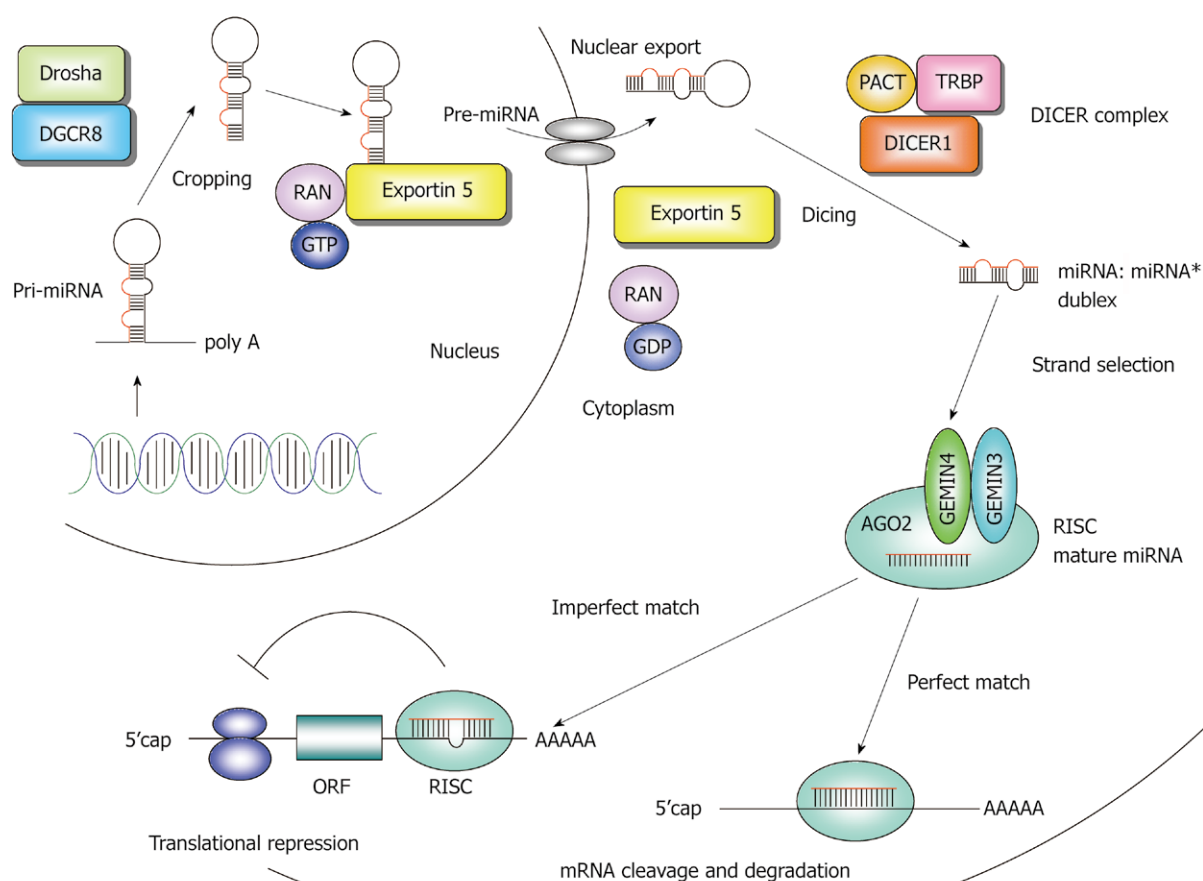


# World Journal of *Gastroenterology*

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Editorial Board of *World Journal of Gastroenterology*  
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Beijing 100025, China  
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*World Journal of Gastroenterology*  
Room 903, Building D, Ocean International Center,  
No. 62 Dongsihuan Zhonglu, Chaoyang District,  
Beijing 100025, China  
Telephone: +86-10-59080039  
Fax: +86-10-85381893  
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## Update on risk scoring systems for patients with upper gastrointestinal haemorrhage

Adrian J Stanley

Adrian J Stanley, Gastrointestinal Unit, Glasgow Royal Infirmary, Glasgow G4 0SF, United Kingdom

Author contributions: Stanley AJ was the sole contributor to this article.

Correspondence to: Dr. Adrian J Stanley, MD, FRCP (Ed), FRCP (Glasg), Consultant Gastroenterologist, Gastrointestinal Unit, Glasgow Royal Infirmary, Castle Street, Glasgow G4 0SF, United Kingdom. [adrian.stanley@ggc.scot.nhs.uk](mailto:adrian.stanley@ggc.scot.nhs.uk)

Telephone: +44-141-2114073 Fax: +44-141-2115131

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### Abstract

Upper gastrointestinal haemorrhage (UGIH) remains a common medical emergency worldwide. It is increasingly recognised that early risk assessment is an important part of management, which helps direct appropriate patient care and the timing of endoscopy. Several risk scores have been developed, most of which include endoscopic findings, although a minority do not. These scores were developed to identify various end-points including mortality, rebleeding or clinical intervention in the form of transfusion, endoscopic therapy or surgery. Recent studies have reported accurate identification of a very low risk group on presentation, using scores which require simple clinical or laboratory parameters only. This group may not require admission, but could be managed with early out-patient endoscopy. This article aims to describe the existing pre- and post-endoscopy risk scores for UGIH and assess the published data comparing them in the prediction of outcome. Recent data assessing their use in clinical practice, in particular the early identification of low-risk patients, are also discussed.

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**Key words:** Upper gastrointestinal haemorrhage; Bleed-

### INTRODUCTION

Upper gastrointestinal haemorrhage (UGIH) continues to be a major cause of hospital admission and mortality throughout the world. A recent United Kingdom national prospective audit of 6750 patients with UGIH reported a median five day length of stay and 10% mortality<sup>[1]</sup>. In that audit, peptic ulcer disease and variceal bleeding accounted for 36% and 11% patients respectively.

Management of UGIH consists of appropriate resuscitation and assessment, with timely endoscopy to diagnose and if necessary treat the underlying lesion. Similar to other common medical conditions, risk scores have been developed to try and identify those at lower or higher risk of poor outcome. Two recent international consensus documents have emphasised the importance of risk assessment in patients with UGIH<sup>[2,3]</sup>.

An ideal risk score is one that is easy to calculate, accurate for relevant outcomes and can be measured early after presentation with UGIH. Most risk scores require endoscopy although others do not. If a low risk group can be identified soon after presentation, it may allow non-admission of this group with arrangements made for out-patient endoscopy. Higher risk groups require in-patient endoscopy for full evaluation and therapy. This



review describes the existing risk scores for UGIH (clinical and endoscopy based) and gives an update on data regarding their use in clinical practice.

## METHODOLOGY

A Medline and PubMed search was undertaken using the keywords: upper gastrointestinal haemorrhage, bleeding, endoscopy, risk assessment and scoring systems. The period covered was 2000-2011 although earlier major publications were used for this review, including those referenced by articles and guidelines within the search period.

It is well recognised that patients with variceal bleeding constitute a specific and high risk group, with outcome largely dependent on the severity of underlying liver disease as assessed by the Childs-Pugh score or model for end stage liver disease (MELD)<sup>[4]</sup>. This review was not designed to describe scores specifically designed for patients with variceal bleeding and will not describe assessment of this subgroup in detail.

The review is split into assessment and comparisons of risk scores for UGIH which require endoscopy, and those which do not (pre-endoscopy scores) which can be calculated early after presentation. Where studies have directly compared scores for specific end-points, the area under the receiver operator curves (AUROC) are given if available. Finally there is a section describing the optimum clinical use of scores, focusing on the important issue of early identification of low-risk patients who may be suitable for discharge or even non-admission.

## RISK SCORES REQUIRING ENDOSCOPY

The most commonly used risk scoring system in UGIH is the Rockall score, which was described in 1996 following analysis of data from a large English audit<sup>[5]</sup> (Table 1). The score was developed to assess the risk of death following presentation with UGIH and incorporates patient age, haemodynamics, comorbidities and endoscopic findings. Due to the importance of underlying liver disease or failure in prognosis, most generic scoring systems for UGIH including the Rockall score incorporate this as a score component.

The American Baylor score was developed in 1993 to predict rebleeding after endoscopic therapy for non-variceal UGIH<sup>[6]</sup>. It includes five clinical and endoscopic variables. The Cedar Sinai predictive index is another American score which was derived after a structured literature review to predict outcome and length of hospital stay after UGIH<sup>[7]</sup>. It includes endoscopic findings, haemodynamics, comorbidities and time from symptoms.

The Spanish Almela score was developed to identify a low risk non-variceal group suitable for out-patient management and includes components from the history, haemodynamics and endoscopic findings<sup>[8]</sup>. An Italian 10 point score was recently developed to predict mortality after non-variceal bleeding<sup>[9]</sup>. Several other endoscopy based guidelines and clinical prediction models

Table 1 Rockall score

Component score	0	1	2	3
Age (yr)	< 60	60-79	≥ 80	-
Haemodynamics:				
Pulse (bpm)	< 100	≥ 100	-	-
Systolic BP (mmHg)	≥ 100	≥ 100	< 100	-
Comorbidities	None	-	IHD, cardiac failure, other major comorbidity	Renal or liver failure, disseminated malignancy
Diagnosis	MW or no lesion and no stigmata	All other diagnosis	Malignant lesions of UGIT	-
Stigmata of haemorrhage	No stigmata or dark spot on ulcer	-	Blood in UGIT, adherent clot, visible/spurting vessel	-

A score of ≤ 2 identifies a low-risk patient suitable for early discharge. UGIT: Upper gastrointestinal tract; IHD: Ischaemic heart disease; MW: M-Weiss tear; GI: Gastrointestinal; BP: Blood pressure.

for UGIH have been reported from America<sup>[10,11]</sup>, Hong Kong<sup>[12]</sup> and Italy<sup>[13]</sup>.

## COMPARISONS OF ENDOSCOPY BASED RISK SCORES

The Rockall score has been externally validated in several countries<sup>[14-17]</sup>. It has been also been shown to be superior to the Baylor and Cedar-Sinai scores in identifying low risk patients among a cohort with non-variceal bleeding<sup>[14]</sup>. In this study, all three scores were better at predicting mortality than rebleeding. The AUROC figures for mortality for the Rockall, Cedar-Sinai and Baylor scores were 0.85, 0.81 and 0.78 respectively, with the corresponding figures for rebleeding 0.68, 0.67 and 0.59. The Italian 10-point score was recently reported to be superior to the Rockall score for predicting 30-d mortality (AUROC 0.81 *vs* 0.66), but this requires external validation<sup>[9]</sup>.

At present, the Rockall score is the most widely used and studied post-endoscopy score to predict outcome. No other endoscopy based score has yet been validated to be of proven superiority in clinical use.

