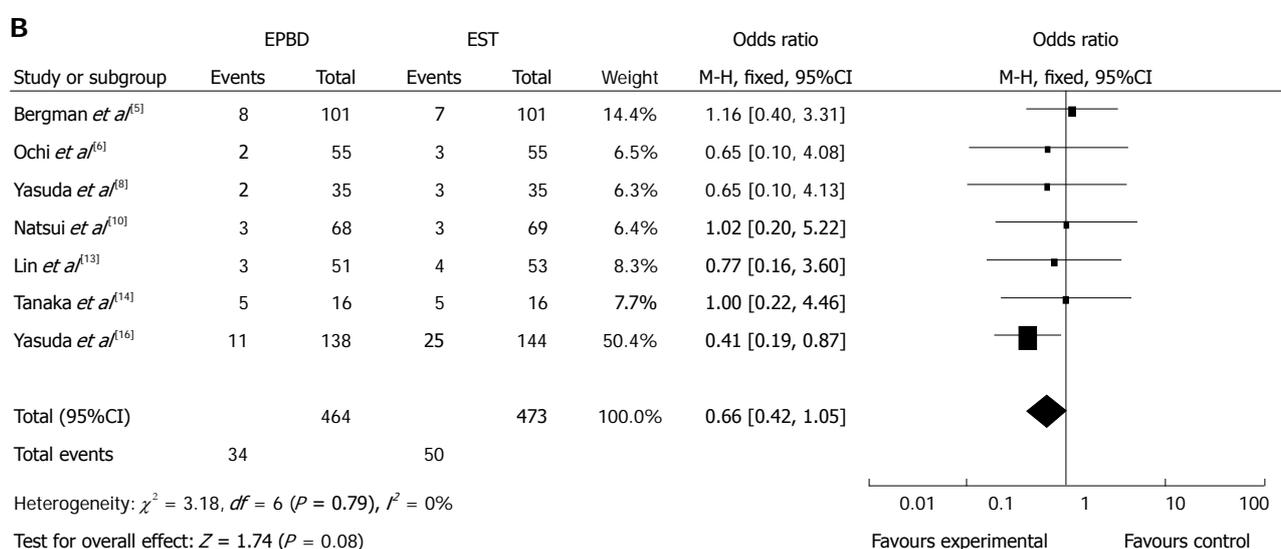
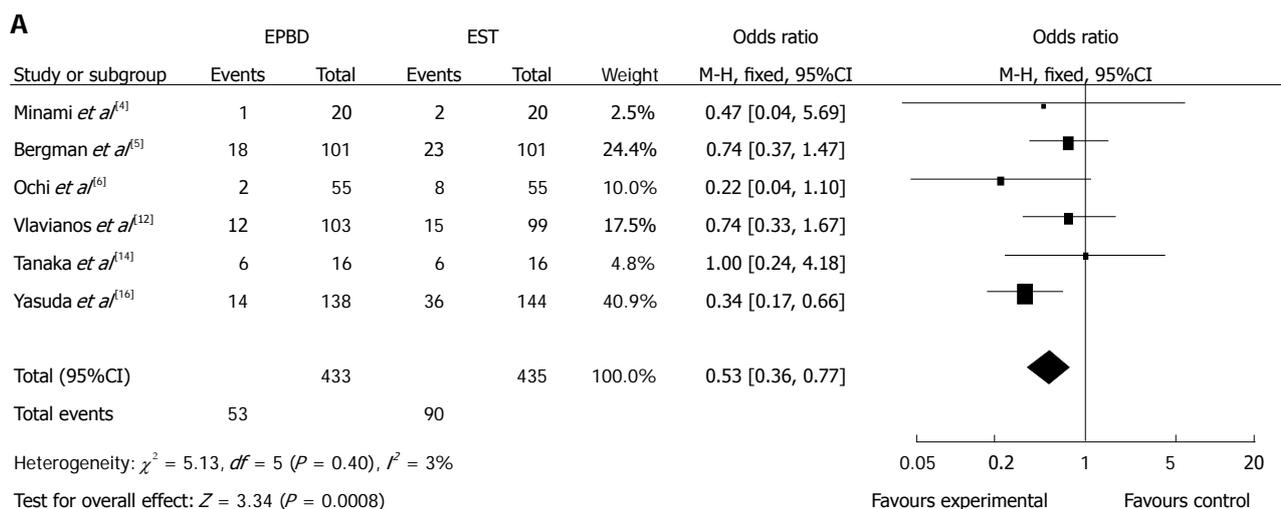


Figure 3 Overall incidence of long-term complications (A) and bile duct stone recurrence rate (B) between endoscopic papillary balloon dilatation and endoscopic sphincter papillotomy. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincter papillotomy.

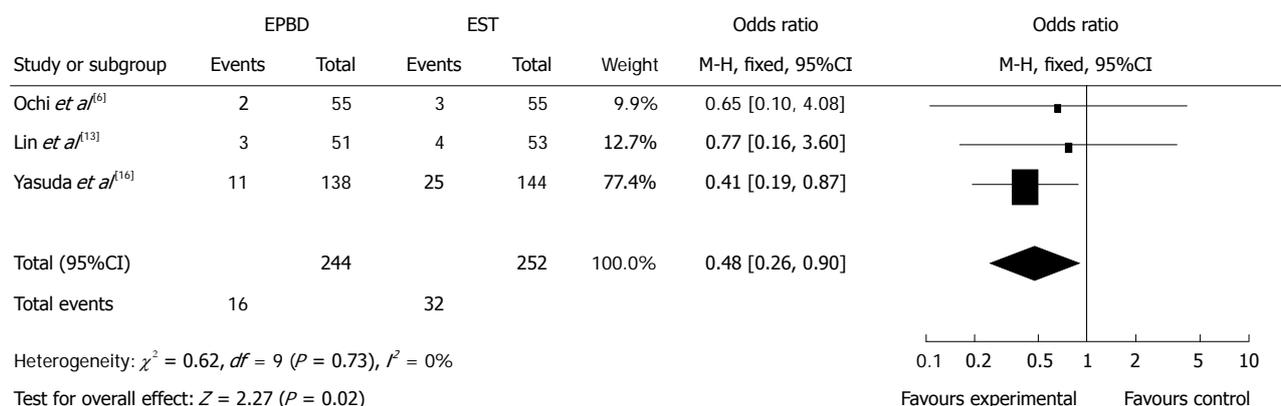


Figure 4 Incidence of bile duct stone recurrence rate between endoscopic papillary balloon dilatation and endoscopic sphincter papillotomy with a follow-up of more than one year. EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincter papillotomy.

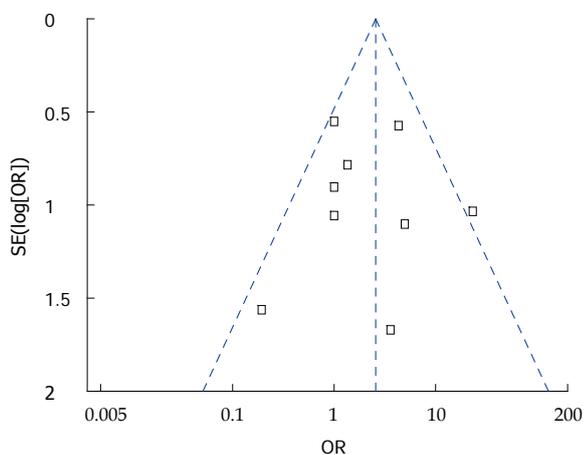
However, allocation concealment was only performed in the 5 studies<sup>[5,9,12,15,16]</sup>. Some outcomes were reported based on the intention to treat using assigned randomization at the beginning of the study<sup>[5,9,11,12,15]</sup>. In other

studies, there was no detail given about randomization. A double-blinding method was not applied in any of the included studies. The main observational outcomes reported were the overall clearance of stones and stone

**Table 1 Overview of study characteristics (mean ± SD)**

| Ref.                                  | Year | Country        | Treatment | Case | Sex   | Age (yr)    | MFT (mo)    | Diameter of stones (mm) | No. of stones | Jadad grade |
|---------------------------------------|------|----------------|-----------|------|-------|-------------|-------------|-------------------------|---------------|-------------|
| Minami <i>et al</i> <sup>[4]</sup>    | 1995 | Japan          | EPBD      | 20   | 13/7  | 64.0 ± 11.2 | 21.5 ± 6.2  | < 12                    | No data       | 4 score     |
|                                       |      |                | EST       | 20   | 9/11  | 71.3 ± 14.0 | 23.1 ± 5.0  | < 12                    |               |             |
| Bergman <i>et al</i> <sup>[5]</sup>   | 1997 | Holland        | EPBD      | 101  | 43/58 | 72.0 ± 11.8 | 6.2         | 10.0 ± 5.5              | 2.0 ± 2.2     | 6 score     |
|                                       |      |                | EST       | 101  | 45/56 | 71.0 ± 11.1 | 6.1         | 9.0 ± 3.5               | 1.0 ± 2.3     |             |
| Ochi <i>et al</i> <sup>[6]</sup>      | 1999 | Japan          | EPBD      | 55   | 34/21 | 62.6 ± 15.9 | 23.0 ± 6.3  | 8.1 ± 3.4               | 2.1 ± 1.9     | 4 score     |
|                                       |      |                | EST       | 55   | 31/24 | 66.3 ± 14.3 | 23.0 ± 6.3  | 8.8 ± 4.2               | 1.7 ± 1.2     |             |
| Arnold <i>et al</i> <sup>[7]</sup>    | 2001 | Germany        | EPBD      | 30   | 11/19 | 54.2 ± 18.5 | No data     | 7.0 ± 3.5               | 1.6 ± 1.1     | 4 score     |
|                                       |      |                | EST       | 30   | 13/17 | 58.5 ± 18.5 |             | 10.0 ± 4.7              | 1.8 ± 1.5     |             |
| Yasuda <i>et al</i> <sup>[8]</sup>    | 2001 | Japan          | EPBD      | 35   | 19/16 | 69.5 ± 7.3  | No data     | 12.4 ± 3.3              | 3.7 ± 2.5     | 4 score     |
|                                       |      |                | EST       | 35   | 14/21 | 69.4 ± 7.5  |             | 12.3 ± 3.2              | 3.3 ± 2.5     |             |
| Bergman <i>et al</i> <sup>[9]</sup>   | 2001 | Holland        | EPBD      | 16   | 12/4  | 73.0 ± 6.8  | No data     | 9.0 ± 2.8               | 2.0 ± 1.5     | 6 score     |
|                                       |      |                | EST       | 18   | 18/0  | 72.0 ± 2.8  |             | 8.0 ± 2.0               | 2.0 ± 1.5     |             |
| Natusi <i>et al</i> <sup>[10]</sup>   | 2002 | Japan          | EPBD      | 70   | 33/37 | 64.5 ± 10.6 | No data     | 9.2 ± 3.2               | 2.7 ± 2.3     | 4 score     |
|                                       |      |                | EST       | 70   | 33/37 | 67.1 ± 8.3  |             | 9.7 ± 2.3               | 2.6 ± 2.3     |             |
| Fujita <i>et al</i> <sup>[11]</sup>   | 2003 | Japan          | EPBD      | 138  | 75/63 | 66.8 ± 1.3  | No data     | 7.0 ± 3.1               | 2.4 ± 2.5     | 5 score     |
|                                       |      |                | EST       | 144  | 92/52 | 61.9 ± 1.2  |             | 7.7 ± 3.4               | 2.4 ± 2.9     |             |
| Vlaviano <i>et al</i> <sup>[12]</sup> | 2003 | England        | EPBD      | 103  | 25/78 | 60.8 ± 1.3  | No data     | No data                 | No data       | 4 score     |
|                                       |      |                | EST       | 99   | 35/64 | 62.0 ± 1.2  |             |                         |               |             |
| Disario <i>et al</i> <sup>[2]</sup>   | 2004 | United States  | EPBD      | 117  | 76/41 | 47.0 ± 19.0 | 1.6 ± 2.9   | 6.0 ± 1.6               | 1.0 ± 16.5    | 6 score     |
|                                       |      |                | EST       | 120  | 89/31 | 54.0 ± 19.0 | 1.6 ± 0.4   | 5.0 ± 2.3               | 1.0 ± 1.5     |             |
| Lin <i>et al</i> <sup>[13]</sup>      | 2004 | Taiwan (China) | EPBD      | 51   | 28/23 | 64.0 ± 10.3 | 16.0 ± 3.0  | 8 ± 6                   | No data       | 4 score     |
|                                       |      |                | EST       | 53   | 31/22 | 65.0 ± 10.0 | 16.0 ± 3.0  | 8 ± 6                   |               |             |
| Tanaka <i>et al</i> <sup>[14]</sup>   | 2004 | Japan          | EPBD      | 16   | 10/6  | 67.2 ± 4.7  | No data     | 10.2 ± 3.5              | 2.0 ± 1.8     | 5 score     |
|                                       |      |                | EST       | 16   | 3/13  | 70.6 ± 6.3  |             | 12.4 ± 6.0              | 2.0 ± 0.5     |             |
| Watanabe <i>et al</i> <sup>[15]</sup> | 2007 | Japan          | EPBD      | 90   | 51/39 | 69.1 ± 13.1 | No data     | 8.1 ± 3.2               | 2.7 ± 2.8     | 4 score     |
|                                       |      |                | EST       | 90   | 49/41 | 70.2 ± 8.1  |             | 7.7 ± 2.3               | 2.5 ± 2.7     |             |
| Yasuda <i>et al</i> <sup>[16]</sup>   | 2010 | Japan          | EPBD      | 138  | 52/92 | 68.5 ± 11.2 | 80.3 ± 15.0 | 6.5 ± 2.1               | 1.0 ± 2.5     | 5 score     |
|                                       |      |                | EST       | 144  | 63/75 | 71.0 ± 10.3 | 81.6 ± 15.2 | 7.0 ± 2.3               | 1.0 ± 3.8     |             |

M: Male; F: Female; MFT: Mean follow-up time; MD: Mean diameter; EPBD: Endoscopic papillary balloon dilatation; EST: Endoscopic sphincter papillotomy.