## PRE-ENDOSCOPIC RISK SCORES

An abbreviated pre-endoscopic or “admission-Rockall” score is often used, omitting the last two (endoscopic) components of the full Rockall score. However there has been debate about its accuracy and general clinical applicability. The Glasgow Blatchford Score (GBS) was developed in 2000 to predict the need for hospital based intervention (transfusion, endoscopic therapy, or surgery) or death following UGIH<sup>[18]</sup> (Table 2). Romagnuolo *et al*<sup>[19]</sup> described a modified GBS (due to unavail-

**Table 2 Glasgow blatchford score**

Admission risk marker	Score
Blood urea (mmol/L)	
6.5-8	2
8-10	3
10-25	4
> 25	6
Hb (g/L)	
Men	
120-130	1
100-120	3
< 100	6
Women	
100-120	1
< 100	6
Systolic BP (mmHg)	
100-109	1
90-99	2
< 90	3
Pulse $\geq$ 100/min	1
History and comorbidities	
Melaena	1
Syncope	2
Hepatic disease <sup>1</sup>	2
Cardiac failure <sup>2</sup>	2

<sup>1</sup>History of or clinical/laboratory evidence of liver disease; <sup>2</sup>History of or clinical/echocardiographic evidence of cardiac failure. A score of zero identifies low-risk patients suitable for non-admission. BP: Blood pressure.

ability of serum urea or history of syncope) from Canadian data which predicted high risk endoscopic stigmata, rebleeding and mortality.

The Cambridge score<sup>[20]</sup> and artificial neural networks (ANNs)<sup>[21,22]</sup> are other reported pre-endoscopic scoring systems. The former requires 14 clinical and laboratory variables and has not been externally validated. The latter require analysis of even more variables using computer software and are only applicable to non-variceal UGIH. Partly for these reasons the scores are not widely used.

## COMPARISONS OF PRE-ENDOSCOPIC RISK SCORES

Six recent studies from United Kingdom and Taiwan have shown the GBS to be superior to the admission Rockall score in predicting need for clinical intervention or death<sup>[18,23-28]</sup>. Interestingly, a large United Kingdom multi-centre study indicated the GBS was also superior to the full (post-endoscopy) Rockall score for predicting these combined outcomes, with AUROC figures for the GBS, full Rockall and admission Rockall scores 0.90, 0.81 and 0.71 respectively<sup>[23]</sup>. Another recent United Kingdom study comparing the GBS and admission Rockall scores for the same end-points has reported similar AUROC figures at 0.92 and 0.75, respectively<sup>[28]</sup>.

In a larger ( $n = 1555$  patients) follow-up publication from the United Kingdom multi-centre study group, AUROC figures for mortality were similar using the GBS, full Rockall and admission Rockall scores at 0.74, 0.79

and 0.76 and respectively<sup>[26]</sup>. An even higher mortality AUROC figure of 0.81 was recently reported using the GBS in a large study from Singapore and Malaysia<sup>[29]</sup>.

The United Kingdom multicentre follow-up study reported similar figures for the GBS and full Rockall scores in predicting need for endoscopic therapy or surgery, with both superior to the admission Rockall score. AUROC figures for this end-point were 0.79, 0.76 and 0.63 respectively. In a recent large study from Hong Kong, the GBS was again shown to be a better predictor of need for endoscopic therapy than the admission Rockall score, with an AUROC of 0.72<sup>[30]</sup>. In this study, the admission Rockall score was unable to predict need for endoscopic therapy.

Superiority of a modified GBS over the admission Rockall score in predicting high risk endoscopic stigmata or rebleeding has been reported from North America<sup>[19]</sup>. The GBS has also been shown to be superior to both the full and admission Rockall scores in predicting need for transfusion (AUROC figures 0.92, 0.75 and 0.69 respectively), presumably because the GBS includes admission haemoglobin as a component variable<sup>[26]</sup>.

Two recent studies assessing relatively complex ANNs have reported them to be superior to the admission Rockall and equivalent to the full Rockall score in predicting endoscopic therapy and superior to the full Rockall score in predicting mortality in non-variceal UGIH<sup>[21,22]</sup>. The larger of these studies revealed AUROC figures of 0.95 and 0.67 in predicting mortality using the ANN and the Rockall score respectively<sup>[22]</sup>. This is an impressive figure, but the complexity of ANNs is a significant limitation.

Whilst these studies suggest that some pre-endoscopic scores are equivalent or better at predicting outcome compared with the full Rockall score, all higher risk patients require in-patient endoscopy to diagnose and possibly treat underlying pathology. However pre-endoscopic scores may allow early identification of a low risk group who may not require in-patient endoscopy. As indicated above, studies from several countries have suggested that the relatively simple GBS is superior to the admission Rockall score in predicting clinically relevant end points. Interestingly the GBS also appears to perform well in comparison to the (post endoscopy) full Rockall score. Other pre-endoscopy scores have either not been externally validated or appear too complex for routine clinical use.

## OPTIMUM CLINICAL USE OF SCORES FOR UPPER GASTROINTESTINAL HAEMORRHAGE

The major existing risk scores for UGIH are summarised in Table 3. It is usually recommended that all patients with UGIH, except a very low-risk group, are admitted and have endoscopy within 24 h<sup>[2,3]</sup>. There is no clear evidence of benefit if endoscopy is undertaken earlier than 24 h, although a small group of patients with massive bleeding and haemodynamic compromise will

**Table 3** Summary of major published risk scores for upper gastrointestinal haemorrhage

Score <sup>[Ref.]</sup>	Endoscopy required?	Number of variables	Suitable for unselected upper GI bleeding patients?
Full Rockall <sup>[5]</sup>	Yes	6 <sup>1</sup>	Yes
Baylor <sup>[6]</sup>	Yes	5	No
Cedars Sinai <sup>[7]</sup>	Yes	6	Yes
Admission Rockall <sup>[5]</sup>	No	4 <sup>1</sup>	Yes
Glasgow Blatchford <sup>[18]</sup>	No	5 <sup>2</sup>	Yes
ANN <sup>[21]</sup>	No	20	No

<sup>1</sup>Comorbidities variable describes 5 specific conditions; <sup>2</sup>History and comorbidities variable describes 4 specific situations. GI: Gastrointestinal; ANN: Artificial neural network.

require emergency endoscopy. The decision on urgent endoscopy in this emergency group is usually based on clinical judgement rather than a specific score, however the recent study from Singapore and Malaysia suggested survival benefit for patients with a GBS of  $\geq 12$  who were endoscoped within 12 h<sup>[29]</sup>. This approach requires further study.

The most helpful use of a score in clinical practice is possibly identification of a low risk group who are suitable for early discharge or even non-admission. Interestingly, most scores seem to perform better in patients at low rather than higher risk<sup>[14]</sup>.

### Early identification of low-risk patients using endoscopy based scores

Patients with a Rockall score of  $\leq 2$  are generally accepted as being at low-risk of poor outcome, but calculation requires endoscopy<sup>[5,31]</sup>. Of the initial cohort used to develop the score, 26% patients met these criteria<sup>[5]</sup>. Longstreth reported safe early discharge using endoscopic and clinical guidelines to identify low risk patients<sup>[32]</sup>. Interestingly 32% of patients defined as low risk in this study had a Rockall score  $> 2$ . Two relatively small randomised studies suggested that early discharge of selected patients deemed “low risk” using endoscopic and clinical parameters did not affect outcome and offers cost savings<sup>[13,33]</sup>.

Local evaluation of the Cedars-Sinai predictive index reported that 70% patients achieved low risk status after endoscopy, and hospital stay was significantly reduced<sup>[7]</sup>. The Almela score identified over one third of non-variceal UGIH patients as suitable for early discharge following endoscopy<sup>[8]</sup>. There were five deaths in this early discharge group, although none were related to UGIH.

Although endoscopic resources vary internationally, it is interesting that the recent United Kingdom national audit revealed that only 52% hospitals had 24 h emergency endoscopy cover and only 50% patients admitted with UGIH had their endoscopy within 24 h<sup>[34]</sup>. At weekends, American and United Kingdom data show that endoscopy is significantly delayed<sup>[35,36]</sup>. Therefore the ability to identify low risk patients prior to endoscopy who may be suitable for out-patient management is very attractive.

### Early identification of low-risk patients using pre-endoscopy scores

A GBS of zero has been reported to have  $> 99\%$  sensitivity in identification of those who do not require intervention, rebleed or die in studies from Hong Kong (China)<sup>[30]</sup>, United States<sup>[37]</sup>, Japan<sup>[38]</sup>, Taiwan (China)<sup>[27]</sup> and United Kingdom<sup>[18,23,25,28]</sup>. The proportion of patients with a GBS of zero in the above studies ranged from 5%–22%, probably due to differences in local populations and healthcare organisation. Several studies have assessed extending the definition of low risk patients suitable for out-patient management to those with GBS  $\leq 1$  or  $\leq 2$ , but safety of this approach requires further study<sup>[24,28,38,39]</sup>.

An admission Rockall score of zero is often cited as identifying low risk patients, and identified 15% patients in the initial report<sup>[4]</sup>. However, studies have shown that up to 18% in this “low risk” group have clinically relevant end-points including endoscopic therapy, rebleeding and death<sup>[23,25,28,36]</sup>. Whilst no score will be perfect in clinical use for identifying low risk patients, most clinicians would prefer to err on the safe side and use a score with high sensitivity, to avoid discharging patients who may require intervention or die.

## CONCLUSION

Risk scores are of critical importance in UGIH, allowing early discharge of low risk patients and appropriate therapy for higher risk patients. The Rockall score is the most widely used and studied post-endoscopy score. The GBS is more accurate than the admission Rockall score for early (pre-endoscopic) prediction of clinically relevant outcomes, and is highly sensitive in identifying low risk patients suitable for out-patient management. Whilst other UGIH risk scores have been described, they require external validation and further comparative studies with the established GBS and Rockall scores.

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## Interrelationship between microsatellite instability and microRNA in gastrointestinal cancer

Hiroyuki Yamamoto, Yasushi Adachi, Hiroaki Taniguchi, Hiroaki Kunimoto, Katsuhiko Noshio, Hiromu Suzuki, Yasuhisa Shinomura

Hiroyuki Yamamoto, Yasushi Adachi, Hiroaki Kunimoto, Katsuhiko Noshio, Hiromu Suzuki, Yasuhisa Shinomura, First Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo 060-8543, Japan

Hiroaki Taniguchi, Division of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Hiromu Suzuki, Department of Molecular Biology, Sapporo Medical University School of Medicine, Sapporo 060-8556, Japan

Author contributions: Yamamoto H conceived the topic, reviewed the literature and prepared the manuscript; Adachi Y, Taniguchi H, Kunimoto H, Noshio K and Suzuki H reviewed and analyzed the literature; and Shinomura Y provided intellectual support.

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Correspondence to: Hiroyuki Yamamoto, MD, FJSM, PhD, First Department of Internal Medicine, Sapporo Medical University School of Medicine, S1W16 Chuo-ku, Sapporo 060-8543, Japan. [h-yama@sapmed.ac.jp](mailto:h-yama@sapmed.ac.jp)

Telephone: +81-11-6112111 Fax: +81-11-6112282

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### Abstract

There is an increasing understanding of the roles that microsatellite instability (MSI) plays in Lynch syndrome (by mutations) and sporadic (by mainly epigenetic changes) gastrointestinal (GI) and other cancers. Deficient DNA mismatch repair (MMR) results in the strong mutator phenotype known as MSI, which is the hallmark of cancers arising within Lynch syndrome. MSI is characterized by length alterations within simple repeated sequences called microsatellites. Lynch syndrome occurs primarily because of germline mutations in one of the MMR genes, mainly *MLH1* or *MSH2*, less frequently *MSH6*, and rarely *PMS2*. MSI is also observed in about 15% of sporadic colorectal, gastric, and en-

dometrial cancers and in lower frequencies in a minority of other cancers where it is often associated with the hypermethylation of the *MLH1* gene. miRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer. As a possible new mechanism underlying MSI, overexpression of *miR-155* has been shown to downregulate expression of *MLH1*, *MSH2*, and *MSH6*. Thus, a subset of MSI-positive (MSI+) cancers without known MMR defects may result from *miR-155* overexpression. Target genes of frameshift mutation for MSI are involved in various cellular functions, such as DNA repair, cell signaling, and apoptosis. A novel class of target genes that included not only epigenetic modifier genes, such as *HDAC2*, but also miRNA processing machinery genes, including *TARBP2* and *XPO5*, were found to be mutated in MSI+ GI cancers. Thus, a subset of MSI+ colorectal cancers (CRCs) has been proposed to exhibit a mutated miRNA machinery phenotype. Genetic, epigenetic, and transcriptomic differences exist between MSI+ and MSI- cancers. Molecular signatures of miRNA expression apparently have the potential to distinguish between MSI+ and MSI- CRCs. In this review, we summarize recent advances in the MSI pathogenesis of GI cancer, with the focus on its relationship with miRNA as well as on the potential to use MSI and related alterations as biomarkers and novel therapeutic targets.

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**Key words:** Microsatellite instability; MicroRNA; DNA mismatch repair; Frameshift mutation; MicroRNA processing

**Peer reviewers:** Dr. John Souglakos, Department of Medical Oncology, University Hospital of Heraklion and Laboratory of Cancer Biology, 71110 Heraklion, Greece; Dr. Jose Perea, De-

partment of Surgery, 12 De Octubre University Hospital, Rosas De Aravaca 82A, 28023 Madrid, Spain

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## INTRODUCTION

A type of genetic instability characterized by length alterations within simple repeated microsatellite sequences, termed microsatellite instability (MSI), occurs in a majority of patients with Lynch syndrome (also known as hereditary nonpolyposis colorectal cancer) by mutations and in a subset of sporadic gastrointestinal (GI) and other cancers by mainly epigenetic changes<sup>[1-10]</sup>. Genetic and epigenetic inactivation of DNA mismatch repair (*MMR*) genes results in the mutator phenotype, mutations in cancer-related genes, and cancer development (Figure 1). MSI underlies a distinctive carcinogenic pathway because MSI-positive (MSI+) cancers exhibit many differences in clinical, pathological, and molecular characteristics relative to MSI-negative (MSI-) cancers irrespective of their hereditary or sporadic origins. The differences in genotype can be explained because deficient *MMR* leads to a strong mutator phenotype with a very specific mutation spectrum. MSI accumulates frameshift mutations in repeated sequences located in coding regions of target tumor suppressor genes. The peculiar genotype of MSI+ cancers also includes specific patterns of gene regulation. MSI+ GI cancers often show an aberrant epigenetic pattern such as hypermethylation of various genes, including the key *MMR* gene, *MLH1*. The differences in genotype and phenotype between MSI+ and MSI- GI cancers are likely to be causally linked to their differences in biological and clinical features. Diagnostic characterization of the MSI status thus has implications in basic and clinical oncology. MiRNAs are small RNA molecules that regulate gene expression at the posttranscriptional level and are critical for many cellular functions<sup>[11-20]</sup>. There is accumulating evidence to support the notion that the interrelationship between MSI and miRNA plays a key role in the pathogenesis of GI cancer.

## MSI BY THE OVEREXPRESSION OF MIR-155 OR MIR-21

Various pathogenic events, including germline and somatic mutations, promoter methylation, and reduced histone acetylation<sup>[21]</sup>, lead to inactivation of core *MMR* proteins. A vast majority of MSI+ cancers can be explained by mutation and/or epigenetic inactivation of the core *MMR* proteins<sup>[22]</sup>. The etiologies of the remaining MSI+ cancers remain poorly understood. In an unselected se-

ries of 1066 colorectal cancer (CRC) patients, 135 (13%) were MSI+<sup>[22]</sup>. Of these, 23 (6%) had germline mutations in one of the *MMR* gene, and 106 (79%) showed methylation of the *MLH1* promoter. Approximately 5% of these MSI+ cancers displayed loss of expression of at least one of the core *MMR* proteins without a well-defined genetic or epigenetic cause<sup>[22]</sup>.

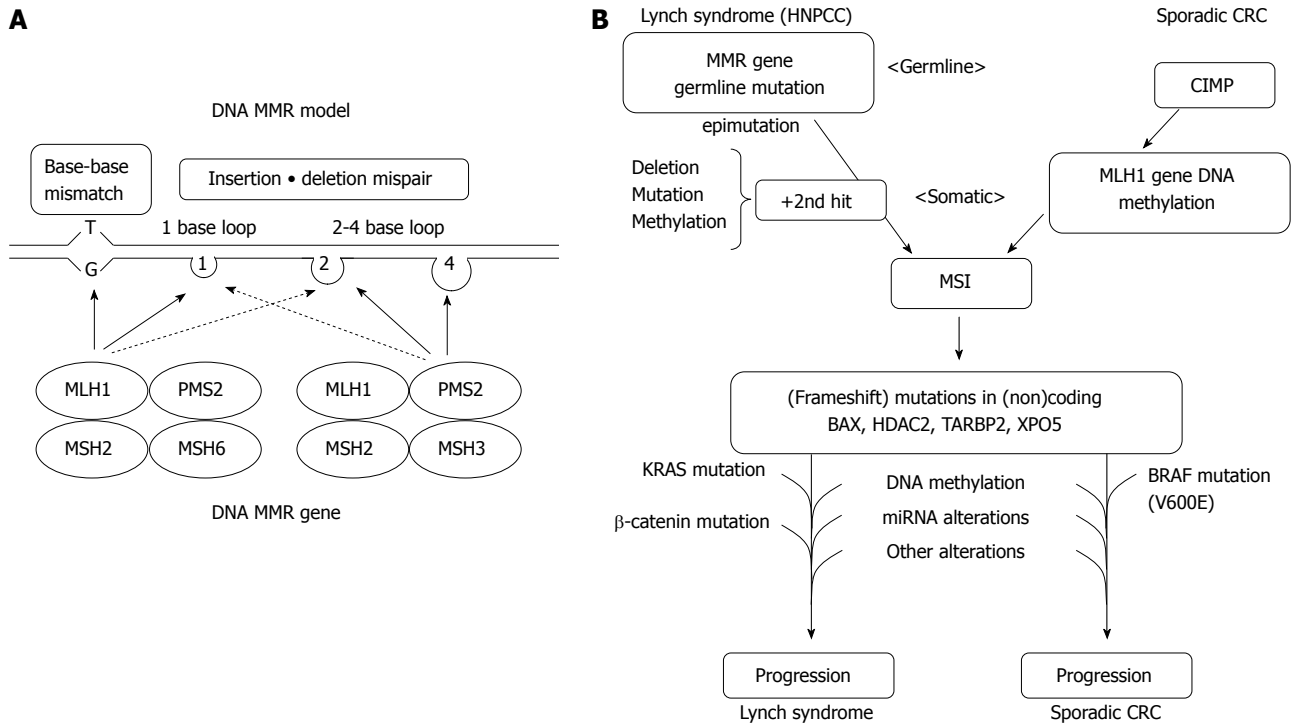
Vareli *et al.*<sup>[23]</sup> have shown that overexpression of *miR-155* significantly downregulates the core *MMR* proteins; namely, *MLH1*, *MSH2*, and *MSH6* in CRC cell lines, thus inducing an MSI. The downregulation of *MLH1* and *MSH2* proteins by *miR-155* lead to destabilization of the respective heterodimeric complex proteins and a mutator phenotype<sup>[24]</sup>. An inverse correlation between *miR-155* overexpression and the expression of *MLH1* and *MSH2* was further demonstrated in human CRC tissues. Most MSI+ cancers without a known cause of *MMR* inactivation show *miR-155* overexpression. However, not all CRCs with increased *miR-155* expression were MSI+. It is also possible that *miR-155* affects other related DNA repair proteins, thus enhancing the phenotypic effect of *MMR* defects. Although further confirmation is required, the results suggest that *miR-155* overexpression is an additional mechanism underlying the development of MSI in cancer (Figure 2)<sup>[23]</sup>.

The reduced expression of a single allele of the adenomatous polyposis coli and transforming growth factor (*TGF*)- $\beta$  receptor I gene has been linked to CRC<sup>[25,26]</sup>. Thus, incomplete repression of *MMR* proteins by *miR-155* is not unique to tumor suppressor genes in cancer. Recently, miRNAs have been suggested to act as transactivating elements involved in allele and gene expression regulation<sup>[27,28]</sup>. These results support the notion that miRNAs play a role in the non-Mendelian regulation of *MMR* genes.