**Figure 5 Funnel plot analysis of included studies.** X-axis [Peto odds ratio (OR)] represents treatment effects. Y-axis (log[Peto OR]) represents the large sample size. Each square represents an individual study. The dashed line represents 95%CI.

clearance at the first attempt, both of which were reported by all studies. Completed data for the other outcomes was not available.

## DISCUSSION

Meta-analysis is an accepted method that can evaluate controversial issues met in clinical practice, by analyzing

high quality randomized controlled trials in quantification. Studies from many different countries were included in this analysis, and the differences between EPBD and EST were evaluated systematically to produce meaningful results.

The results indicated that EST increased the overall clearance of stones compared to EPBD, but that the stone clearance at first attempt was similar. It is generally acknowledged that EPBD is less likely to clear big stones than EST because its inflation into the papilla is smaller. In our analysis, however, we have defined the stone diameter as part of the exclusion criteria. Some studies were excluded if the smallest stone was larger than 12 mm<sup>[4]</sup>, 15 mm<sup>[6]</sup>, 20 mm<sup>[7]</sup>, or 14 mm<sup>[11]</sup>. Two studies were excluded because the total number of stones was more than 10<sup>[6]</sup> or 5<sup>[7]</sup>. Stone extraction basket usage was more frequent when patients were treated with EPBD than EST, especially when the diameter of stones was larger than 8 mm<sup>[4]</sup>, 10 mm<sup>[5,6]</sup>, or 12 mm<sup>[10]</sup>.

In the matter of safety, our results suggest that there was no significant difference in short-term complications between EPBD and EST. However, the incidence of long-term pancreatitis was higher after EPBD. This phenomenon may be due to the poor outflow of pancreatic juice caused by hydrops or spasm of the OS after EPBD. The risk of pancreatitis after EPBD is also a highly disputed issue, especially in America. EPBD has been abandoned by many endoscope doctors because they believe

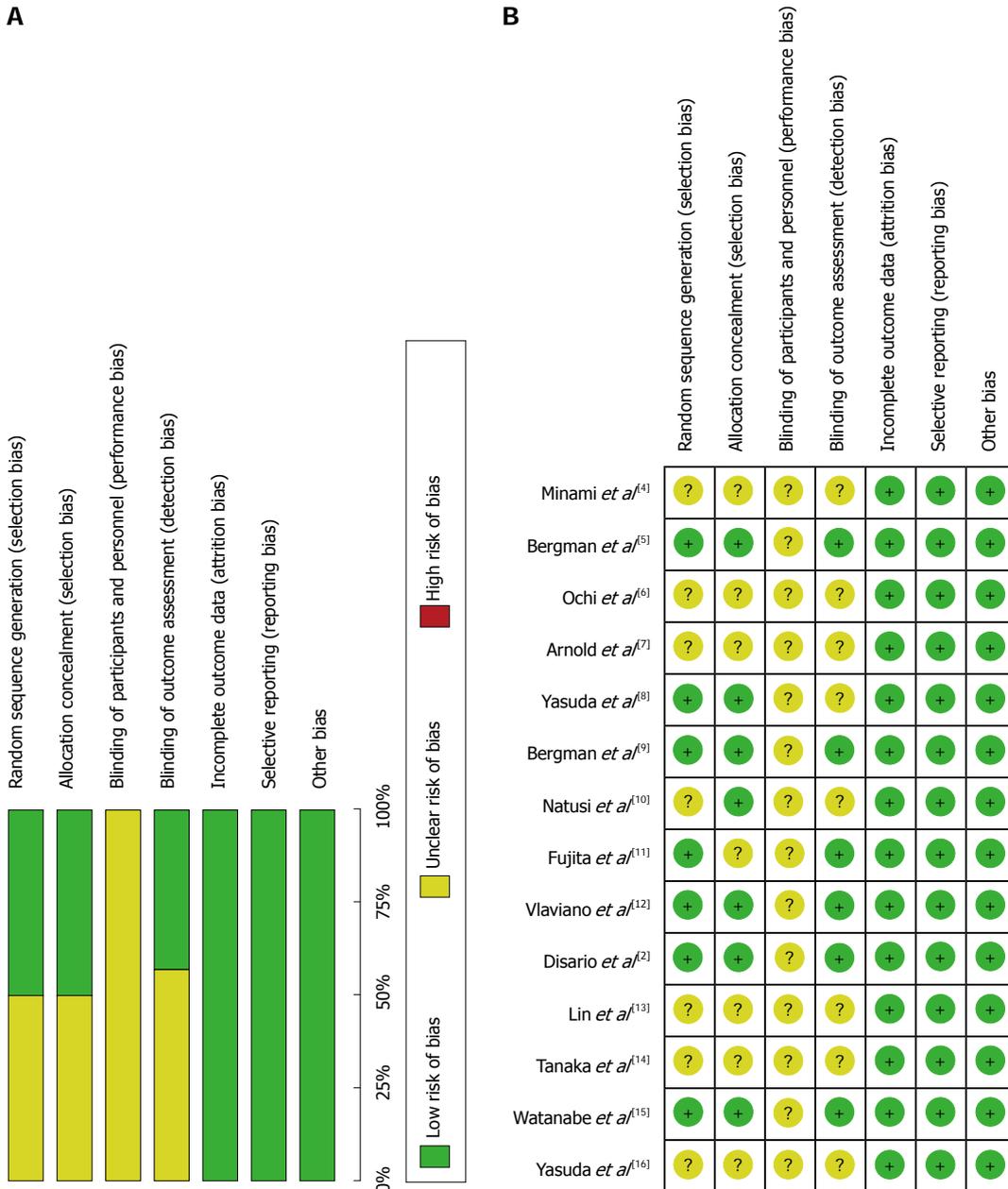


Figure 6 Risk of bias summary (A) and bias assessment for each individual study (B).

that this technology increases risk of long-term pancreatitis<sup>[17]</sup>. Conversely, most researchers in Asia and Europe maintain that there is no direct consequence between pancreatitis risk and EPBD and report that pancreatitis usually occurs in the light or medium stage<sup>[5,6,10,13,14]</sup>. A retrospective study by Tsujino *et al*<sup>[18]</sup> showed that among 1000 patients treated by EPBD, only 48 of them (4.8%) experienced light to moderate pancreatitis and only one patient (0.1%) experienced serious pancreatitis, but was fully recovered 8 mo after treatment.

Compared to EST, the incidence of bleeding during operation was significantly lower in EPBD, possibly due to the protection of the sphincter of Oddi during EPBD that is not incised directly as in EST. This advantage promotes the application of EPBD for patients with poor blood clotting abilities. A previous study of 98 patients

who were diagnosed with CBD stones experiencing liver cirrhosis was carried out by Tsujino *et al*<sup>[18]</sup>. In that report, bleeding only occurred in one patient after the EPBD procedure, even though 31 of these patients were in C-stage of Child-Pugh. Moreover, when faced with atypical anatomy, such as duodenal diverticulum or Billroth-II gastrectomy history, EPBD is more suitable than EST because EPBD can expand the OS more easily. In addition, Bergman *et al*<sup>[9]</sup> has reported that for patients with Billroth-II gastrectomy history, the amount of bleeding was significantly decreased after EPBD (EPBD *vs* EST: 2% *vs* 17%,  $P = 0.03$ ).

Based on the results of this analysis, compared to EST, EPBD decreased the incidence of long-term cholecystitis and the recurrence of stones in CBD, but there were no significant differences in the duration of long-

term cholangitis or recurrence of stones. Some research has suggested that EST could lead to some serious side effects, such as exhaustive, non-reversible loss of function of the OS, although pneumobilia and bile regurgitation exists in nearly 50% of patients and biliary bacterial disease exists in nearly all patients<sup>[19]</sup>. Some complications may be continuously affected by these conditions, such as with acute cholecystitis and cholangitis. Our analysis showed discordant results with long-term cholangitis, but this may be due to the limited sample size and short follow-up.

A study by Natsui *et al.*<sup>[20]</sup> determined that EST was correlated with bacterial colonization in bile duct, which could lead to chronic inflammation and fibrosis of the bile duct system. Research by Kurumado *et al.*<sup>[21]</sup> suggested that destruction of the sphincter of Oddi could even increase the risk of bile duct cancer. A retrospective study by Kojima *et al.*<sup>[22]</sup> found that recurrence of CBD stones was higher after EST than EPBD (17.0% *vs* 6.8%,  $P < 0.05$ ), and the sphincter of Oddi function was protected in 70% patients after EPBD, decreasing the recurrence of stones. Another study reported an 8.8% recurrence rate of stones in 837 patients after 4.4 years of follow-up where mechanical lithotripsy usage and subsequent cholelithiasis were considered risk factors for recurrence<sup>[18]</sup>.

This meta-analysis was limited by two factors. First, there was some study selection bias that could not be excluded easily. Secondly, the definition of short-term and long-term complications was not completely consistent. For example, complications that occurred during 24 h<sup>[12]</sup>, 15 d<sup>[9,14]</sup>, and 30 d<sup>[2]</sup> were all included in short-term complications, while long-term complications included 6 mo<sup>[5]</sup>, 12 mo<sup>[8,12]</sup>, 16 mo<sup>[13]</sup>, 23 mo<sup>[6]</sup>, 30 mo<sup>[4,10]</sup>, 72 mo<sup>[14]</sup>, and 81 mo<sup>[16]</sup>. These uncontrollable factors may have affected the accuracy of this study to some extent.

In summary, in a comparison of the two treatments, EPBD and EST, the overall stone clearance rate of EPBD was lower and the incidence of pancreatitis with EPBD was higher. EPBD has the advantage of decreasing the incidence of long-term complications, and for patients who have a high risk of bleeding, EPBD may be more suitable.

## COMMENTS

### Background

To date, endoscopic sphincterotomy (EST) and endoscopic papillary balloon dilatation (EPBD) are the primary choices for the removal of common duct stones. As a replacement for EST, EPBD is a less complicated procedure, has fewer short-term complications, such as bleeding and perforation, and protects the function of the Oddi sphincter. However, a heated debate still remains as to which is the better procedure.

### Research frontiers

In this report, a meta-analysis was performed to evaluate the curative effects and safety of EST and EPBD by systematically reviewing newly published randomized trials.

### Innovations and breakthroughs

The meta-analysis showed that in comparison with EST, patients in the EPBD group were younger (OR = -1.16, 95%CI: -1.49 to -0.84,  $P < 0.01$ ), had lower

rate of total stone clearance (OR = 0.64, 95%CI: 0.42 to 0.96,  $P = 0.03$ ), had a greater use of stone extraction baskets (OR = 1.91, 95%CI: 1.41 to 2.59,  $P < 0.01$ ), experienced a higher incidence of pancreatitis (OR = 2.53, 95%CI: 1.55 to 4.13,  $P = 0.0002$ ) and a significantly lower incidence of bleeding (OR = 0.12, 95%CI: 0.04 to 0.34,  $P < 0.01$ ) and acute cholecystitis (OR = 0.41, 95%CI: 0.20 to 0.84,  $P = 0.02$ ). The total long-term complication rate was also significantly reduced in the EPBD group (OR = 0.53, 95%CI: 0.36 to 0.77,  $P = 0.0008$ ). These findings also suggest that in patients who experience a greater risk of bleeding, EPBD may be more suitable.

### Applications

A greater understanding of the advantages and disadvantages of EST and EPBD treatments for the removal of common biliary duct stones allows clinicians to make appropriate treatment decisions for patients.

### Peer review

The authors performed a systematic meta-analysis to investigate the curative effect and safety between EST and EPBD. Compared with EST, in spite of the overall stone clearance rate of EPBD is lower, and the incidence of pancreatitis in the EPBD is higher. EPBD has the advantages to decrease the incidence of long-term complications. For patients experiencing a greater risk of bleeding, EPBD may be more suitable.

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## Foreign body impaction in the sigmoid colon: A twenty euro bet

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Supported by János Bolyai Research Grant, to Veres G; A Hungarian Scientific Research Fund Grant, No. OTKA-K 105530

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Received: July 23, 2012 Revised: October 26, 2012

Accepted: November 14, 2012

Published online: June 28, 2013

### Abstract

Foreign body ingestion is a common clinical problem in early childhood. However, it may occur even in adults, unknowingly. Most ingested foreign bodies entering the stomach pass through the gastrointestinal tract uneventfully. Here we report on a 13-year-old boy who presented with chronic abdominal pain, weight loss and occult gastrointestinal bleeding for 6 mo. Colonoscopy was negative; however, a ballpoint pen was impacted in the sigmoid region. Subsequently, the child admitted swallowing a pen as a 20-euro bet 6 mo previously. Crohn's disease is a chronic relapsing inflammatory gastrointestinal disease. It is often difficult to diagnose due to the fact that there is no single pathognomonic sign or symptom. This case is a description of an adolescent with chronic gastrointestinal symptoms due to a foreign body. Therefore, an ingested foreign body should be included in the differ-

ential diagnostic procedure related to gastrointestinal symptoms.