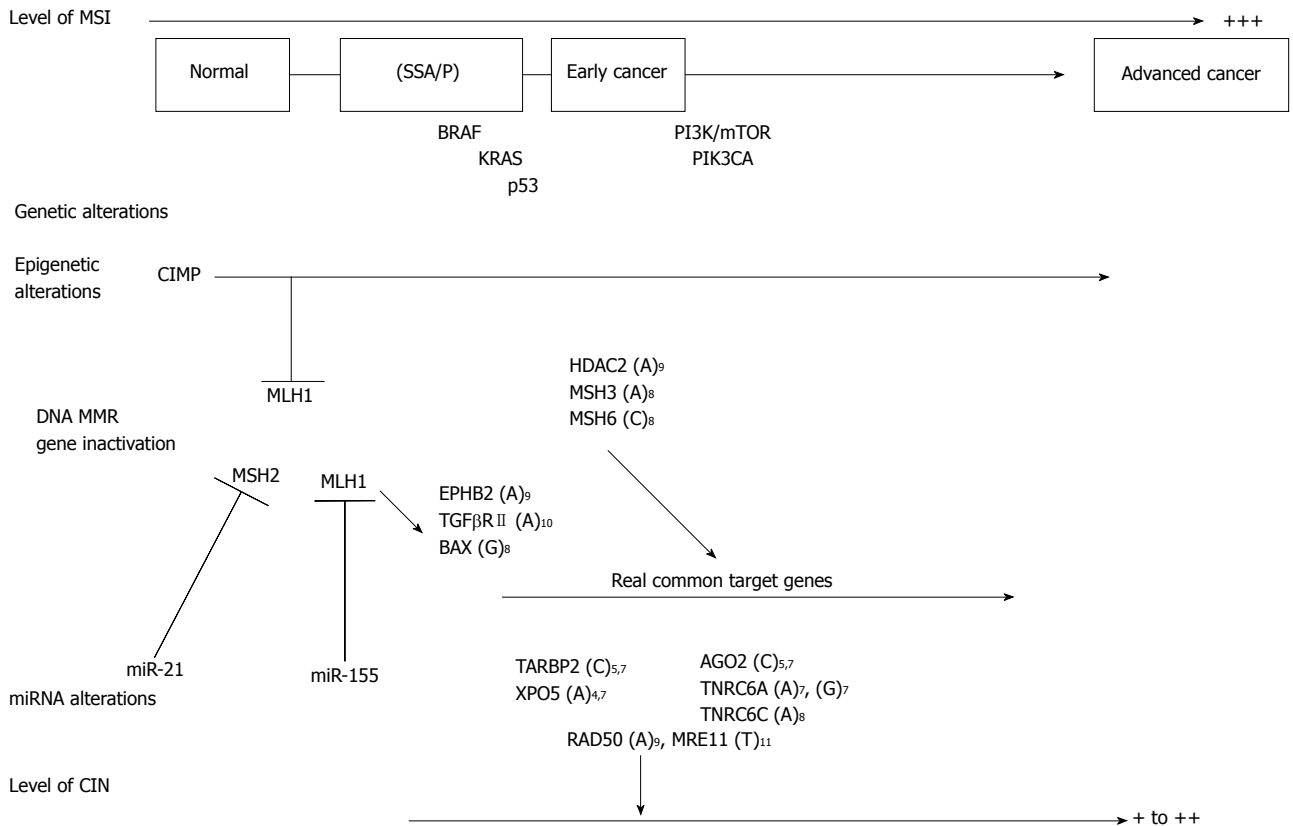
*miR-21* is overexpressed in various types of human cancers, including CRC<sup>[29]</sup>. Valeri *et al.*<sup>[30]</sup> reported that *miR-21* directly targets the 3' untranslated region (UTR) of *MSH2* and *MSH6* mRNA, resulting in downregulation of protein expression. The inverse correlation between *miR-21* overexpression and *MSH2* expression was shown in CRC tissues. Cells that overexpress *miR-21* showed significantly reduced 5-fluorouracil (5-FU)-induced G2/M damage arrest and apoptosis that is characteristic of defective *MMR*. Because *miR-21* expression could increase in cell lines continuously exposed to 5-FU<sup>[31]</sup>, cancer cells may develop a secondary resistance to 5-FU through *miR-21* overexpression. Thus, *miR-21*-dependent downregulation of *MSH2-MSH6* may be responsible for both primary and secondary resistance to 5-FU.

## TARGET CANCER-RELATED GENES OF MSI

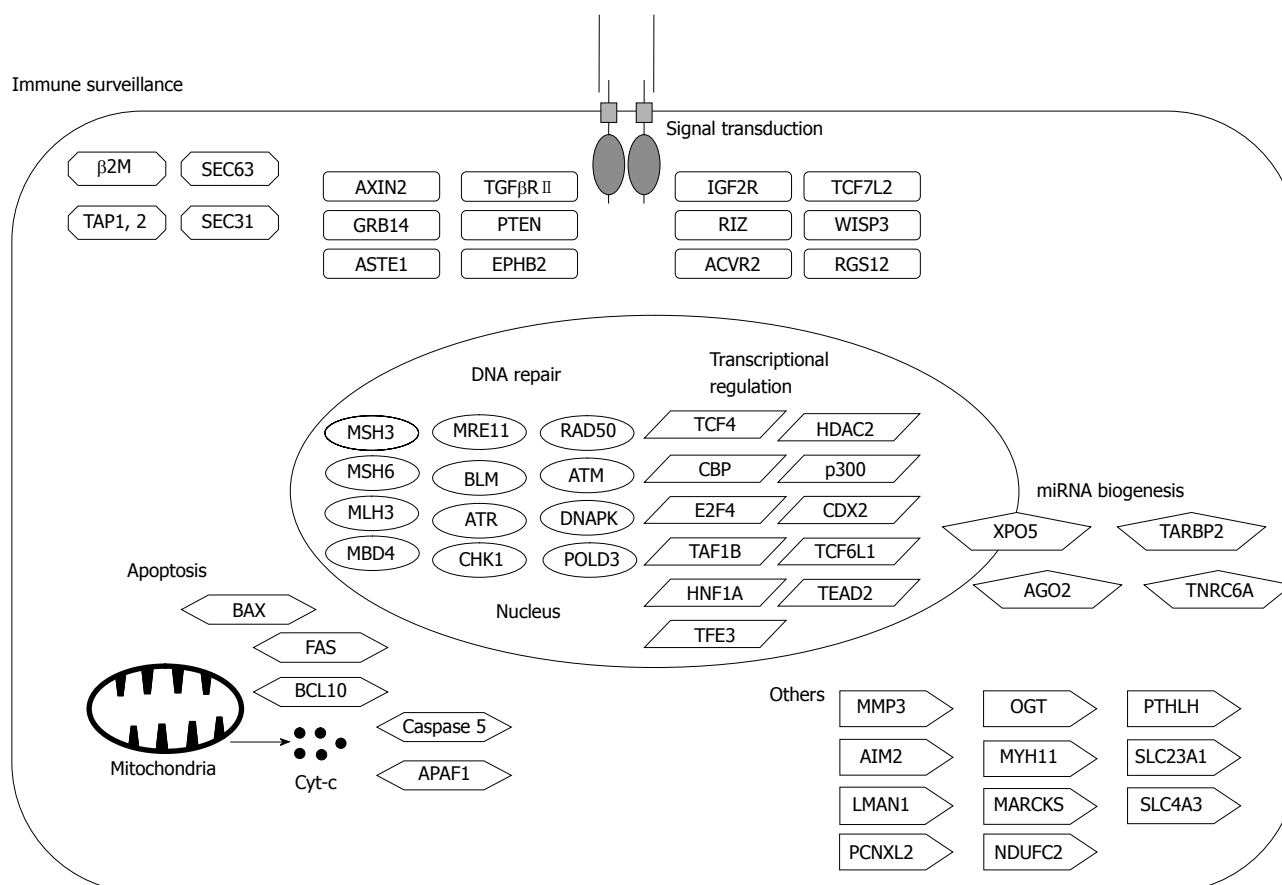
The instability in cancer-related genes at coding microsatellites causes frameshift mutations and functional inactivation of affected proteins, thereby providing a selective growth advantage to deficient *MMR* cells<sup>[32]</sup>. For instance, *TGF- $\beta$  receptor II* and the pro-apoptotic gene *BAX* are



**Figure 1 A model of DNA mismatch repair and molecular pathways for microsatellite instability+ colorectal cancers.** A: A model of the proposed mechanism of mismatch repair (MMR) proteins, illustrating patterns of relevant heterodimerization; B: The models for colorectal cancer (CRC) carcinogenesis are presented in parallel for Lynch syndrome and sporadic cases. HNPCC: Hereditary nonpolyposis colorectal cancer; MSI: Microsatellite instability; CIMP: CpG island methylator phenotype; MSI: Microsatellite instability.



**Figure 2 Cancer progression of sporadic microsatellite instability+ colorectal cancers.** The model for microsatellite instability (MSI)+ colorectal cancer progression is presented based on levels of MSI and chromosomal instability (CIN), and genetic, epigenetic and miRNA alterations. SSA/P: Sessile serrated adenomas/polyps; CIMP: CpG island methylator phenotype; MMR: Mismatch repair.



**Figure 3 Representative target genes in microsatellite instability+ gastrointestinal cancers.** A number of cancer-related genes mutated in microsatellite instability+ gastrointestinal cancers have been reported. The relevance of each mutation is not necessarily proven.

frequently inactivated by slippage-induced frameshift mutations in mononucleotide tracts present in their gene coding regions<sup>[33,34]</sup>. These findings have provided proof for the causative link between MSI and mutations in cancer-related genes, and they were also convincing examples of the differences between the mutator and suppressor pathways for cancer. These genes have also been mutated in cancers in the suppressor pathway, but at decreased frequencies and not by slippage-linked frameshifts<sup>[35,36]</sup>.