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**Key words:** Foreign bodies; Pen; Differential diagnosis; Crohn's disease; Child

Müller KE, Arató A, Lakatos PL, Papp M, Veres G. Foreign body impaction in the sigmoid colon: A twenty euro bet. *World J Gastroenterol* 2013; 19(24): 3892-3894 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3892.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3892>

### INTRODUCTION

Foreign body ingestion is a common problem in everyday emergency practice, primarily in children. Fortunately, the majority of the ingested foreign objects pass through the gastrointestinal tract without complications. Foreign body ingestion may present without symptoms<sup>[1]</sup>, and in some cases results in perforation with gastrointestinal bleeding or an obstruction<sup>[1]</sup>. Rarely, an abscess or a fistula occurs<sup>[1]</sup>. It has been reported to mimic other diseases (renal stone or irritable bowel syndrome)<sup>[1]</sup>; however, there are no literature data concerning differential diagnostic difficulties of Crohn's disease (CD) and a foreign body.

CD is a chronic relapsing inflammatory gastrointestinal disease. It is often difficult to diagnose due to the fact that there is no single pathognomonic sign or symptom. Here we report on a case with chronic abdominal symptoms, weight loss and occult bleeding suggesting CD. However, at colonoscopy, a swallowed pen was impacted in the sigmoid region causing the aforementioned symptoms.

### CASE REPORT

A 13-year-old boy was admitted to the First Depart-

ment of Paediatrics at Semmelweis University with 10-kg weight loss in the last 6 mo and intermittent colicky abdominal pain. Past medical history revealed that 6 mo previously he was admitted to hospital for 2 d due to acute abdominal pain and vomiting. He was given intravenous fluids and his complaints improved. However, anorexia persisted, abdominal pain returned intermittently, and he lost 10 kg during this period. Stool blood test was positive twice, while stool culture, parasite and assays for *Clostridium difficile* toxins A and B were all negative. Considering the age of the patient, weight loss, chronic abdominal pain and positive stool blood test, as well as negative stool culture, inflammatory bowel disease was suspected and he was referred to our clinic.

Physical examination revealed normal vital signs without any clinical abnormalities. His abdomen was soft, non-tender, without guarding or palpable masses. There was normal sphincter tone, no perianal abscess, skin tag or fistula at rectal examination. Laboratory tests showed normal red blood cell count, white blood cell count and thrombocyte count. C-reactive protein, total protein and albumin were within the normal limits. Abdominal ultrasound showed slight wall thickening in the descending colon. There was no family history of inflammatory bowel disease.

The upper endoscopy was negative. There were no ulcers, no sign of bleeding, and no antral or bulbar lymphonodular hyperplasia. The terminal ileum and the colon appeared normal, confirmed by multiple mucosal biopsies at histology. Surprisingly, at withdrawal of the colonoscope, an impacted foreign body (plastic half-ball pen, Figure 1) was observed embedded in the sigmoid region. The surrounding mucosa was inflamed, with no visible mucosal vessels. The plastic, numbered ballpoint pen could not be removed by Dormia set and polypectomy snare despite several attempts. After the diagnostic procedures, the patient admitted swallowing a half-plastic pen as a 20-euro bet with his friend 6 mo ago. At first, he thought there might have been a connection between the swallowed pen and his symptoms as he had a hospital admission for 2 d due to acute abdominal pain and vomiting (see above). Later, he was embarrassed and hoped that these two events were just a coincidence. He never disclosed his bet.

Plain abdominal X-ray and abdominal ultrasound were performed the next day after endoscopy, but the foreign body could not be visualized. The pen passed through the colon spontaneously hence the control colonoscopy showed no foreign body after 5 d. The patient had no symptoms during 21 mo of follow-up; his weight gain is normal, and there is no occult bleeding.

## DISCUSSION

We report on a case of an ingested foreign body as a twenty-euro bet. To our knowledge, this is the first description of a swallowed object causing chronic gastrointestinal symptoms in a paediatric patient. However,



Figure 1 Half-plastic pen impacted in the sigmoid region.

ingested foreign bodies are a common event in the paediatric population.

The first recorded paediatric foreign body ingestion was described by Frederick the Great in 1692<sup>[2]</sup>. Most accidentally ingested foreign bodies go undetected and pass through without any incident. However, 10%-20% require endoscopic removal and 1% or less require surgical intervention<sup>[3]</sup>. In general, the passing of an ingested foreign body depends on the anatomic conditions of the gastrointestinal tract and on factors related to the ingested foreign body. Long, thin objects as seen in our case are less likely to pass the pylorus or the duodenum<sup>[2]</sup>. The presenting features vary according to the site and include pancreatitis, hepatic abscess, appendicitis, intussusceptions and irritable bowel syndrome<sup>[1,2]</sup>.

There are only a few reports of foreign bodies imitating CD in the English literature. Ioannidis *et al*<sup>[4]</sup> reported on a case of incidental toothpick ingestion which caused an ileum fistula and mimicked CD. In addition, a patient presented with recurrent, subacute obstruction and right iliac mass mimicking the presentation of CD<sup>[5]</sup>. Subsequent lower endoscopy revealed small bowel phytobezoar which passed spontaneously.

To our knowledge, only one paediatric case of an ingested foreign body mimicking CD has been reported<sup>[6]</sup>; nevertheless, this was an acute event. A 7-year-old boy presented with a 2-wk history of cramping abdominal pain and low grade fever. Colonoscopy revealed an oedematous, friable rectosigmoid junction with a solitary fistula or ulcer. Hydrocortisone enemas were prescribed with minimal improvement. Seven days later his condition became more serious, and a computed tomography scan revealed a right iliopsoas abscess. Repeated colonoscopy showed a toothpick in the lumen of the rectosigmoid colon.

Foreign bodies usually cause acute symptoms of perforation, obstruction or gastrointestinal bleeding. However, our patient had chronic symptoms. This is probably due to the lack of perforation or obstruction. Meanwhile, his symptoms were similar to the reported cases, in which perforation or fistula was diagnosed.

We were presented with a healthy, young adolescent with chronic abdominal pain, weight loss and occult bleeding - classic presenting features of paediatric inflam-

matory bowel disease. Our working hypothesis was CD, though the laboratory tests were negative. Colonoscopy revealed a plastic, half-ball pen embedded in the sigmoid mucosa. The patient concealed swallowing a half-pen as a 20-euro bet with his friend. His subsequent hospital admission due to vomiting and acute abdominal symptoms may be explained as due to gastric irritation. Later the foreign body passed through the upper gastrointestinal tract and impacted in the sigmoid region. We speculate that occult bleeding was caused by the chronic mucosal irritation around the embedded pen. Anorexia and abdominal pain can be explained by the increased bowel peristalsis around the logged pen<sup>[7]</sup>.

The value of imaging studies for an ingested foreign body seems to be questionable based on our case. Hence, plain abdominal X-ray (plastic pen) and abdominal ultrasound (sigmoid localization) could not identify the pen. Nevertheless, the role of imaging studies is crucial to determine the inflammatory reaction in and around the bowel wall and to exclude findings requiring surgical intervention<sup>[7]</sup>.

The majority of ingested foreign bodies that reach the stomach pass through the alimentary tract without complication. If not, the management of ingested foreign bodies is dependent on their size, shape, material and location. After imaging studies, endoscopy should be considered as the crucial step in management since it is a potent and safe diagnostic tool. On the other hand, surgical treatment is mandatory in the presence of complications such as abscesses and fistulas<sup>[7]</sup>. In our patient, we attempted the removal of the embedded pen, but due to the increased possibility of perforation, we did not prevail. Subsequently, the pen was spontaneously passed after the first endoscopy as a result of the previous attempts of removal.

In summary, we report on an adolescent patient with

chronic gastrointestinal symptoms due to a swallowed plastic pen that mimicked CD. Therefore, an ingested foreign body should be included in the differential diagnostic procedure related to chronic gastrointestinal symptoms.

## ACKNOWLEDGMENTS

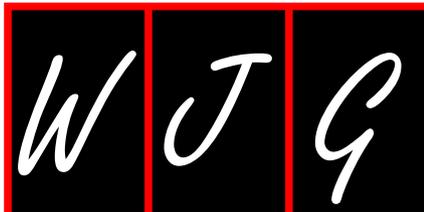
We thank Alexandra Horvath for her language editing.

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## Early-stage primary signet ring cell carcinoma of the colon

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Received: November 22, 2012 Revised: April 15, 2013

Accepted: May 18, 2013

Published online: June 28, 2013

**Core tip:** Primary signet ring cell carcinoma of the colorectum detected at an early stage is very rare; most cases are detected at an advanced stage. Therefore, its prognosis is poorer than that of ordinary colorectal cancer. We report a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

Kim JH, Park SJ, Park MI, Moon W, Kim SE. Early-stage primary signet ring cell carcinoma of the colon. *World J Gastroenterol* 2013; 19(24): 3895-3898 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3895.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3895>

### Abstract

Primary signet ring cell carcinoma of the colorectum detected at an early stage is very rare; most cases are detected at an advanced stage. Therefore, its prognosis is poorer than that of ordinary colorectal cancer. A 56-year-old Korean man was seen at this hospital for management of signet ring cell carcinoma of the colon. Colonoscopic examination revealed a II a-like, ill-defined and flatly elevated 9-mm residual tumor in the cecum. Endoscopic mucosal resection was performed. Pathological examination of the resected specimen revealed signet ring cell carcinoma that had invaded the lamina propria without venous or perineural invasion. Abdominal computed tomography (CT) and positron CT showed no evidence of primary lesions or distant metastasis. An additional laparoscopic right-hemicolectomy was performed; no residual tumor or lymph node metastasis was found. We report a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

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**Key words:** Colon carcinoma; Signet ring cell carcinoma; Primary carcinoma; Early stage; Endoscopic mucosal resection

### INTRODUCTION

Primary signet ring cell carcinoma is a rare type of colorectal cancer comprising 0.1%-2.6% of all colorectal cancers<sup>[1]</sup>. Because clinical symptoms tend to occur late in the course of signet ring cell carcinoma, most cases are usually detected at an advanced stage<sup>[2]</sup>; therefore, its overall survival rate is reported to be poorer than that of ordinary colorectal adenocarcinoma<sup>[3,4]</sup>. Early diagnosis is important to improve outcomes; however, there is little known about the early stages of signet ring cell carcinoma. In this case report, we describe a case of primary signet ring cell carcinoma of the colon detected at an early stage and provide a review of the literature.

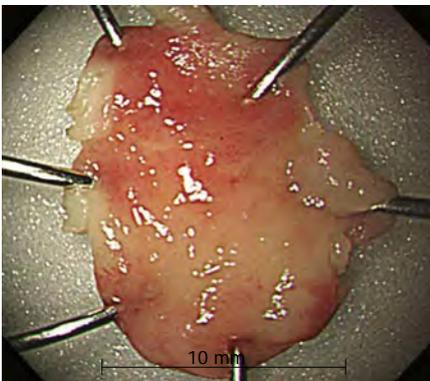
### CASE REPORT

A 56-year-old Korean man was seen in the gastroenterology clinic at this hospital for management of signet ring cell carcinoma of the colon.

The patient had been healthy up to the time of his presentation. Ten days before his evaluation at this hospital, the patient saw a gastroenterologist at another hospital and underwent colonoscopy for a health checkup. Colonoscopic examination revealed a II a-like, ill-defined



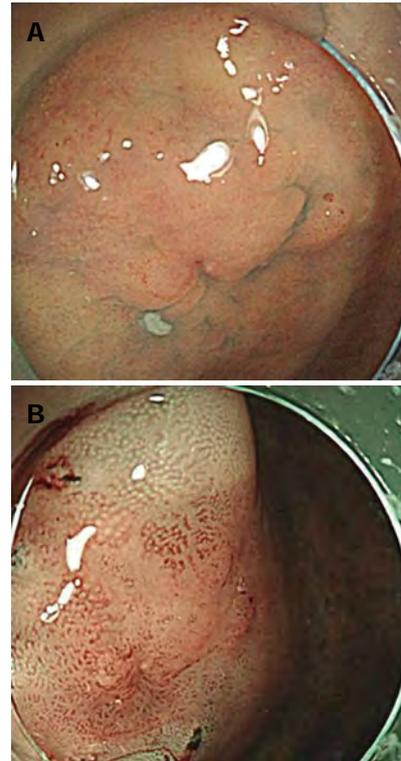
**Figure 1** Initial colonoscopic findings (from another hospital). Colonoscopic examination revealed a II a-like, ill-defined and flatly elevated 5-mm tumor in the cecum (arrow).



**Figure 3** Resected specimen by endoscopic mucosal resection. The resected specimen was 10 mm in diameter.

and flatly elevated 5-mm tumor in the cecum (Figure 1). In addition, several polyps were observed in the ascending colon extending to the transverse colon. Snare polypectomy of the tumor in the cecum was performed incompletely, and a biopsy of several polyps was also conducted. Pathological examination of a biopsy specimen of the cecal tumor showed signet ring cell carcinoma, while biopsy specimens of several polyps showed tubular adenoma with low grade dysplasia. The patient was referred to the gastroenterology clinic at this hospital on October 6, 2009.

At presentation, the patient was active and was experiencing no symptoms. He took no medications and had no known allergies to medications. He drank alcohol, had a history of smoking (20 packs per-year), and did not use illicit drugs. His past history was unremarkable, and there was no family history of cancer. On examination, his body weight was 59.0 kg and height was 154 cm; the vital signs and remainder of the physical examination were normal. The level of carcinoembryonic antigen was 2.26 ng/mL (reference value, < 3.4). Results of a complete blood count, plasma levels of electrolytes, tests of coagulation and kidney and liver function, and a urinalysis were normal. One day later, upper endoscopic examination revealed no primary lesions, and colonoscopic examination revealed a II a-like, ill-defined and flatly elevated



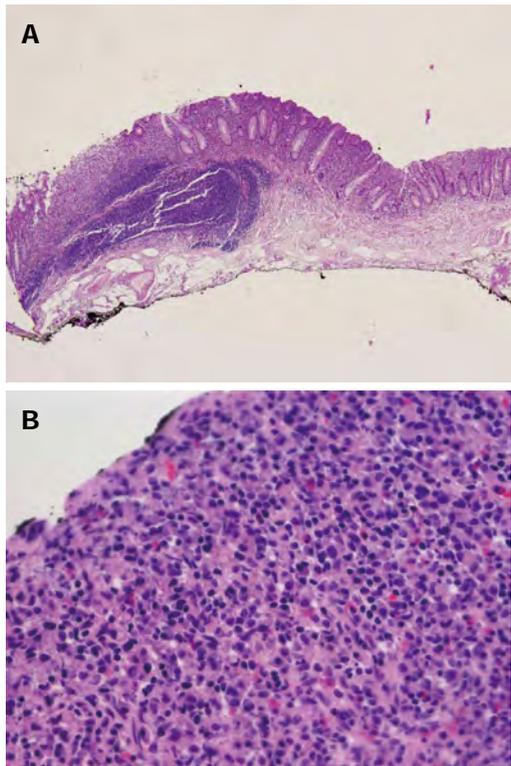
**Figure 2** Colonoscopic findings at this hospital. A: Colonoscopic examination revealed a II a-like, ill-defined and flatly elevated 9-mm residual tumor in the cecum; B: Narrow band imaging shows the lesion more clearly.

9-mm residual tumor in the cecum (Figure 2). In addition, several lateral spreading tumors were observed in the ascending colon extending to the transverse colon. Endoscopic mucosal resection (EMR) of the residual tumor in the cecum was performed. The tumor was successfully removed en bloc by EMR without complications. The resected specimen was 10 mm in diameter (Figure 3). Histologically, the tumor was composed of carcinoma with lymphoid hyperplasia and, favored signet ring cell carcinoma that had invaded the lamina propria without venous or perineural invasion (Figure 4). The cut end of the resected specimen was examined for safety. The other tumors were removed by polypectomy; histologically, the specimens were composed of tubular adenoma with low grade dysplasia. Abdominal computed tomography (CT) and CT scanning with positron-emission tomography (PET-CT) showed no evidence of primary lesions or distant metastasis. Two weeks later, the patient underwent an additional laparoscopic right-hemicolectomy, because of the high incidence of peritoneal metastasis associated with signet ring cell carcinoma and the possibility of recurrence of several lateral spreading tumors. The resected specimen revealed no residual carcinoma at the EMR site and showed no lymph node metastasis (Figure 5).

The patient has been in good health for two years and has shown no recurrence after the operation.

## DISCUSSION

Primary signet ring cell carcinoma of the colon and



**Figure 4** Scanning view of the endoscopic mucosal resection site. Histologically, the resected specimen showed diffusely infiltrated signet ring cells in the lamina propria without venous or perineural invasion. A: hematoxylin/eosin staining,  $\times 40$ ; B: hematoxylin/eosin staining,  $\times 400$ .

rectum was first described by Laufman and Saphir in 1951<sup>[5]</sup>. Its characteristics include more advanced stages at presentation<sup>[1,4,6]</sup>, younger age at presentation<sup>[3,4]</sup>, chiefly peritoneal dissemination<sup>[1,4,7]</sup>, lymphatic spread<sup>[5,6]</sup>, few liver metastases<sup>[1,4,8]</sup>, and poor prognosis<sup>[1,4,6-8]</sup>. Because its clinical symptoms develop late, most cases are usually detected at an advanced stage. Bonello *et al.*<sup>[9]</sup> described three factors for this delay in diagnosis: (1) the rarity of the tumor; (2) intramucosal tumor spread with relative sparing of the mucosa, accounting for minimal symptoms and heme-negative stools; and (3) radiographic tumor resemblance to inflammatory processes. Because most cases are detected at an advanced stage, the prognosis of such tumors is dismal. Median and mean survival times are reported as 20 and 45 mo, respectively<sup>[6-8]</sup>, and 5-year survival rates are between 9% and 36%<sup>[1,6,8]</sup>. Makino *et al.*<sup>[10]</sup> found that all 17 patients with stage 0/I disease were alive at the latest follow-up evaluation, and the 5-year survival rate of patients with T2 disease was 75.0%. Therefore, to improve outcome, early diagnosis is very important. The patient in this case report was diagnosed with primary signet ring cell carcinoma of the colon at an early stage, and a thorough workup did not reveal any other sites of involvement and the tumor invaded only lamina propria without venous or perineural invasion. A review of the literature revealed that there are only 27 cases of primary signet ring cell carcinoma of the colon and rectum detected at an early stage, including the patient in this case report<sup>[11-17]</sup>. Of these 27 patients, 22 were males



**Figure 5** Gross findings. The mucosal surface of the cecum showing an ill-defined irregularly-shaped scar (probably the endoscopic mucosal resection site). The remaining mucosa showing multiple polyps and previous polypectomy sites.

and 5 were females, with a mean age of 57.1 years (range: 6-79 years). The mean size of the tumor was 15.7 mm (range: 2-45 mm). Regarding the location of the tumors, 14 cases were in the right-side of the colon, 7 cases were in the left-side of the colon, and 6 cases were in the rectum. Macroscopically, 10 cases were polypoid type, 4 cases were flat type, and 13 cases were depressed type. Microscopically, 20 cases had submucosal invasion and 7 cases had intramucosal invasion. Makino *et al.*<sup>[10]</sup> reported that all 69 patients with scirrhous and polypoid tumors had stage T3 or T4 disease, while 83.3% of patients with superficial tumors had stage T0 or T1 disease. In this case report, the patient's tumor was located in the mucosa, and the gross shape of the tumor was Ila-like, ill-defined and flatly elevated. Thus, for early detection of signet ring cell carcinoma of the colorectum, we believe that careful observation of easily overlooked lesions such as superficial tumors, flatly elevated, or flatly depressed lesions during colonoscopic examinations is important.

Some authors have suggested an association between ulcerative colitis and signet ring cell carcinoma of the colorectum<sup>[5,18]</sup>. Ojeda *et al.*<sup>[19]</sup> reported that 7 of 60 patients (12%) had both ulcerative colitis and primary colorectal signet ring cell carcinoma. Anthony *et al.*<sup>[1]</sup> found that 4 of 29 patients (14%) had a previous history of inflammatory bowel disease. The diagnosis in 2 of these patients was ulcerative colitis; the other 2 patients were diagnosed with Crohn's disease. The patient in this case report had no inflammatory bowel disease. Although the role of chronic inflammatory bowel disease in the development of this tumor is still undetermined, regular colonic examination of patients with history of chronic inflammatory bowel disease could be important.

A positive family history is a risk factor for ordinary colorectal cancer. However, in accordance with some studies, a positive family history may not be a predictive factor for signet ring cell carcinoma<sup>[2,4]</sup>. This finding could be attributed to the relatively small number of cases or could represent variability of this type of tumor. The patient in this case report had no family history of colorectal cancer.

Little is known about the early stages of signet ring cell carcinoma. It is unclear whether signet ring cell carcinoma develops from a pre-existing adenomatous polyp or as a so-called *de novo* carcinoma. To our knowledge, only 3 cases of an association between signet ring cell carcinoma and adenomatous polyps have been reported. Hamazaki *et al.*<sup>[12]</sup> reported a case of a 6-year-old boy with a signet ring cell carcinoma in a polyp of the colon, Nakamura *et al.*<sup>[14]</sup> described a case of a 4.5 cm rectal adenoma with multiple foci of signet ring cell carcinoma, and Tandon *et al.*<sup>[20]</sup> reported a case of a sigmoid colon adenoma with a focus of signet ring cell carcinoma. The patient in this case report had several lateral spreading tumors in the ascending colon extending to the transverse colon; however, histologically, none of the specimens were composed of foci of signet ring cell carcinoma. Although controlled studies about the association of adenomas and signet ring cell carcinomas are lacking, it could be possible that progression of an adenoma to signet ring cell carcinoma is occurring.

In the present case report, we have described a rare case of primary signet ring cell carcinoma of the colon detected and treated at an early stage, and provided a review of the literature.

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## Severe irinotecan-induced toxicity in a patient with *UGT1A1\*28* and *UGT1A1\*6* polymorphisms

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Author contributions: Xu JM designed research; Wang Y analyzed the pharmacokinetic and clinical data; Ge FJ and Lin L collected clinical data; Liu ZY analyzed pharmacokinetic data; Xu JM and Sharma MR wrote the paper; all authors read and approved the final manuscript.

Supported by National Natural Science Foundation Project, Grants No. 30971579; and Capital Development Foundation, No. 2007-2029

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Received: March 8, 2013 Revised: April 24, 2013

Accepted: May 8, 2013

Published online: June 28, 2013

*7A1\*6* polymorphism (G/A). The patient was treated with FOLFIRI for 9 cycles and underwent two irinotecan dose reductions according to pharmacokinetic data regarding exposure to the active metabolite, SN-38. Simultaneous heterozygous *UGT1A1\*28* and *UGT1A1\*6* polymorphisms may produce higher exposure to SN-38 and a higher risk of adverse effects related to irinotecan. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both *UGT1A1\*28* and *UGT1A1\*6* polymorphisms.

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**Key words:** Irinotecan; Toxicity; *UGT1A1\*28*; *UGT1A1\*6*; Polymorphism

**Core tip:** This is the first reported case. This patient with heterozygous *UGT1A1\*28* and *UGT1A1\*6* polymorphisms experienced two dose reductions of irinotecan due to severe toxicity according to pharmacokinetic analyses of SN-38 and SN-38 glucuronide levels. It seems that this patient benefited from a longer treatment duration, suggesting that irinotecan dose individualization for mutant metastatic colorectal cancer patients with heterozygous *UGT1A1\*28* or *UGT1A1\*6* polymorphisms may be warranted.

### Abstract

Many studies have demonstrated the impact of *UGT1A1* on toxicity of irinotecan. In particular, patients bearing *UGT1A1\*28* (TA 7/7) have a higher risk of severe neutropenia and diarrhea. Based on this, prescribers of irinotecan are advised that patients with *UGT1A1\*28* (TA 7/7) should start with a reduced dose of irinotecan, although a particular dose is not specified. Research in Asian countries has shown a lower incidence of *UGT1A1\*28* (TA 7/7), while *UGT1A1\*6* (A/A) is more often found and is associated with severe irinotecan-related neutropenia. We report here a case of a metastatic colorectal cancer patient who is heterozygous for the *UGT1A1\*28* polymorphism (TA 6/7) as well as the *UG-*

Xu JM, Wang Y, Ge FJ, Lin L, Liu ZY, Sharma MR. Severe irinotecan-induced toxicity in a patient with *UGT1A1\*28* and *UGT1A1\*6* polymorphisms. *World J Gastroenterol* 2013; 19(24): 3899-3903 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3899.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3899>

### INTRODUCTION

The FOLFIRI regimen, which is composed of 5-fluorouracil (5-FU), leucovorin, and irinotecan, is a commonly

used treatment regimen for metastatic colorectal cancer. UGT1A polymorphisms have been the focus of irinotecan pharmacokinetics (PK) and toxicity research since UGT1A enzymes play a key role in the glucuronidation of the active metabolite of irinotecan, SN-38, to the inactive SN-38G<sup>[1-3]</sup>. There have been a number of studies to examine the associations of the *UGT1A1*, *UGT1A7*, and *UGT1A9* genotypes and severe irinotecan-induced toxicity, particularly diarrhea and neutropenia<sup>[4-7]</sup>. The data strongly suggest that the *UGT1A1\*28* genotype is associated with severe irinotecan-induced diarrhea and neutropenia<sup>[7-9]</sup>, which led to a change in the irinotecan United States label to recommend dose reduction in patients with lower UGT1A1 activity. The incidence rates of severe neutropenia and diarrhea (grade 3/4) in patients homozygous for *UGT1A1\*28* (TA 7/7) in Western and Eastern populations were > 30%<sup>[10,11]</sup>. Interestingly, there are no polymorphisms at the *UGT1A1\*6* locus reported in the Western population. However, studies in Asian countries indicated that there is a common (35.8%-38.9%) single nucleotide polymorphism at the *UGT1A1\*6* (G→A) locus that is associated with severe irinotecan-related neutropenia<sup>[12-14]</sup>. The effects of other *UGT1A7* and *UGT1A9* polymorphisms on irinotecan-related toxicity remain unclear<sup>[4,12]</sup>. To determine the optimal dose of irinotecan in the FOLFIRI regimen, we are conducting a prospective and multicenter clinical trial in which the dose of irinotecan is adjusted for the specific *UGT1A* genotypes in patients with metastatic colorectal cancer (NCT01523431).