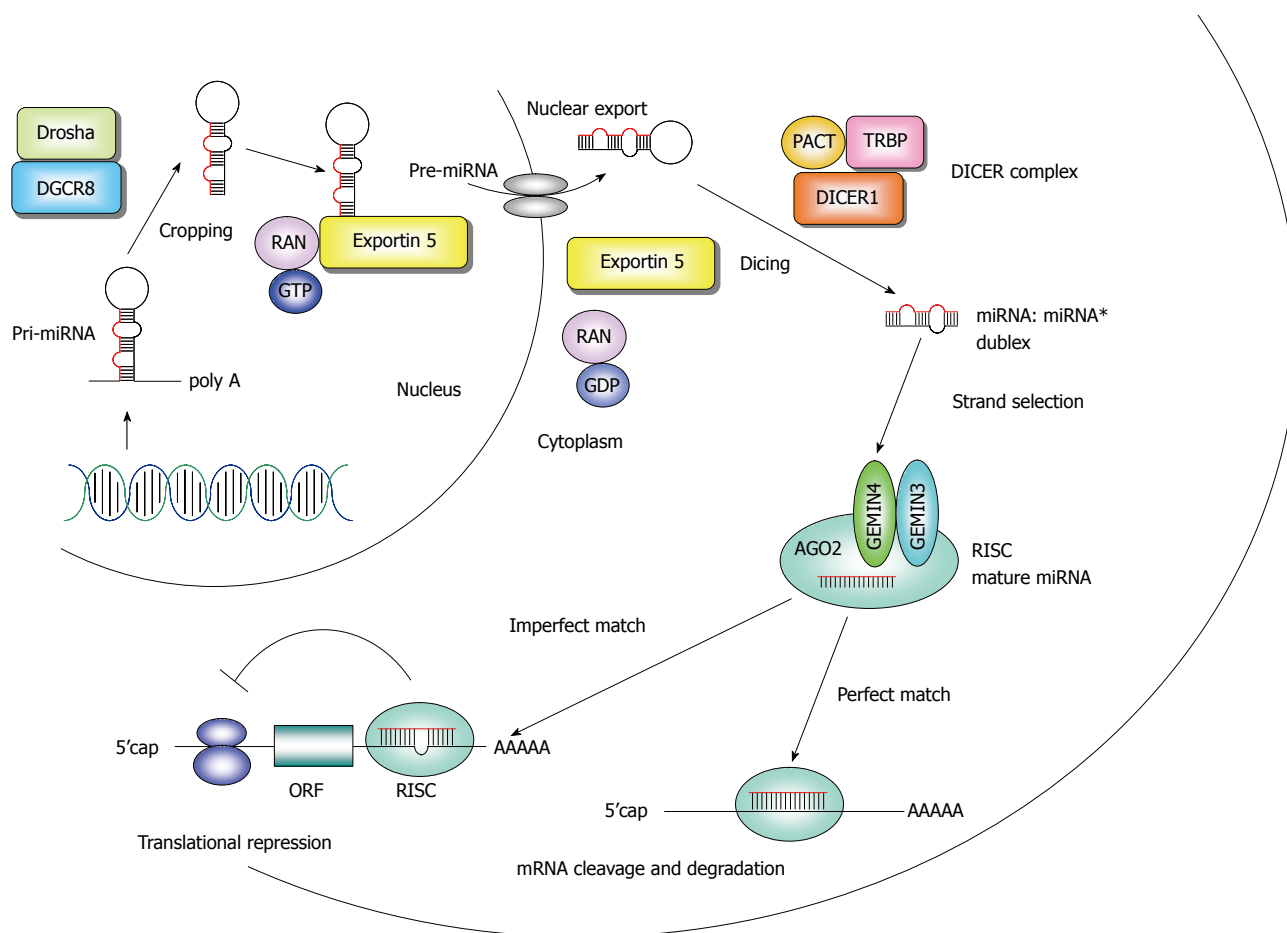
A number of cancer-related genes mutated in MSI+ cancers have been reported (Figure 3). Mutations that promote cancer cell growth are assumed to be the driving force during MSI+ carcinogenesis and are designated as real common target genes (Figure 2)<sup>[37]</sup>. Mutations of microsatellite-harboring genes that do not contribute to carcinogenesis are designated as bystander genes. However, it is not always clear which mutations are “driver mutations” and which are “passenger mutations”<sup>[9,38]</sup>. The Selective Targets database (SelTarbase) (<http://www.seltarbase.org>) of human mononucleotide-microsatellite mutations and their potential impact to carcinogenesis and immunology has been developed<sup>[39]</sup>. The database includes a comprehensive database of all human coding, untranslated, non-coding RNA and intronic mononucleotide repeat tracts and is useful for basic and clinical oncology.

Because MSI+ cancers accumulate many mutations, disruption of cell growth and survival regulation can be

accomplished in different cancers by mutations in different genes of the same signaling pathways<sup>[40]</sup>. Therefore, genes with infrequent and/or monoallelic mutations should not be regarded as irrelevant. Thus, the relevance of microsatellite-specific mutations in MSI+ cancers can be proven only when there is supporting evidence for functionality irrespective of mutation incidence<sup>[2]</sup>.

Target cancer-related genes of MSI+ cancers can be functionally categorized as tumor suppressors and genes involved in DNA repair, cell cycle, cell proliferation, apoptosis and others (Figure 3). Interestingly, every human *MMR* gene except *MLH1* contains a mononucleotide repeat of at least A7<sup>[41]</sup>. Thus, frameshift mutations of *MSH3* and *MSH6* led to the concept of “the mutator that mutates the mutator” (Figure 2)<sup>[42]</sup>.

The spectrum of mutations of target genes could affect cancer biology, therapeutic response, and prognosis of patients. Most putative MSI target genes have been proposed mainly on the basis of high mutation frequency detected within their coding regions. However, genes containing microsatellites that are located within noncoding regulatory regions, such as introns, promoters, and 5' and 3' UTRs, could be also mutated in MSI+ cancers. Alterations within untranslated mononucleotide repeat tracts can alter transcription level or transcript stability. It has been suggested that some intronic repeat mutations in genes, such as *ATM*, *MYB*<sup>[43]</sup>, and *MRE11*<sup>[44]</sup>, play a



**Figure 4** MiRNA biogenesis and genes mutated in microsatellite instability+ gastrointestinal cancers. A consequence of perfect complementarity between miRNA and mRNA is mRNA cleavage and degradation. Imperfect alignment represses gene translation. ORF: Open reading frame; RISC: RNA-induced silencing complexes.

role in MSI carcinogenesis<sup>[45]</sup>. Decreased matrix metalloproteinase (MMP)-3 expression due to insertions and/or deletions in the *MMP-3* promoter region led to a decrease in the levels of the active MMP-9 form, which may explain the less invasive potential of MSI+ cancer cells<sup>[46]</sup> and the propensity for a good prognosis in the case of MSI+ CRCs<sup>[47]</sup>.

Genomic copy number changes are frequently observed in cancers. It is well known that MSI+ cancers show less genomic copy number changes and are mostly diploid<sup>[48]</sup>. However, genes responsible for chromosomal instability (CIN) could be mutated in MSI+ cancers, and these defects may be selected during the course of cancer progression (Figures 2 and 3)<sup>[49]</sup>. Furthermore, mutations of *RAD50* and *MRE11* are reportedly associated with defects in nonhomologous end-joining, leading to chromosomal alterations during cancer progression<sup>[45]</sup>.

Altered histone modifications that affect chromatin structures are also involved in carcinogenesis<sup>[49]</sup>. Epigenetic modifier genes could also be MSI target genes. Rope-ro *et al.*<sup>[50]</sup> detected frameshift mutations in the histone deacetylase (*HDAC*) 2 gene in MSI+ GI cancers. This *HDAC2* mutation made mutation-positive cancer cells more resistant to the antiproliferative and proapoptotic

effects of certain HDAC inhibitors such as trichostatin A, but not to others such as butyric acid and valproic acid. Since HDAC inhibitors may serve as therapeutic agents for cancer, these findings support the use of *HDAC2* mutation status in future pharmacogenetic treatment<sup>[50]</sup>.

## MIRNA PROCESSING MACHINERY GENES AS MSI TARGET GENES

MiRNAs are small noncoding RNAs that regulate gene expression at the posttranscriptional level and are critical in many biological processes and cellular pathways (Figure 4)<sup>[11-20]</sup>. MiRNA expression profiles of human cancers have been characterized by an overall mature miRNA downregulation<sup>[51-53]</sup>. The causes of the aberrant miRNA expression patterns in cancer involve DNA copy number amplification or deletion<sup>[54]</sup>, inappropriate transactivation, transcriptional repression by oncogenic and/or other factors<sup>[55]</sup>, failure of miRNA post-transcriptional regulation<sup>[56]</sup>, and genetic mutation<sup>[57]</sup> or transcriptional silencing associated with hypermethylation of CpG island promoters<sup>[58-62]</sup>.

The control of the miRNA biosynthesis pathway is important in the spatiotemporal pattern of miRNA expression in cells. Thus, impaired miRNA processing pathways



may themselves be targets of genetic and/or epigenetic disruption in cancer<sup>[63,64]</sup>. Recently, it has been reported that mitogenic signaling can be translated into changes in cell viability and proliferation through the miRNA biogenesis pathway. A *TAR RNA-binding protein 2* (*TARBP2*) gene encodes TRBP, an essential functional partner of DICER1 (Figure 4)<sup>[65,66]</sup>. *TARBP2* is phosphorylated under normal growth conditions, which increases the stability of *TARBP2* and *DICER1*<sup>[67]</sup>. Upon growth factor stimulation, MAPK/ERK pathway increases *TARBP2* phosphorylation, leading to a coordinated increase in levels of growth-promoting miRNA and a decrease in the expression of *let-7* tumor suppressor miRNA. In contrast, pharmacological inhibition of MAPK/ERK resulted in an anti-growth miRNA profile<sup>[67]</sup>. These results further suggest the important role of miRNA processing mediated by *TARBP2* in preservation of a normal, untransformed cell state<sup>[17]</sup>.

Melo *et al.*<sup>[68]</sup> have found truncating heterozygous mutations in *TARBP2* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers (Figure 4). *TARBP2* mutations diminished TRBP protein expression, resulting in impaired miRNA processing and enhanced cellular transformation. The TRBP impairment was associated with a secondary defect in *DICER1* activity by destabilization of the *DICER1* protein. Thus, *TARBP2* mutations may explain overall miRNA downregulation in a subset of MSI+ cancers. Because the restoration of efficient miRNA production by the reintroduction of TRBP can suppress cancer cell growth, these findings are important for the development of new therapeutic strategies for the treatment of cancer<sup>[68]</sup>.

## MUTATIONS OF THE *EXPORTIN 5* GENE

Because of the nuclear retention of certain precursor miRNAs (pre-miRNAs), mature miRNA expression levels are not consistent with pre-miRNA expression levels in various human cancer cell lines<sup>[17]</sup>. Thus, defects in the nuclear export of pre-miRNAs may be one of the mechanisms underlying the global impairment of mature miRNAs in human cancer. The *exportin 5* (*XPO5*) mediates nuclear export of pre-miRNA (Figure 4). Melo *et al.*<sup>[69,70]</sup> have identified inactivating heterozygous mutations of *XPO5* in MSI+ cancer cell lines and in primary sporadic and hereditary MSI+ GI cancers. The mutant form of *XPO5* does not comprise a C-terminal region that is important for the formation of the pre-miRNA/*XPO5*/Ran-GTP ternary complex. Thus, the *XPO5* defect trapped certain pre-miRNAs in the nucleus, reduced miRNA processing, and impaired miRNA-target inhibition. It is important to note that the restoration of *XPO5* functions rescued the disturbed export of critical tumor-suppressive pre-miRNAs, which results in tumor suppression<sup>[69]</sup>.

Interestingly, although the heterozygous *XPO5* mutation decreased accumulation of a fraction of detectable miRNAs, many others were not affected. It seems that *XPO5* does not bind to pre-miRNAs universally but has

certain substrate preferences, which are possibly mediated by sequence or structure<sup>[38]</sup>. Strategies directed toward stimulating the activity of miRNA processing factors and restoring the production of mature growth inhibitory miRNAs may have therapeutic value<sup>[69]</sup>.