### CASE REPORT

A 72-year-old male patient with an adenocarcinoma at the hepatic flexure of the colon underwent right hemicolectomy. Several mo later, the patient developed metastases in the liver, bilateral lungs and mediastinal lymph nodes. Liver biopsy confirmed metastatic disease from colon cancer. Routine genotyping showed that the patient was heterozygous for the *UGT1A1\*28* polymorphism (TA 6/7) as well as the *UGT1A1\*6* polymorphism (G/A). The patients had normal liver function and renal function. He was treated with the standard FOLFIRI regimen: a 90-min intravenous (*iv*) infusion of irinotecan (Coptosar, Pfizer) (180 mg/m<sup>2</sup>); an *iv* infusion of leucovorin (400 mg/m<sup>2</sup>); followed by 5-FU by *iv* bolus (400 mg/m<sup>2</sup>) and continuous *iv* infusion (2400 mg/m<sup>2</sup>) over 46 h; this regimen was repeated every 2 wk. Concurrently, a 5-mL heparinized blood sample was collected before irinotecan administration, at 1 and 1.5 h during the infusion and at 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24, 36, and 48 h after the termination of the drug infusion. After the first treatment cycle, the patient suffered grade 4 neutropenia, grade 3 diarrhea, grade 2 fatigue, and grade 2 mucositis. The area under the curve (AUC) of SN-38, the active metabolite of irinotecan, was 929 ng/mL per hour (Figure 1), which was 4-fold that of the mean AUC for wild-type patients treated with the standard FOLFIRI regimen

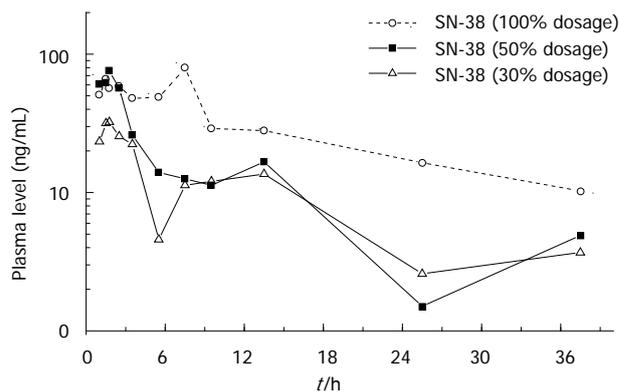


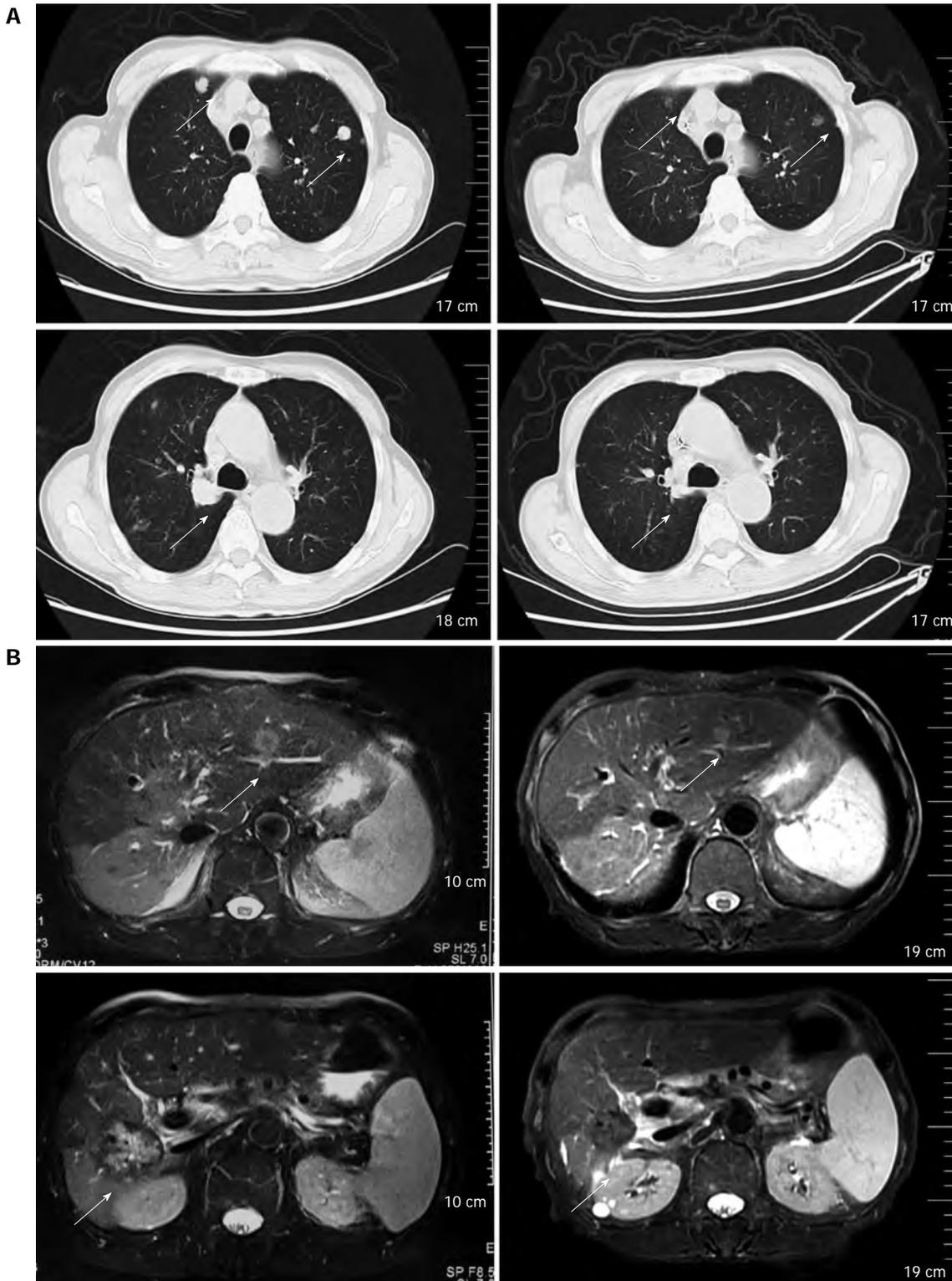
Figure 1 Plasma concentration-time profiles of SN-38 in different dose levels of irinotecan.

Table 1 Area under the curve of irinotecan, SN-38 and SN-38G for this patient at various doses of irinotecan, compared with a group of patients with wild type UGT1A1 at the standard dose of irinotecan

| Irinotecan dose (mg/m <sup>2</sup> )   | AUC <sub>irinotecan</sub> (ng/mL per hour) | AUC <sub>SN-38</sub> (ng/mL per hour) | AUC <sub>SN-38G</sub> (ng/mL per hour) | AUC <sub>SN-38G/AUC<sub>SN-38</sub></sub> |
|--|--|---------------------------------------|--|---|
| 180                                    | 26838                                      | 929                                   | 3000                                   | 3.2                                       |
| 90                                     | 12790                                      | 476                                   | 2014                                   | 4.2                                       |
| 54                                     | 9488                                       | 328                                   | 2096                                   | 3.4                                       |
| UGT1A1 wild type patients <sup>1</sup> |  |                                       |  |   |
| 180                                    | 6321 ± 3993                                | 234 ± 185                             | 645 ± 353                              | 3.3 ± 2.1                                 |

<sup>1</sup>Treated with standard FOLFIRI regimen at our cancer center (n = 38, mean ± SD). AUC: Area under the curve.

at our cancer center. The AUC ratio of glucuronidated SN-38G/SN-38 was 3.23, which was similar to that of wild-type patients (Table 1). Therefore, we reduced the irinotecan dose by 50% (to 90 mg/m<sup>2</sup>) but maintained the doses of 5-FU and leucovorin in subsequent cycles. The second round of pharmacokinetic analysis showed that the AUC of SN-38 was 476 ng/mL per hour, which was more than 2-fold that of the mean AUC for wild-type patients. After the second cycle, there was no neutropenia and only grade 1 diarrhea. Moreover, computerized tomography (CT) and magnetic resonance imaging scans showed a partial response in the lung and liver metastases, respectively, and the response was confirmed after two additional cycles (Figure 2). However, after the fifth cycle, the patient developed recurrent neutropenia and additional doses of irinotecan were held. His Eastern Cooperative Oncology Group performance status had worsened from 0 to 1. He received two cycles of capecitabine (2000 mg/m<sup>2</sup> divided *bid* for 2 wk on, 1 wk off) as maintenance therapy, and his CT showed stable disease (SD). However, the patient discontinued therapy for 1 mo because of grade 3 mucositis and grade 3 diarrhea. A few mo later, he experienced disease progression in the liver and lung and his weight had decreased from 54.5 to 47 kg over 3 mo. We decided to reinstate the FOLFIRI regimen. The irinotecan dose was reduced by



**Figure 2** Computerized tomography scan (A) and magnetic resonance imaging (B). Before treatment (left figure) and after treatment (right figure) of the lung and the liver metastases, respectively; the response was confirmed after two additional cycles.

70% (to 54 mg/m<sup>2</sup>), while the 5-FU dose was reduced by 30%. The third round of pharmacokinetic analysis showed that the AUC of SN-38 was 328 ng/mL per hour, which is close to the mean AUC with a standard dose in wild-type patients. After two additional cycles, he had SD but experienced grade 3 neutropenia and grade 2

diarrhea. CT scan showed disease progression after two additional cycles of chemotherapy.

## DISCUSSION

Recent studies in Asian countries have indicated that

the polymorphism in UGT1A1\*6 has a similar effect as UGT1A1\*28 on irinotecan-induced toxicity and PK<sup>[12,15,16]</sup>. However it is unclear whether simultaneous heterozygous UGT1A1\*28 and UGT1A1\*6 (TA 6/7 + G/A genotype) polymorphisms may have significantly more side effects and impact on PK of SN38. Irinotecan PK are determined by multiple metabolizing enzymes, whereas the saturation of enzymatic reactions is affected by other factors, such as age and creatinine clearance<sup>[17]</sup>. The patient in the present study, who carried both polymorphisms, experienced serious toxicity after one cycle of a standard irinotecan dose, which corresponded to an SN-38 exposure (AUC) that was 4-fold higher than in wild-type patients. It has been reported that low dose irinotecan-induced toxicity is not associated with UGT1A1 polymorphisms<sup>[18]</sup>. In the present study, however, toxicity recurred even after reducing the irinotecan dose by 50%, and the AUC of SN-38 was still 2-fold higher than the mean in wild-type patients who received standard dose irinotecan. Even after a 70% dose reduction, the AUC of SN-38 was close to the mean in wild-type patients who received standard dose irinotecan. This observation suggests that the PK were still affected by the polymorphisms in the UGT1A1 gene even at relatively low doses. Whether dosing and AUC may be associated with efficacy is still unclear; however, data from Toffoli *et al*<sup>[7]</sup> suggest that higher doses and higher AUC may be associated with higher efficacy in patients with mutant metastatic colorectal cancer.

When faced with intolerable toxicity, oncologists typically reduce the dose, delay treatment, or discontinue treatment, any or all of which may reduce the treatment duration and affect the progression-free survival and overall survival of patients. The patient in this report underwent two irinotecan dose reductions during the course of 9 treatment cycles with FOLFIRI, as well as 2 cycles of capecitabine as maintenance therapy. The duration of disease control, including breaks, was 7 mo, which suggests that the patient benefited from the dose reductions. The AUC ratio of SN-38G/SN-38 did not decrease with additional treatment cycles, suggesting that patients with UGT1A1 polymorphisms may not experience the irinotecan-induced inhibition of UGT1A1 and corresponding decrease in the AUC ratio of SN-38G/SN-38 that has been observed by Hirose *et al*<sup>[16]</sup> in wild-type patients. To our knowledge, the present case is the first report to adjust the irinotecan dose twice based on the patient's UGT1A1 genotype and according to PK characteristics. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both UGT1A1\*28 and UGT1A1\*6 polymorphisms and to determine how this genotype may influence efficacy.

In summary, simultaneous heterozygous UGT1A1\*28 and UGT1A1\*6 polymorphisms may produce higher exposure to SN-38 and a higher risk of adverse effects related to irinotecan. Additional studies will be necessary to determine the optimal starting dose of irinotecan for patients with both UGT1A1\*28 and UGT1A1\*6 polymorphisms.

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**P-Reviewer** Ladero JM    **S-Editor** Gou SX  
**L-Editor** O'Neill M    **E-Editor** Xiong J.



## Pathological diagnosis is maybe non-essential for special gastric cancer: Case reports and review

Wu Song, Chun-Yu Chen, Jian-Bo Xu, Jin-Ning Ye, Liang Wang, Chuang-Qi Chen, Xin-Hua Zhang, Shi-Rong Cai, Wen-Hua Zhan, Yu-Long He

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Supported by The Science and Technology Development Project of Guangdong Province, No. 2011B031800240 and No. 2012B031800389

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Received: November 21, 2012 Revised: April 17, 2013  
Accepted: May 8, 2013  
Published online: June 28, 2013

### Abstract

Histopathological results are critical for the diagnosis and surgical decision regarding gastric cancer. However, opposite opinions from radiology and pathology can sometimes affect clinical decisions. The two cases reported in this article were both highly suspected as gastric cancer by clinical manifestations and radiologic findings, although both showed negative results in the first biopsy examination. One was confirmed as gastric cancer by the time of the 6<sup>th</sup> biopsy, while the other was still negative even after 8 biopsies. With a definite pathologic result and the agreement of the patient for the latter case, both of them finally received surgery. Postoperative pathological examination revealed findings that were the same as Borrmann type IV gastric cancer. We believed that duplicate biopsies under ra-

diologic guidance were necessary for highly suspected gastric cancer cases in the absence of a definite pathology result, and patients should be under close follow-up. We propose that, if gastric cancer is highly suspected when typical radiology changes of widely diffuse gastric parietal lesions suffice to exclude lymphoma and other similar situations, and even in absence of a positive biopsy result, a diagnostic laparotomy under laparoscopy and even radical gastrectomy may be reasonably performed by an experienced gastric cancer center with the agreement of the patient after being decided by a multidisciplinary discussion team.

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**Key words:** Gastric cancer; Pathology; Diagnosis; Borrmann type IV

**Core tip:** Histopathological diagnosis is the diagnostic gold standard of gastric cancer required by medical ethics and practice guideline. However, cases with repeated suspected false negative pathological results always concern medical practitioners a great deal. This article might illustrate a possible standard process for these cases. We propose that, if gastric cancer is highly suspected when typical radiology changes of widely diffuse gastric parietal lesions suffice to exclude lymphoma and other similar situations, and even in absence of a positive biopsy result, surgery could be performed with the agreement of the patient after being decided by a multidisciplinary discussion team.