## MUTATED MIRNA MACHINERY PHENOTYPE AS A NEW CANCER PHENOTYPE

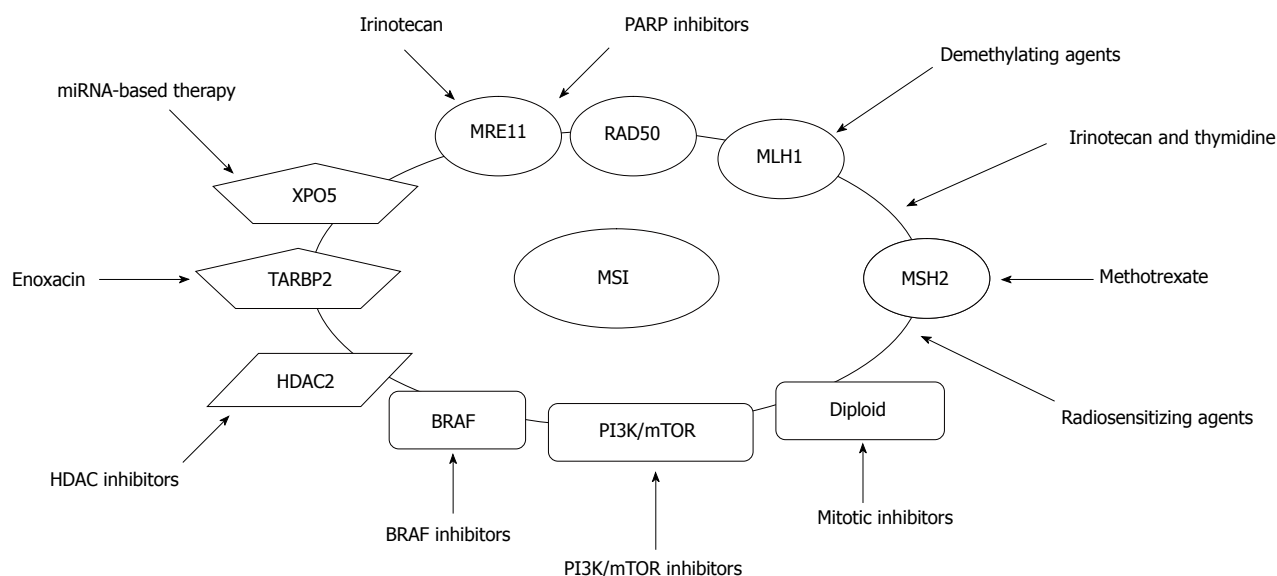
Recent works have suggested that the other component of the miRNA biogenesis pathway, *DICER1*, is a haploinsufficient tumor suppressor<sup>[71,72]</sup>. Therefore, it appears that at least three components of the miRNA biogenesis pathway are haploinsufficient tumor suppressors, with *TARBP2* and *XPO5*, but not *DICER1*, mutations prevalent in MSI+ cancers<sup>[38]</sup>. In addition, the miRISC components *AGO2*, *TNRC6A*, and *TNRC6C* can also be mutated in MSI+ cancers (Figure 4)<sup>[73]</sup>, although the functional significances remain to be determined<sup>[38,70]</sup>.

From these observations, a new cancer phenotype known as mutated miRNA machinery phenotype (MMMP) has been proposed for MSI+ CRCs having mutations in the miRNA machinery genes and the deregulated miRNAome. Although larger studies are required to fully characterize and validate this feature as a criterion for classification, a broader miRNAome-modifying approach may be effective for cancer patients with MMMP<sup>[15]</sup>.

## TRANSCRIPTOMIC DIFFERENCES BETWEEN MSI+ AND MSI- CRCs

As molecular markers, gene expression profiles are being developed for many cancers. Array technology has identified a number of genes that are expressed differentially between MSI+ and MSI- CRCs<sup>[74-76]</sup>. By using supervised analysis of cDNA microarray data, Giacomini *et al.*<sup>[77]</sup> identified a robust expression signature distinguishing MSI+ and MSI- CRC cell lines. By using high-density oligonucleotide microarrays, Kruhoffer *et al.*<sup>[78]</sup> constructed a gene signature that distinguished MSI+ and MSI- CRCs. The authors further constructed a signature that distinguished sporadic and hereditary cases of MSI+ CRCs. Identification of a signature for MMR deficiency would be relevant, both biologically and clinically<sup>[78]</sup>.

As for miRNAs, Lanza *et al.*<sup>[79]</sup> analyzed 16 MSI+ and 23 MSI- CRCs for genome-wide expression of miRNA and mRNA. On the basis of combined miRNA and mRNA expression, a molecular signature comprising 27 differentially expressed genes, including 8 miRNAs, could correctly distinguish MSI+ and MSI- CRCs. Among the differentially expressed miRNAs, various members of the oncogenic *miR-17-92* family were significantly upregulated in MSI- cancers. Among these, *miR-17-5p*, *miR-20*, *miR-25*, *miR-92-1*, *miR-92-2*, *miR-93-1*, and *miR-106a* were significantly upregulated in MSI- when compared with



**Figure 5 Targeted therapies based on molecular alterations in microsatellite instability+ colorectal cancers.** Microsatellite instability+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations. MSI: Microsatellite instability; HDAC: Histone deacetylase; PI3K/mTOR: Phosphoinositide 3-kinase/mammalian target of rapamycin; XPO5: Exportin 5.

MSI+ CRCs. Because members of the *miR-17-92* family act as oncogenes, these results may explain, at least in part, the less aggressive behavior of MSI+ CRCs when compared with their MSI- counterparts.

Earle *et al*<sup>[80]</sup> analyzed 22 MSI-H, including 6 Lynch syndrome, 8 MSI-L, and 25 MSS CRCs for a selected panel of 24 miRNAs. Relative expression of *miR-26b*, *miR-31*, *miR-92*, *miR-155*, *miR-196a*, and *miR-223* were significantly different among MSI subgroups, and *miR-31* and *miR-223* were overexpressed in CRCs of patients with Lynch syndrome. These findings indicate that miRNA expression in CRC is associated with MSI status, including Lynch syndrome and MSI-L, and that miRNAs may play significant roles in these MSI subgroups in addition to having possible effects on cancer characteristics.

Slattery *et al*<sup>[81]</sup> analyzed 70 CRCs for 866 miRNAs using microarrays. At the 1.5-fold level, 143 miRNAs were differentially expressed in MSI+ CRCs. *miR-139-3p*, *miR-223*, and *miR-370* were upregulated and *miR-24-2*, *miR-424*, *miR-552*, and *let-7g* were downregulated at a level of 1.5-fold or greater in MSI+ CRCs when compared with MSI- CRCs.

Thus, differentially expressed miRNAs are likely to be relevant, both biologically and clinically, although their functional significances remain to be determined. High levels of *miR-21* in the stroma of CRCs reportedly predict short disease-free survival in stage II CRC patients; however, the levels are not associated with the MSI status<sup>[82]</sup>.

## DEFECTIVE MMR AS A NOVEL THERAPEUTIC TARGET

MSI+ cancers may be managed more effectively with novel targeted therapies based on molecular alterations (Figure 5). A combination of treatments that target both primary

alterations of DNA MMR gene and secondary alterations, such as frameshift mutations of target genes, may be also effective. A synthetic lethal relationship, where the simultaneous inhibition of two different regulatory pathways leads to cell death, is a recent therapeutic strategy<sup>[83]</sup>. Therefore, identification of synthetic lethal interactions with MMR deficiency could potentially lead to the identification of specific therapeutic targets<sup>[84]</sup>. The inhibition of poly (adenosine diphosphate-ribose) polymerase (PARP) is a potential synthetic lethal therapeutic strategy for the treatment of cancers with specific DNA repair defects, such as a BRCA1 or BRCA2 mutation<sup>[85]</sup>.

A subset of MSI+ CRCs may also be suitable for this strategy. A novel PARP inhibitor, ABT-888, showed preferential activity on MSI+ CRC cell lines harboring mutations in both *MRE11* and *RAD50* genes when compared with MSI- cell lines that were wild type for both genes<sup>[86]</sup>. Recently, Vilar *et al*<sup>[87]</sup> reported that MSI+ CRCs deficient in double strand break (DSB) repair due to *MRE11* mutations show a higher sensitivity to PARP-1 inhibition. A phase II study assessing the efficacy of a PARP-1 inhibitor, olaparib, in CRCs stratified by MSI status is ongoing. Further clinical studies regarding combinations of a PARP-1 inhibitor with other DSB-inducing therapies, such as radiation or irinotecan, are warranted in MSI+ CRCs with *MRE11* mutations. Although these results need to be confirmed in other settings, they suggest that specific mutations such as *MRE11* can be used to exploit the concept of synthetic lethality in MSI+ cancers, which has been successful in BRCA1-mutant breast cancers<sup>[88]</sup>.

Methotrexate reportedly induces oxidative DNA damage and is selectively lethal to cancer cells with MSH2 defects<sup>[84,89]</sup>. Thus, a synthetic lethal relationship between deficient MSH2 and treatment with methotrexate led to a phase II trial, incorporating measurement of 8-oxoG

DNA lesions as a biomarker, in metastatic CRC patients with germline mutation or loss of MSH2.

Because MSI+ CRCs often harbor a near diploid stable karyotype, these cancers may be sensitive to mitotic inhibitors, such as taxanes and kinesin-5 inhibitors<sup>[90]</sup>. To determine the effect of CIN and MSI on the efficacy of the microtubule-stabilizing agent paclitaxel/EPO960, a phase II study called CIN and Anti-Tubulin Response Assessment in CRC is ongoing. It is assumed that MSI+ CRC patients will benefit more than MSI- CRC patients.

Gene expression signatures can also be used for new MSI+ cancer therapies. Fourteen of the 164 compounds were shown to target MSI+ cancer cell lines using combined gene expression data sets and a connectivity map<sup>[91]</sup>. Rapamycin, LY-294002, 17-(allylamino)-17-demethoxygeldanamycin, and trichostatin A were the most convincing candidate compounds. MSI+ cell lines with *MLH1* hypermethylation were preferentially targeted by rapamycin and LY-294002 when compared with MSI- cells. These results underscore the relevant role of the PI3K/AKT/mTOR pathway and its therapeutic application in MSI+ cancer, although its clinical significance needs confirmation.

MiRNA-based cancer therapy is limited mainly to targeting a single miRNA<sup>[92,93]</sup>. However, if most cancers are characterized by a defect in miRNA production and global mature miRNA downregulation<sup>[51-53]</sup>, restoration of the global miRNAome may be an attractive approach in cancer therapy. Melo *et al.*<sup>[94]</sup> have found that the small molecule enoxacin, a fluoroquinolone used as an antibacterial compound, enhances the miRNA-processing machinery by binding to TRBP. Enoxacin was shown to inhibit the growth of a variety of cancer cells. The enhanced miRNA-processing activity by enoxacin did not depend on general fluoroquinolone activity but on the unique chemical structure of enoxacin. These results highlight the key role of disturbed miRNA expression in carcinogenesis, and suggest the potential of novel miRNA-based cancer therapy to restore the disrupted miRNAome of cancer cells.

Finally, it remains to be determined whether *XPO5* mutations can be exploited therapeutically. Since it is difficult to directly restore *XPO5* activity, restoring miRNA accumulation by alternative methods may be a more realistic strategy. Given that only a few deregulated tumor suppressor miRNAs appear to be critical for the tumor-promoting effect of *XPO5* mutations, it may be possible to supply those miRNAs exogenously as miRNA duplexes that would not need to undergo nuclear export. It may also be possible to find a subset of important target genes of deregulated tumor suppressor miRNAs, which may be responsive to inactivation by conventional pharmacological methodologies or novel biologics.

## CONCLUSION

The biological and clinical implications of MSI in GI cancers continue to develop. Recent findings, such as overexpression of *miR-21* and *miR-155* and mutations of *TARBP2* and *XPO5* in MSI+ GI cancers, further suggest

the important interrelationship between MSI and miRNA in MSI carcinogenesis. The clinicopathological, genetic, epigenetic, prognostic, and therapeutic characteristics of MSI+ cancers are becoming clear, but remain to be fully determined. Analysis of MSI status in cancer patients is warranted as a screening for Lynch syndrome; it could be a potential predictive marker of response to chemotherapy. Since molecular targeting therapeutics are being used in clinical settings and trials, it seems important to clarify if molecular target genes are differentially regulated between MSI+ and MSI- cancers and if the MSI status has the prognostic or predictive significance in metastatic CRC. Further analysis is required to gain insight into MSI carcinogenesis, for a better understanding of disease pathogenesis, and for the development of new diagnostic and/or therapeutic approaches targeting essential pathogenetic alterations.

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## Toxic hepatitis in occupational exposure to solvents

Giulia Malaguarnera, Emanuela Cataudella, Maria Giordano, Giuseppe Nunnari, Giuseppe Chisari, Mariano Malaguarnera

Giulia Malaguarnera, Emanuela Cataudella, Maria Giordano, Mariano Malaguarnera, Research Centre "The Great Senescence", University of Catania, 95100 Catania, Italy  
 Giuseppe Nunnari, Department of Infective Diseases, Hospital "Garibaldi di Nesima", University of Catania, 95100 Catania, Italy

Giuseppe Chisari, Microbiology and Clinical Microbiology, Department of Biomedical Science, Hospital Policlinico, University of Catania, 95100 Catania, Italy

**Author contributions:** Malaguarnera G, Cataudella E and Giordano M performed and wrote the paper; Nunnari G and Malaguarnera M designed and performed the review; and Chisari G contributed data.

**Correspondence to:** Emanuela Cataudella, MD, Research Centre "The Great Senescence", University of Catania, Via Mesina, 829, 95100 Catania, Italy. [emacata@hotmail.it](mailto:emacata@hotmail.it)

Telephone: +39-95-7262008 Fax: +39-95-7262011

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### Abstract

The liver is the main organ responsible for the metabolism of drugs and toxic chemicals, and so is the primary target organ for many organic solvents. Work activities with hepatotoxins exposures are numerous and, moreover, organic solvents are used in various industrial processes. Organic solvents used in different industrial processes may be associated with hepatotoxicity. Several factors contribute to liver toxicity; among these are: species differences, nutritional condition, genetic factors, interaction with medications in use, alcohol abuse and interaction, and age. This review addresses the mechanisms of hepatotoxicity. The main pathogenic mechanisms responsible for functional and organic damage caused by solvents are: inflammation, dysfunction of cytochrome P450, mitochondrial dysfunction and oxidative stress. The health impact of exposure to solvents in the workplace remains an interesting and worrying question for professional health work.

### INTRODUCTION

Some studies have suggested that exposure to organic solvents may induce liver toxicity<sup>[1,2]</sup> because most chemicals are metabolized in the liver and toxic metabolites generated through the metabolism are the main cause of liver damage.

Work activities with hepatotoxin exposure are numerous and include chemists, dry cleaners, farm workers, painters, health care workers, nurses, and printers. Organic solvents are used in various industrial processes such as spray painting, paint manufacturing, degreasing, metal processing, aeronautical and auto manufacturing maintenance and manufacturing, as well as various chemical storage facilities. Exposure to hepatotoxins can occur through intentional or accidental ingestion in food or absorption of toxic contaminants through the skin. Contamination includes the ingestion of water, skin absorption *via* water baths, and volatilization of solvents, and heated bathrooms with a shower of water.

Although a number of industrial chemicals are known

to be hepatotoxins, liver disease from occupational exposure is rarely suspected or diagnosed<sup>[3]</sup>.

Three conditions must be fulfilled for the diagnosis of professional toxic hepatitis: (1) Liver damage should take place after occupational exposure to a substance; patient occupational history and the workplace in question is necessary; (2) Liver enzymes must increase to at least double the upper limit of normal levels; and (3) Tertiary conditions, such as other causes of liver disease, must be excluded<sup>[4,5]</sup>.

The most important factors contributing to toxicity liver are protein binding, species differences, points of binding inside the liver intracellular, nutritional condition, genetic factors, interaction with medications in use, alcohol abuse and interaction, and age. For the age factor, it has been shown that age susceptibility clearly plays a role. For instance, neonatal rats are less susceptible to carbon tetrachloride and bromobenzene toxicity as compared to adult animals<sup>[6]</sup>.

The hepatotoxic effects of some of the solvents were recognized as early as 1887. Very little is known about the frequency of occupational liver injury by solvents. It is still difficult to assess the damage from exposure due to difficult controls in the workplace<sup>[7-9]</sup>. Clinical presentation of occupational liver disease may be acute/subacute or chronic, but is often insidious.

Occupational toxic hepatitis can be divided into three types: hepatocellular, cholestatic and mixed (Table 1).

Liver damage is likely to be more severe in the hepatocellular type than in the cholestatic or mixed type; a patient with elevated bilirubin levels in hepatocellular liver injury indicates serious liver disease.

Patients with the cholestatic or mixed type are likely to develop chronic disease more frequently than those with the hepatocellular type.

The solvents suspected to be responsible for liver occupational disease are: dimethylformamide (DMF), dimethylacetamide (DMA), trichloroethylene (TCE), tetrachloroethylene, carbon tetrachloride, xylene, toluene, and chloroform, whose organoleptic properties and main uses are schematically presented in Table 2.

The solvents that follow are the most extensively used in the chemical industry.

## DIMETHYLFORMAMIDE

DMF is an organic compound with the formula  $(\text{CH}_3)_2\text{NC(O)H}$  that takes the form of a colorless, water-soluble liquid. Pure dimethylformamide is odorless, whereas technical grade or degraded dimethylformamide often has a fishy smell due to the impurity of dimethylamine. Its name is derived from the fact that it is a derivative of formamide, the amide of formic acid.

Dimethylformamide has been termed the universal solvent and is used commercially as a solvent for vinyl resins, adhesives and epoxy formulations (the latter for use in laminated printed circuit boards); for purification and/or separation of acetylene, acid gases and aliphatic hydro-

Table 1 Clinical-diagnostic types of occupational toxic hepatitis

Type of disease	ALT	ALP	$\gamma$ -GT	Bilirubin	Bile acids
Hepatocellular	> 2 ULN	N	> 2	Elevated levels	Elevated levels
Cholestatic	N	> 2 ULN	> 4	Normal or moderate level	Elevated levels
Mixed	> 2 ULN	$\geq$ ULN	> 2	Normal or moderate level	Elevated Levels

ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; GT: Glutamyl transferase; ULN: Upper limit of normal; N: Normal value. Normal value, ALT 9-63 U/L; ALP 38-126 U/L;  $\gamma$ -GT 7-50 U/L; Bilirubin 0.20-1.5 mg/dL; Bile acids < 10  $\mu\text{mol/L}$ .

carbons; and in the production of polyacrylic or cellulose triacetate fibres and pharmaceuticals. It is also used as a catalyst in carboxylation reactions; in organic synthesis; as an industrial paint; as a carrier for gases, and in inks and dyes in printing and fibre-dyeing applications<sup>[10-12]</sup>.

It is widely used for resins and polar polymers and in applications such as protective coatings, films, printing inks and adhesives. It is also used in the pharmaceutical industry in the formulation of pesticides, and in the manufacture of synthetic leathers<sup>[13,14]</sup>.

Occupational exposure to dimethylformamide may occur in the production of organic chemicals, resins, fibers, paints, inks and adhesives. Exposure can also occur during the use of ink coatings, adhesives, in the synthetic leather industry, and in the repair of aircraft.

In 100 workers occupationally exposed to this solvent for at least one year (mean exposure of 5 years; range = 1-15 years), a statistically significant incidence of hepatic impairment was found, as indicated by elevated gamma-glutamyl transpeptidase levels and digestive disturbances<sup>[15]</sup>.

Symptoms of irritation occurring during work with DMF include watery eyes, dry throat, and coughing. The exposed workers also reported a reduced sense of smell and dry coughs. Workers exposed to DMF also reported facial flushing and palpitations after ingesting alcohol. This condition is related to alcohol intolerance, characterized by a disulfiram-type reaction.

## DIMETHYLACETAMIDE

DMA is an organic compound with the formula  $\text{CH}_3\text{C(O)N(CH}_3)_2$  that is widely used in the synthetic fiber and resin industries<sup>[16]</sup>.

It is colorless, water miscible, has a high boiling point, and is commonly used as a polar solvent in organic chemistry, as a solvent for vinyl resins, cellulose derivatives, polyacrylonitrile, linear polyesters and styrene. It is also used as in catalyst and solvent elimination, cyclization, alkylation reactions and halogenations.

DMA is a widely used solvent in acrylic and elastane fiber spinning, and is also used as: a solvent in the production of X-ray contrast media; a solvent in the production process of antibiotics like cephalosporins (such as cefadroxil, cefalexin and cefradine); and a solvent and

Table 2 Main features of the solvents described

Solvents	Organoleptic properties	Main uses	Main mode of absorption
Dimethylformamide	Water-soluble liquid	Production of organic chemicals, resins, fibers, paints, inks and adhesives	Inhalation
	Colorless	Vinyl resins, adhesives and epoxy formulations	Skin
	Odorless	Purification and/or separation of acetylene	
	Polar polymer	Production of polyacrylic or cellulose triacetate fibres and pharmaceuticals	
		Industrial paint	
		Protective coatings, films, printing inks and adhesives	
		Pharmaceutical industry	
Dimethylacetamide	Colorless	Formulation of pesticides	
	Water miscible	Organic chemistry	Inhalation
	High boiling point	Vinyl resins	Skin
	Polar	Cellulose derivatives	Gastric
	Greasy	Polyacrylonitrile	
		Linear polyesters and styrene	
		Production process of antibiotics like cephalosporins	
Trichloroethylene	Non-flammable liquid	Production of X-ray contrast media	
	Clear	Manufacture of polyimide resins, polysulfones and cellophane	
	Pleasant smell	Volatile anesthetic (in the past)	Inhalation
	Volatile	Food industry (e.g., the decaffeination of coffee and the preparation of flavoring extracts from hops and spices)	Skin
	Organic compound		Gastric
Tetrachloroethylene	Colorless liquid		
	Volatile	Dry cleaning and metal cleaning, veterinary anthelmintic, textile industry, automotive and other metalworking industries, dry-cleaning industry	Inhalation
	High stabile		Skin
Carbon tetrachloride	Non-flammable		Gastric
	Liquid	Refrigerant	Inhalation
	Easily evaporates	Pesticide	Skin
Xylene	Sweet smell		Gastric
	Unpleasant smell (> 10 ppm)		
	Flammable liquid	Resins, gums, rubber cleaners, degrease paints, lacquers, varnishes, adhesives, cements, inks, gasoline	Inhalation
Toluene	Light-colored or colorless, strong odor		Skin
	Refractive liquid	Paints, coatings, synthetic fragrances, adhesives, inks, cleaning agents, pharmaceuticals, dyes, cosmetic nail products, and synthesis of organic chemicals	Inhalation
	Colorless		Skin
Chloroform	Flammable soluble in water		
	Pungent odor		
	Colorless	Pharmaceutical industry	Inhalation
	Sweet-smelling	Dyes and pesticides	
	Dense liquid	Reagent	
		Anesthetic	

reaction medium in the manufacture of polyimide resins, polysulfones and cellophane.