Song W, Chen CY, Xu JB, Ye JN, Wang L, Chen CQ, Zhang XH, Cai SR, Zhan WH, He YL. Pathological diagnosis is maybe non-essential for special gastric cancer: Case reports and review. *World J Gastroenterol* 2013; 19(24): 3904-3910 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3904.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3904>

## INTRODUCTION

Gastric cancer is one of the most common cancers of the digestive system, and shows the highest morbidity and mortality among all digestive malignancies in China. Early detection, diagnosis, and treatment are of significant importance in improving patient cure rate and 5-year survival. Gastroscopic biopsy remains one of the primary means for the screening of the disease, and at the same time the gold standard of diagnosis<sup>[1]</sup>. However, biopsy accuracy relies on samples obtained from gastrofiberscopy so much that doctors would be confused when a pathologic examination showed a negative result inconsistent with radiologic findings and other results. Thus, treatment is often delayed due to medical ethics considerations, which require a definite pathological diagnosis. Both cases we are reporting in this article were negative in repeated biopsies but suggested as gastric cancer by radiologic and gastrofiberscopy examination. We intend to demonstrate the clinical decisions for similar patients.

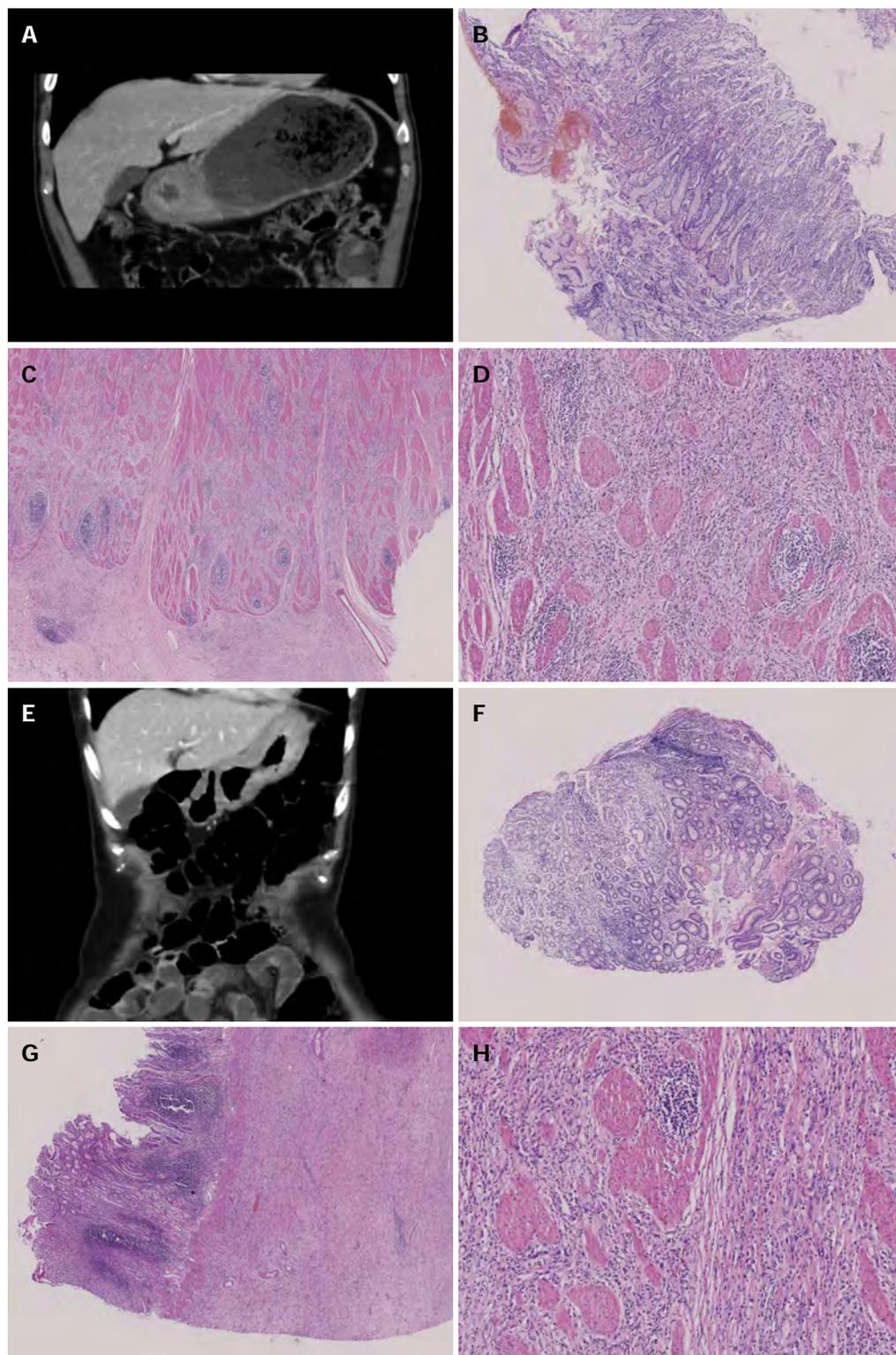
## CASE REPORT

### Case 1

A 50-year-old male presented on May 15, 2009, and was admitted due to recurrent upper abdominal discomfort associated with acid reflux, hiccups, and weight loss for 3 mo. Two months previously, it was suggested that he have an abdominal computed tomography (CT) scan in another hospital, and an obviously thickened antral wall was discovered. The CT report considered a differential diagnosis of gastric cancer and primary gastric lymphoma. The patient then had his first gastroscopy examination. Gastroduodenoscopy did not find any apparent ulcer of the stomach, but did show stenosis of the antrum and pyloric. The biopsy result was antral mucosal inflammation. Finally, the patient was treated as gastritis for 2 mo, but no alleviation of discomfort was achieved. Thus, he came to our hospital for further treatment and was suggested to have further examinations after admission. General blood tests that included alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), tumor marker-CA125 (CA125), tumor marker-CA19-9 (CA19-9), and squamous cell carcinoma antigen revealed nothing abnormal. A second abdominal CT scan in our hospital found circular thickening of the antrum stomach wall and retention of gastric contents (Figure 1). Possible gastric cancer was considered. But after a consultation of biopsy slides of the previous scan, we had the same comments. Therefore, a second gastroscope biopsy was conducted in our hospital, only to find chronic inflammation of antral mucosa and local fibrous tissue proliferation, rather than any malignant cells. Since the patient was highly suspected for Borrmann type IV gastric cancer by our multidisciplinary discussion team (MDT), a third biopsy was performed with endoscopic ultrasound as guidance. We sampled 15 tissue cores by electric biopsy forceps through holding in the thickening antrum stomach wall. Endoscopic ultrasonography also supported that the thickening antral

wall was a typical change of malignant lesions. However, pathological diagnosis reported chronic inflammation of the antral mucosa without any atypical cells (Figure 1). The patient was discharged for another month of continuous gastritis treatment, which failed to bring about any improvements. One month later, the patient came back for a fourth gastroscope biopsy, but with immunohistochemical examination this time. As before, the report showed chronic inflammation of the antral mucosa and local fibrous tissue proliferation. Immunohistochemistry results were: suspicious atypical cells with M-CEA ( $\pm$ ), S-100 (-), actin (-), Syn (-), CD117 (-), CD56 (-), glandular epithelium CK (-), small lymph L26 (+), CD79a (+), the LCA (+), CD3 (+), the tissue cells CD68 (+) and plasma cells CD38 (+). Although there was no definite pathologic result of gastric cancer, Borrmann type IV gastric cancer was strongly suspected. Therefore a further examination of positron emission tomography-CT (PET-CT) was performed and reported. It showed uneven diffuse thickening of the antrum stomach wall, with antrum metabolic imaging not supporting this malignant change and no abnormal metabolic lesions or standard uptake value (SUV) occurring in the other tissues. Conventional medical management seemed to be ineffective and the patient gradually felt worse and worse. On July 29<sup>th</sup>, he underwent alimentary tract barium meal examination, and the result indicated it was most probably primary gastric lymphoma or antrum cancer with submucosal infiltration, besides eosinophilic gastritis. The patient was discharged from hospital again because we failed to get a positive biopsy result supporting the diagnosis of cancer. On August 30<sup>th</sup>, one month after discharge, he was asked to have an abdominal CT and gastroscopy examination, which was the suggestion from the MDT last time. The sixth gastroscopy biopsy result confirmed it was gastric antrum signet-ring cell (Figure 1) carcinoma, accompanied with immunohistochemical stain results: signet ring-like cells CK (+), CEA (+). Abdominal CT examination reported gastric antrum cancer with perigastric lymph nodes enlargement. A surgery decision was made soon after the confirmation.

During laparotomy, we found the antropylorus where lesions located was thickened, and the perigastric lymph nodes, especially those of the lesser curvature, were obviously enlarged from the 0.5 to 2.5 cm. In addition, multiple metastatic nodules were detected on the omentum. There were no findings of metastasis to other organs within the abdominal cavity and no dissemination of the peritoneum was observed. Radical distal gastrectomy (D2 lymphadenectomy) was therefore performed. Postoperative pathological examination showed that gastric poorly differentiated adenocarcinoma infiltrated the whole gastric wall, and with myenteric nerve invasion (Figure 1) and lymph nodes metastasis (12/27). The patient was finally diagnosed as having antrum signet ring cell carcinoma (T3N2M0, III B period, Borrmann type IV). Eight courses of postoperative adjuvant chemotherapy treatment followed by the XELOX plan were initiated since



**Figure 1 Computerized tomography and pathological findings.** A: Diffuse thickened antral detected by computerized tomography (CT) scan; B: Repeated false negative slides without cancer cells detected for the previous 5 biopsies [hematoxylin-eosin (HE) stain,  $\times 40$ ]; C: Positive slide with cancer cells observed by the 6<sup>th</sup> biopsy (HE stain,  $\times 100$ ); D: Postoperative slide with cancer cells observed, many signet ring-like cells can be observed in the upper-right quadrant of the slide (HE stain,  $\times 200$ ); E: Diffuse thickened fundic and gastric body detected by CT scan; F: Repeated false negative slides without cancer cells detected for 8 biopsies (HE stain,  $\times 40$ ); G: Postoperative slide with cancer cells observed and the whole gastric wall infiltrated (HE stain,  $\times 100$ ); H: Fibrosis stomach wall, scattered or small focal distributed signet ring-like cells could be observed (HE stain,  $\times 200$ ).

the fourth week after surgery. Our follow-up data show that the patient is still alive.

### Case 2

A 52-year-old female had been suffering from abdominal

dull pain with intermittent melena and weight loss for 11 mo, and her medical history showed nothing special. Before she came to us, she had been in a local hospital three times for the same complaint, and received five gastrofiberscopy examinations with biopsies, since the doctors strongly suspected she had gastric cancer. Unfortunately, every biopsy result remained negative, as did the upper gastrointestinal barium meal examination. The barium meal examination regarded it as gastritis due to the imaging findings: a thick mass of the gastric body mucosa folds was detected but no signs of niche or filling defect, gastric motility and tension were well, and barium could go through the pyloric canal smoothly. However, views of the gastrofiberscopy reports were unified: hypertrophy of gastric body mucosal folds; gastric cancer, and duodenal ulcers was considered. Due to the previous ineffective medical management and being unqualified for any further examination or treatment at the local hospital, the patient was referred to our hospital. General blood tests, including tumor markers AFP, CEA, CA125, CA19-9 and squamous cell carcinoma antigen, were performed after admission, which all showed results within the normal ranges. Gastric endoscopic ultrasonography reported a differential diagnosis of adenoma and lymphoma due to the thickened stomach wall of the fundus and gastric body. The patient was suggested to have a sixth biopsy. Under a gastroduodenoscopy, this time in our hospital, we found the gastric mucosa had obvious congestion, edema, was stiff, and the gastric folds were thicker and harder than normal. A biopsy was then performed. Pathological findings showed that no adenocarcinoma was found with hematoxylin-eosin stain, a small amount of abnormal glands were noticed under cytokeratin immunohistochemical stain, M-CEA (-), and a particularly high positive rate of Ki-67 was not seen in the samples. Pathology failed to diagnose the cancer. An abdominal CT examination revealed a diffusely thickened and stiff stomach wall of the gastric fundus and body, a fixed stomach shape, obvious changes to the mucosa of the thickened stomach wall during the contrast enhanced phase, and a number of enlarged lymph nodes in the lesser curvature and retroperitoneal area (Figure 1). The CT report stated possible lymphoma. However, gastric cancer was highly suspected according to our experience and the patient's clinical manifestation. A seventh deep layer biopsy was therefore carried out, but only to disappointingly show a negative result again. After deliberation by the MDT, including experts from departments of imaging, ultrasound diagnosis, pathology, surgery, and internal medicine, we insisted it to be Borrmann type IV stomach adenocarcinoma that showed hypertrophic gastric lesions, and that conventional biopsy methods would always display a low positive rate. We decided to take endoscopic ultrasound guided fine-needle aspiration for the eighth biopsy. We made two punctures deep into the submucosa and muscularis at the stomach wall of the lower segment of the gastric body for samples where the endoscopic ultrasound showed disappearing gastric structure.

Unfortunately, our pathologist told us that cancer cells were still not detected in the samples, only normal gastric mucosa epithelium and some lamina propria glands. A liquid-based cytology test was also negative (Figure 1). However, according to our experience, Borrmann type IV stomach cancer was strongly suspected and surgery was recommended after an MDT discussion. Naturally, the patient refused to have surgery without a definite histological diagnosis. So a PET-CT examination was followed. It reported that the stomach wall of the gastric fundus and body were obvious thickened to a maximum of 1.5 cm. Abnormal  $^{18}\text{F}$ -fluorodeoxyglucose ( $^{18}\text{F}$ -FDG) uptake was also observed, with a maximum SUV of approximately 3.1. The thickened gastric wall and slightly active metabolic imaging indicated the possibility of poorly differentiated adenocarcinoma. Considering that the patient's clinical symptoms were aggravating, surgery was again recommended, and she agreed to undergo surgery and a laparotomy was performed.

During the laparotomy, we found the stomach wall was thickened overall and the tumor, as large as 14 cm × 9 cm, had invaded the serosa, accompanied by the enlargement of the perigastric lymph nodes. The center of the transverse colon, omentum, the spleen, and partial left adrenal were all invaded. Enlarged retroperitoneal lymph nodes were palpable. Adhesion or ascites was not detected, and there were no findings of metastasis to the rest organs in the abdominal cavity. So we decided to perform gastric cancer extended radical mastectomy (radical total gastrectomy, splenectomy, partial resection of the left adrenal, D2 lymphadenectomy, and Roux-en-Y esophagojejunostomy). Postoperative pathological examination showed that the stomach wall tissue was fibrotic with small focal distributed signet ring-like cells (Figure 1), CK (+), M-CEA (+), CK20 multifocal (+), poorly differentiated adenocarcinoma infiltrated the whole stomach wall, no cancer cells were found in the Splenic tissue, transverse colon, surgical margins or omentum, lymph nodes metastasis (0/32). She was diagnosed with gastric signet ring cell carcinoma (T4aN0M0, II B stage, Borrmann type IV). As with the former case, the patient was suggested to receive postoperative adjuvant chemotherapy treatment, and is still alive.

## DISCUSSION

Histopathological diagnosis is the diagnostic gold standard, as well as the treatment basis of gastric cancer due to its high specificity and negative predictive value<sup>[2,3]</sup>. Since a definite preoperative pathological result is required by medical ethics and practice guidelines, we could not carelessly make clinical decisions only according to our experience. But it is definite that there no test methods that can be 100% confirmed for a diagnosis. Thus, when the biopsy results didn't agree with the patient's clinical manifestations and other examination results (such as CT, endoscopic ultrasound, PET-CT, *etc.*, in our article), both patients were suggested to have repeated

**Table 1** Gastroscope statistics from the database of the First Affiliated Hospital *n* (%)

| Items       | Total | F (-) 1 <sup>st</sup> | (+) 1 <sup>st</sup> | F (-) 2 <sup>nd</sup> | (+) 2 <sup>nd</sup> | F (-) ≥ 3 | (+) ≥ 3 |
|-------------|-------|-----------------------|---------------------|-----------------------|---------------------|-----------|---------|
| Borrmann IV | 194   | 21 (10.8)             | 173 (89.2)          | 10 (5.2)              | 11 (5.7)            | 6 (3.1)   | 4 (2.1) |
| Early type  | 165   | 11 (6.7)              | 154 (93.3)          | 1 (0.6)               | 10 (6.1)            | 0 (0.0)   | 1 (0.6) |
| Other types | 1388  | 4 (0.3)               | 1384 (99.7)         | 0 (0.0)               | 4 (0.3)             | 0 (0.0)   | 0 (0.0) |
| Total       | 1747  | 36 (2.1)              | 1711 (97.9)         | 11 (0.6)              | 25 (1.4)            | 6 (0.3)   | 5 (0.3) |

F (-) 1<sup>st</sup>: False negative diagnosis of the 1<sup>st</sup> biopsy; (+) 1<sup>st</sup>: True positive diagnosis of the 1<sup>st</sup> biopsy; 2<sup>nd</sup>: The 2<sup>nd</sup> biopsy; ≥ 3: More than 3 times of biopsies.

gastroscopic biopsies to create a proper medical plan for MDT discussion.

Even as the only diagnostic gold standard for gastric cancer, gastroscopy biopsies still have a certain percentage of false negative (positive) rates. A prospective study on 1331 cases by Tatsuta *et al*<sup>[2]</sup> reported a false negative rate of 3.7% and a false-positive rate of 0.6% for gastroscopy biopsies. The data derived from a statistics of the patients' postoperative pathology, autopsy and clinical follow-up etc. According to the study, early gastric cancer, Borrmann type IV gastric cancer, and leiomyosarcoma were the main causes of false negative cases, and the false positive situations were all caused by active ulcer. Therefore, if the biopsy outcomes were suspected to be false negative (positive), repeated examinations for confirmed diagnosis are necessary. That was why we were so hesitated to make surgery decisions without a pathological diagnosis for both cases. However, at the same time, we were very anxious that the increasing number of biopsies would naturally increase the risk of bleeding at the biopsy site, rather than improve accuracy. A study by Choi *et al*<sup>[3]</sup> thus recommended 3-4 biopsies from visible tissue through endoscopy to make a correct pathologic diagnosis. When 6 or more biopsy specimens were obtained, diagnostic accuracy would reach 100%. But they also admitted the special difficulty for Borrmann type IV gastric cancer and insisted that if the negative results were accompanied with a malignant impression under endoscopic examination, a re-biopsy should be performed with careful targeting, which were the measures we performed in the cases reported. Similarly, statistics from the database of our hospital, including 1747 gastric cancer patients (Table 1), revealed a general false negative rate of 2.1% for the initial pathological diagnosis, but a second biopsy followed by multiple and deep sampling could mostly bring a definite outcome. However, we found it difficult to have a positive result, even with careful targeting, if the second biopsy was still negative. Among the false negative cases, Borrmann type IV gastric cancer covered the highest false negative rate, and the cases we reported were the most notable two. Borrmann type IV gastric cancer often calls for several biopsies before a confirmatory diagnosis, due to its peculiar biological properties in which a submucosal spread of malignant cells is present without a mucosal lesion. Malignant cells could not be obtained by subsequent repeated endoscopic biopsy. However, repeated biopsies with the guide of CT and ultrasound might increase the accuracy<sup>[4-7]</sup>. Ahn *et al*<sup>[4]</sup> insisted that if cases were strongly

suspected malignant prior to surgery, and surgery is being considered, endoscopic stomach mucosal resection should be recommended despite any development of stomach perforation or complications. In fact, the risk and uselessness of repeated biopsies were our inevitable concern. Thus, further noninvasive measures, though not the gold standard but of great importance, were under consideration for diagnosis. Upper gastrointestinal imaging, especially for the double contrast barium-air test, is one of the most commonly-used methods for the diagnosis of gastric cancer. Park *et al*<sup>[8]</sup> even pointed out that upper gastrointestinal imaging was superior to gastrofiberscopy for the diagnosis and localization of Borrmann type IV gastric cancer. Practically, alimentary tract barium meals were suggested in case 1 and the result supported this point. But most doctors would hardly be persuaded by this, especially when it is controversial in comparison to other, more credible, results (such as pathology and CT). In addition, PET-CT was suggested and performed in both patients. However, the use of PET-CT in the diagnosis of gastric cancer and evaluation of lymph nodes metastasis is still controversial<sup>[9]</sup>. The reported diagnosis accuracy varies from 60% to 95% in different studies<sup>[10-13]</sup>. For cases in which ordinary CT did not prompt a diagnosis of gastric cancer, we could not carelessly make a diagnosis of cancer simply according to the high gastrointestinal <sup>18</sup>F-FDG uptake<sup>[14,15]</sup>. Since such situations could also happen in normal physiological conditions, gastritis as well as stomach ulcers could also show false positive results. Furthermore, being related to the rich mucin content, Borrmann type IV gastric cancer is often associated with a false negative result. It should be distinguished from mucinous adenocarcinoma, signet ring cell carcinoma, and poorly differentiated adenocarcinoma that are also low in FDG uptake<sup>[9,11]</sup>. The two cases we reported also suggest the limitations of PET-CT in the diagnosis of Borrmann type IV gastric cancer. Case 1 was with a false negative result, though the result of case 2 proved to be true postoperative. However, interfering by the CT report of possible lymphoma and the negative pathologic results, even the MDT felt the decision was tough.

Actually, reasonable treatments determined by a pathologic result were a real concern of doctors, and the reason for the repeated biopsies. Borrmann type IV gastric cancer usually shows an extensive diffuse thickened stomach wall through a CT scan, but so do stomach sarcoma, gastrointestinal stromal tumors, hiatal hernia, gastric lymphoma, benign gastric ulcer, benign tumor, and

gastritis. Combined medical methods such as endoscopic ultrasound, and upper gastrointestinal contrast, would make it easier for the differentiation of most of the diseases mentioned, although gastric lymphoma was the one we mainly concerned about. Since gastric lymphoma also originated from the submucosa, it would firstly invade and grow with a wide lesion. Therefore, gastric lymphomas often have false negative biopsy results, and are also difficult to differentiate from gastric cancer through CT and endoscopic ultrasound. The differential diagnosis of gastric cancer from primary gastric lymphoma is especially important, since gastric cancer mainly depends on surgical treatment combined with radiotherapy and chemotherapy as a supplement at present. However, it is not the first choice for primary gastric lymphoma. Patients of mucosa associated lymphoid tissue lymphoma could mostly be cured simply by *Helicobacter pylori* (*H. pylori*) eradication, local radiotherapy, or chemotherapy<sup>[16]</sup>. For invasive gastric lymphomas, such as the diffuse large B-cell lymphoma patients, if they were at an early stage, chemotherapy combined with local radiotherapy would be suggested instead of surgical treatment. Patients' life quality would be improved during the course of treatment<sup>[17]</sup>. Surgical therapy would be suggested only when meeting with the failure of gastric retention treatment, focal lesion, or uncontrollable complications such as serious perforation and bleeding<sup>[18]</sup>. Therefore, both patients of our reported cases, with imaging findings of diffuse gastric wall thickening, were suggested to have immunohistochemistry tests at the same time as the biopsy, so that we could make a conclusion according to the immunohistochemistry result and the specific lymphoma phenotype<sup>[19]</sup>. <sup>18</sup>F-FDG PET-CT also played an important role in the differentiation and diagnosis of gastric lymphoma, but it is more valuable for a therapeutic evaluation<sup>[20]</sup>, while CT and endoscopic ultrasonography were superior in evaluating gastric lesions and lymph node status<sup>[7,21]</sup> and promising a more accurate guide for the biopsy. Fan *et al.*<sup>[22]</sup> concluded that gastric cancer lesions often covered less than 50% of the stomach wall under a CT scan, but usually more than 75% if it were gastric lymphoma. Furthermore, enlarged perigastric lymph nodes tend to be in one area, but for gastric lymphoma there would be multiple enlarged areas. Interesting, they pointed out that this might indicate a diagnosis of lymphoma if it were associated with the enlargement of the retroperitoneal lymph nodes inferior renal hilus<sup>[22]</sup>. However, it seemed unsuitable for our cases. Needless to say, a confirmed diagnosis of gastric lymphoma also depends on the biopsy results. But similar findings under gastrofiberscopy of superficial ulcers or protuberant submucosal mass in the stomach often lead to a misdiagnosis of adenocarcinoma or benign ulcers. According to a study by Guzička-Kazimierzczak *et al.*<sup>[23]</sup>, endoscopic ultrasound-guided biopsies, multiple biopsies, and *H. pylori* tests would contribute to a diagnosis. However, it seemed not helpful for our cases either.

The two difficult cases we reported were both highly suspected as Borrmann type IV gastric cancer by the

MDT preoperative, but cancer cells could not be detected in early biopsies. A confirmed diagnosis of cancer was mainly as a result of the deterioration of the clinical course. Since, for case 1, the patient was finally pathologically diagnosed as having cancer only in after the sixth biopsy after more than 6 mo from admittance, while the case 2 patient had to undergo surgery to find the truth, because her clinical symptoms were aggravating.

In conclusion, a definite preoperative pathological diagnosis of Borrmann type IV gastric cancer is difficult in many cases due to its characteristic submucosa originated development process. Repeated endoscopic biopsies and deep biopsy guided by CT and endoscopic ultrasonography that reaches the proper area of the stomach may be necessary. Compared with histopathology radiology (such as CT, PET-CT, upper gastrointestinal imaging), ultrasonography, and endoscopy are not the diagnostic gold standard for gastric cancer, and diagnostic value can not be ignored. Especially when cancer cells could not be detected despite repeated biopsies, the results of the methods mentioned above should be considered together with the clinical manifestations of patients. For those with radiology performance of widely diffuse lesions of the stomach wall, it may be highly characterized by Borrmann type IV gastric cancer. But surgery decisions should be carefully made in case of primary gastric lymphoma, which might benefit more from a non-surgical treatment method. We propose that, if gastric lymphoma could be excluded for such cases, even in the absence of any positive biopsy results supporting the diagnosis of gastric cancer, a diagnostic laparotomy and even radical gastrectomy may be reasonably performed by an experienced gastric cancer center with the agreement of the patient after being decided by a multidisciplinary discussion team.

## ACKNOWLEDGMENTS

We thank Peng ZP and Chen WF from the pathology department of the First Affiliated Hospital of Sun Yat-sen University for pathological support and Xu CC from the Zhongshan Ophthalmic Center of Sun Yat-sen University for editorial assistance.

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P- Reviewers Guo JM, Rodriguez DC S- Editor Gou SX  
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## Traumatic rupture of a type IVa choledochal cyst in an adult male

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Author contributions: Duan YF and Yang B managed the patient and wrote the manuscript; Duan YF and Zhu F revised the manuscript; all authors have read and approved the final version to be published.

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Received: February 16, 2013 Revised: March 26, 2013

Accepted: April 10, 2013

Published online: June 28, 2013

### Abstract

Choledochal cyst (CC) is a rare, congenital anomaly of the bile ducts. We describe a 26-year-old male patient who was transferred to our hospital with a reported traumatic rupture of cystic liver lesions following a fall. At the time of injury, the patient experienced severe abdominal pain. He was found to have peritonitis and abdominal hemorrhage, which is quite rare. Laparotomy revealed 3000 mL fluid consisting of a mixture of blood, bile and inflammatory effusion in the peritoneal cavity. The liver, gallbladder, spleen, stomach, duodenum, small intestine, and colon appeared normal. A large cystic mass was discovered near the porta hepatis. This mass, which connected to the hepatic bifurcation and gallbladder had a 5 cm rupture in the right wall with active arterial bleeding. Abdominal computed tomography (CT) and emergency laparotomy revealed rupture of a huge type IVa CC. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction. The postoperative course was uneventful and the patient was discharged on the 12<sup>th</sup> day of hospitalization. Four weeks after surgery,

abdominal CT scan showed pneumatosis in the intrahepatic bile duct, and intrahepatic dilatation which decreased following adequate biliary drainage. The patient has remained well in the close follow-up period for 9 mo.

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**Key words:** Biliary tract; Choledochal cyst; Trauma; Rupture; Peritonitis; Hemorrhage

**Core tip:** Choledochal cyst (CC) is a rare, congenital anomaly of the bile ducts. We describe a young man who was transferred to our hospital with a reported traumatic rupture of liver cystic lesions. The patient was found to have peritonitis and abdominal hemorrhage, which is quite rare. Abdominal computed tomography and emergency laparotomy revealed rupture of a huge type IVa CC. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction.

Duan YF, Yang B, Zhu F. Traumatic rupture of a type IVa choledochal cyst in an adult male. *World J Gastroenterol* 2013; 19(24): 3911-3914 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i24/3911.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i24.3911>

### INTRODUCTION

Choledochal cyst (CC) is a rare congenital disease characterized by single or multiple dilations of the intra and/or extrahepatic biliary tree. There is a higher incidence of CC in females. In CC, spontaneous perforation is observed in 1.8%-7% of all cases. Nevertheless, reports of traumatic rupture of CC are extremely rare. Herein, we describe a young man who was transferred to our

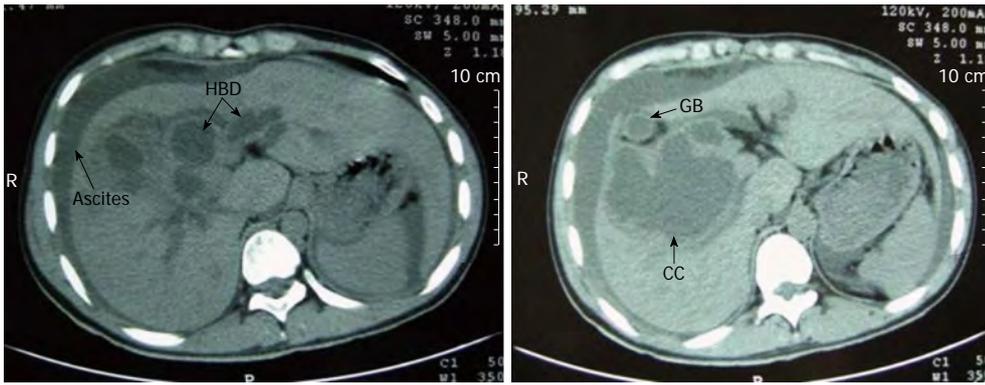


Figure 1 Abdominal computed tomography shows ascites, a normal-sized gallbladder, dilatation of the intrahepatic bile duct, and a huge choledochal cyst. GB: Gallbladder; HBD: Intrahepatic bile duct; CC: Choledochal cyst.

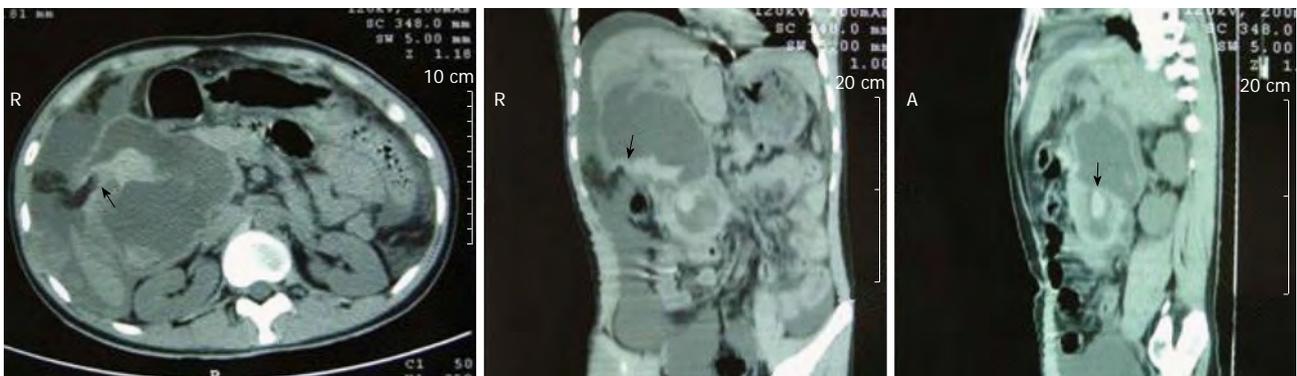


Figure 2 Abdominal computed tomography shows a rupture in the right wall of the choledochal cyst with hemorrhage (arrow).

hospital with a reported traumatic rupture of liver cystic lesions. The patient was found to have peritonitis and abdominal hemorrhage. Abdominal computed tomography (CT) and emergency laparotomy revealed rupture of a huge type IVa choledochal cyst. The patient was successfully managed by primary cyst excision, cholecystectomy, and Roux-en-Y end-to-side hepaticojejunostomy reconstruction.

## CASE REPORT

A 26-year-old male patient fell in a bathroom and suffered an impact to the abdomen. At the time of injury, the patient experienced severe abdominal pain. He was immediately admitted to a local hospital. On ultrasound examination, there was moderate free fluid in the peritoneal cavity, especially around the liver, and multiple cystic lesions in the liver. A plain radiograph of the chest was normal, and a plain radiograph of the abdomen revealed no gastrointestinal perforation. Abdominal paracentesis was performed with bloody fluid aspirated from the peritoneal cavity.

Two hours after injury, he was transferred to our hospital with a reported traumatic rupture of liver lesions. The patient's past medical history was unremarkable with the exception of transient and recurrent mild abdominal pain in childhood. His blood pressure was 100/65 mmHg

and heart rate was 126 beats/min. Physical examination revealed tenderness and guarding of the whole abdomen. The results of laboratory examination were as follows: white blood cells:  $9.88 \times 10^9/L$ , neutrophils: 69.4%, hemoglobin: 105 g/L, and hematocrit: 0.31.

An urgent abdominal CT scan revealed ascites and a normal-sized gallbladder, dilatation of the intrahepatic bile duct, and a 13 cm × 10 cm × 9 cm cyst with hemorrhage in the common bile duct region, extending from the porta hepatis down to the level of the pancreatic head (Figures 1 and 2). He was taken emergently to the operating room with a presumed diagnosis of CC rupture.

Laparotomy revealed 3000 mL fluid consisting of a mixture of blood, bile and inflammatory effusion in the peritoneal cavity. The liver, gallbladder, spleen, stomach, duodenum, small intestine, and colon appeared normal. A large cystic mass was discovered near the porta hepatis and was dissected circumferentially. This mass, which connected to the hepatic bifurcation and gallbladder had a 5 cm rupture in the right wall with active arterial bleeding. It was confirmed to be a congenital CC, type IVa, according to Todani's classification<sup>[1]</sup>.

Because intraperitoneal inflammation was moderate, a complete cyst excision with cholecystectomy and Roux-en-Y end-to-side hepaticojejunostomy reconstruction were performed. The postoperative course was uneventful and the patient was discharged on the 12<sup>th</sup> day of hos-

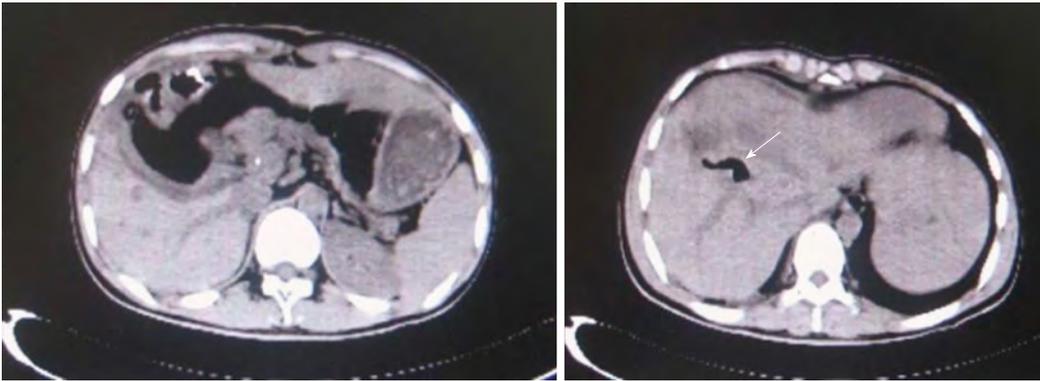


Figure 3 Abdominal computed tomography shows pneumatosis in the intrahepatic bile duct after cyst excision, cholecystectomy and Roux-en-Y hepaticojejunostomy reconstruction (white arrow). The intrahepatic dilatation tends to reduce in size following sufficient biliary drainage.

pitalization. Histological sections of the surgical margins and the operative specimen did not reveal malignancy. The patient has remained well in the close follow-up period for 9 mo. Four weeks after surgery, abdominal CT scan showed pneumatosis in the intrahepatic bile duct, and intrahepatic ductal dilatation, which was reduced following adequate biliary drainage (Figure 3).

## DISCUSSION

Choledochal cyst is a rare congenital disease characterized by single or multiple dilatations of the intra and/or extrahepatic biliary tree. Although the incidence in the Western population is 1 in 100000-150000 live births, it is remarkably higher in Asian countries, particularly in Japan, where these cysts can be found in up to 1 in 1000 live births<sup>[2]</sup>. The incidence is higher in females, with a male/female ratio of 1:3<sup>[3]</sup>. These cysts are typically a surgical problem of infancy and childhood, however, the diagnosis is delayed until adulthood in nearly 20% of patients<sup>[4]</sup>. In CC, spontaneous perforation is observed in 1.8%-7% of all cases<sup>[5]</sup>. Nevertheless, reports of traumatic rupture of CC are extremely rare. As far as we know, only a few cases have been described in the literature<sup>[6-13]</sup>.

The classical triad of jaundice, right upper quadrant mass, and abdominal pain is present in only a minority of patients<sup>[14]</sup>. Other presenting features of CC are cholangitis, pancreatitis, and biliary peritonitis from cyst rupture<sup>[5]</sup>. However, our patient was found to have peritonitis and abdominal hemorrhage, which is quite rare.

Ultrasound is the initial examination of choice in suspected CC<sup>[15]</sup>. Nevertheless, inherent limitations (*e.g.*, gas in the bowel, intraperitoneal inflammation, *etc.*) and the radiologist's skill may affect diagnosis. CT is required when the distal common bile duct is not visualized due to bowel gas. This imaging technique is excellent for detecting cystic lesions in the right upper abdomen, assessing their range and providing information regarding the impact of CC on the surrounding structures. Magnetic resonance cholangiopancreatography (MRCP) is the non-invasive imaging modality of choice for biliary pathology, and may offer as much as endoscopic retrograde chol-

angiopancreatography. However, MRCP is not suitable in patients who are unable to hold their breath for a few seconds, a requisite for breath-hold MRCP sequences.

Surgical treatment of CC should be recommended to reduce the risk of serious complications, such as cholangitis, pancreatitis, rupture, portal hypertension, cirrhosis, and cholangiocarcinoma<sup>[15]</sup>. In patients with type IV cysts without preoperative or intraoperative evidence of liver cirrhosis or biliary malignancy, resection of the entire portion of the extrahepatic cystic dilatation is currently recommended<sup>[16,17]</sup>. Reconstruction at the hepatic duct bifurcation is indicated. In rare cases where cyst rupture occurs, previous reports have speculated that a portal dissection and biliary reconstruction may be hazardous in the presence of bile peritonitis. These reports recommended primary management with temporary external drainage, by percutaneous transhepatic cholangiodrainage or by open placement of a T-tube<sup>[18,19]</sup>. Once the patient has recovered and has been thoroughly evaluated, a complete cyst excision, cholecystectomy, and hepaticojejunostomy can be performed<sup>[20]</sup>. However, this requires long-term maintenance of a T-tube and a second laparotomy. Furthermore, complications may occur in the interim. However, bile is aseptic, and even if bile drains into the abdominal cavity from a CC rupture, the possibility of this condition developing into generalized peritonitis is slight because such peritonitis will be chemical. In our patient, chronic inflammation induced thickening of the CC and fibrous adhesion with adjacent tissues, thus no shrinkage of the cyst cavity was observed following rupture of the CC which was excised. In recent years, due to progress in imaging diagnosis, it has become possible to diagnose the condition early. Therefore, in our opinion, treatment methods must also change. The case reported here demonstrated the feasibility of primary cyst excision and biliary reconstruction.

In conclusion, traumatic rupture of type IVa CC in an adult male is rare. A thorough preoperative diagnostic workup, with CT and/or MRCP, will guide surgeons to an appropriate operative strategy. Primary complete cyst excision, cholecystectomy and Roux-en-Y hepaticojejunostomy reconstruction are the preferred management options.

## ACKNOWLEDGMENTS

The authors thank Dr. Xiao-Dong Li and Dr. Yan Tan for their technical support.

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ISSN 1007-9327



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