The hepatic toxicity of DMA is well known in animals, with reports of an increase in liver weight, steatosis, hepatic focal cystic degeneration, transaminasemia, biliary hyperplasia and centrilobular single cell necrosis<sup>[17]</sup>.

It was discovered that a worker in a polyurethane plastic producing plant who was accidentally exposed to DMA mixed with ethylenediamine developed chemical induced hepatitis with several toxic features<sup>[18]</sup>.

There was another report of a male worker in a factory of synthetic stretch fabric who was exposed to mixed solvents, including DMA, in a confined space continuously for 4-6 h/d for three days developing hepatic injury with other clinical manifestations of acute DMA intoxication. Toxic hepatitis following excessive skin exposure to DMA was reported among workers from a new production line of acrylic fiber<sup>[19,20]</sup>.

## TRICHLOROETHYLENE

TCE is a volatile organic compound with the chemical formula C<sub>2</sub>HCl<sub>3</sub>. A chlorinated hydrocarbon, it is used as an industrial solvent, and takes the form of a clear non-flammable liquid with a sweetish smell resembling chloroform.

Until 1975, it was used as a volatile anesthetic (however, it produced depression of the central nervous system) and inhaled obstetrical analgesic in millions of patients, as well as an extractant in food-processing. It is now used for vapor degreasing and as a solvent<sup>[21]</sup>.

TCE is a solvent for a wide variety of organic materials, but is also used in the food industry for the decaffeination of coffee and the preparation of flavoring extracts from hops and spices.

Higher concentrations can cause tachypnea, and many types of cardiac arrhythmias which are exacerbated by epinephrine (adrenaline).

TCE has also been used as an inhaled patient controlled analgesic agent, mainly for the treatment of trigeminal neuralgia.

It was found that 10% of workers exposed to TCE became jaundiced with massive hepatic necrosis<sup>[22]</sup>.

The data in humans, although limited, clearly suggests a toxic effect on human livers. Case reports describe TCE as inducing hepatitis and liver necrosis<sup>[23]</sup>.

## TETRACHLOROETHYLENE

Also, known under the name tetrachloroethene, tetrachloroethene is a chlorocarbon with the formula  $\text{Cl}_2\text{C}=\text{CCl}_2$ . It is a colorless liquid that is volatile, highly stable, and nonflammable, and mainly used as a solvent in dry cleaning and metal cleaning. It is also used for veterinary anthelmintic, processing and finishing in the textile industry, as an extraction solvent, grain fumigant, heat-exchange fluid, and in the manufacture of fluorocarbons.

Tetrachloroethylene is an excellent solvent for organic materials. It is also used to degrease metal parts in the automotive and other metalworking industries and appears in certain consumer products including spot removers, paint strippers, silicone lubricants, and food<sup>[24,25]</sup>. Its toxicity presents itself as different effects in the central nervous system, kidneys and liver. Symptoms of toxicity from exposure include fatigue, dizziness, headache, vomiting and nausea, signs of hepatic or renal failure, and pulmonary edema<sup>[26]</sup>.

Tetrachloroethylene causes irritation of the eyes and nose mucosal. Severe exposure can lead to behavior alteration, coma and death<sup>[24,26]</sup>.

## CARBON TETRACHLORIDE

Carbon tetrachloride, also known by numerous other names, is an organic compound with the formula  $\text{CCl}_4$  that takes the form of a clear liquid that very easily evaporates. Most carbon tetrachloride that finds its way into the environment is therefore found as a gas.

Carbon tetrachloride does not burn easily. It normally has a sweet smell, but this can change to a more unpleasant odor when the concentration of carbon tetrachloride reaches 10 parts per million parts of air (ppm).

In the 20th century, carbon tetrachloride was commonly used as a dry cleaning solvent, as a refrigerant, and in lava lamps<sup>[27]</sup>. One specialty use of carbon tetrachloride was by stamp collectors to reveal watermarks on the backs of postage stamps without damaging the stamp. However, once it became apparent that carbon tetrachloride exposure had severe adverse health effects, safer alternatives such as tetrachloroethylene were found for these applications, and its use in these roles declined from about 1940 onward.

Carbon tetrachloride was used as a pesticide to kill insects in stored grain but, in 1970, it was banned in consumer products in the United States. Before the Montreal Protocol, large amounts of carbon tetrachloride were

used to produce the Freon refrigerants R-11 (trichlorofluoromethane) and R-12 (dichlorodifluoromethane). However, these refrigerants were identified as playing an important role in ozone depletion, and therefore their use was banned. Carbon tetrachloride is still used to manufacture less destructive refrigerants however.

Carbon tetrachloride is one of the most powerful solvents toxic to the liver, and is widely used in scientific research to assess liver damage and hepatoprotective agents<sup>[28]</sup>.

Indeed, carbon tetrachloride has been known for many years to be toxic to the liver. It has been shown to produce hepatic damage including necrosis and fatty degeneration in various experimental animal species<sup>[29,30]</sup>. Several experiments have also shown that single doses can cause areas of necrosis in the liver within minutes<sup>[31,32]</sup>, as well as liver enzyme abnormalities known to indicate liver damage<sup>[33,34]</sup>.

## XYLENE

Xylene is a clear, light-colored or colorless, flammable liquid which evaporates rapidly and is also called "xytol," "dimethylbenzene," or "mixed xylenes". Its odor is strong and sweetish like other aromatic solvents.

Xylene may be found in: solvents for gums, resins, rubber cleaners, degreaser paints, lacquers, varnishes, adhesives, cements, epoxy resins, inks, dyes, and gasoline.

The xylene in commercial use is composed of a mixture of the three isomers ortho-xylene, meta-xylene, and para-xylene; the meta-isomer predominates in these mixtures. O-Xylene and m-xylene are clear, colorless, flammable liquids that have characteristically sweet, balsam-like odors. At low temperatures, the para-isomer occurs in the form of clear, colorless plates<sup>[35]</sup>.

## TOLUENE

Toluene occurs as a colorless, flammable, refractive liquid that is slightly soluble in water, has a sweet and pungent odor, and has the chemical formula  $\text{C}_6\text{H}_5\text{CH}_3$ <sup>[36]</sup>.

It is used to produce benzene and as a solvent in paints, coatings, synthetic fragrances, adhesives, inks, and cleaning agents. Toluene is also used in the production of polymers used to make nylon, plastic soda bottles, polyurethanes, and for pharmaceuticals, dyes, cosmetic nail products, and the synthesis of organic chemicals<sup>[36]</sup>. Exposed to toluene may occur from breathing ambient or indoor air, from the use of common household products (paints, paint thinners, adhesives, synthetic fragrances and nail polish), and cigarette smoke. The deliberate inhalation of paint or glue may result in high levels of exposure to toluene, as well as other chemicals, in solvent abusers<sup>[36]</sup>.

Toluene exposure may also occur in the workplace, especially in occupations such as printing or painting, where toluene is frequently used as a solvent. Automobile emissions are the principal source of toluene in the ambient air. Toluene may be released to the ambient air



during the production, use, and disposal of industrial and consumer products that contain toluene. Levels of toluene measured in rural, urban, and indoor air averaged 1.3, 10.8, and 31.5 micrograms per cubic meter ( $\mu\text{g}/\text{m}^3$ ), respectively<sup>[36]</sup>.

Toluene is metabolized by the liver; however, the liver does not appear to be a primary target for toluene toxicity. Indeed, the central nervous system (CNS) is the primary target for toluene toxicity in both humans and animals for acute and chronic exposures. CNS dysfunction (which is often reversible) and narcosis have been frequently observed in humans acutely exposed to low or moderate levels of toluene by inhalation; symptoms include fatigue, sleepiness, headaches, and nausea. CNS depression and death have occurred at higher levels of exposure.

A study of printing factory workers who were exposed to toluene at a concentration of less than 200 ppm showed minimal changes to liver enzymes<sup>[37]</sup>. A study by Svensson *et al*<sup>[38]</sup> looked at 47 rotogravure workers occupationally exposed to toluene and showed elevation of liver enzymes and chemical hepatitis.

Hepatotoxicity has been observed in the literature in individuals exposed to xylene and toluene<sup>[39]</sup>.

## CHLOROFORM

Chloroform is a volatile organic compound that takes the form of a colorless liquid with a non-irritating odor and a slightly sweet taste. It will burn only when it reaches very high temperatures. In the past, chloroform was used as an inhaled anesthetic during surgery, but it is not used for that purpose today. Today, chloroform is used to make other chemicals and can also be formed in small amounts when chlorine is added to water. Other names for chloroform are “trichloromethane” and “methyl trichloride”.

It is produced as a byproduct of water chlorination and the bleaching of paper. Chloroform may also be elicited as a vehicle exhaust. In 1923, Meyer and Pessoa showed the toxicity of chloroform to the human liver<sup>[40]</sup>.

## MECHANISMS OF HEPATOTOXICITY

The pathophysiologic mechanisms of hepatotoxicity are still being explored, but are characterized by organic and functional damage of the liver. The principal alterations are: (1) Disruption of the hepatocyte; with a decrease in ATP levels. Disassembly of actin fibrils at the surface of the hepatocyte with blistering and rupture of the membrane; (2) Disruption of the transport proteins; toxins may affect transport proteins at the canalicular membrane and can interrupt bile flow. It also detects interruption of transport pumps; (3) Cytolytic T-cell activation: the covalent binding of a toxin to the P-450 enzyme acts as an immunogen, activating T cells and cytokines and stimulating a multifaceted immune response; (4) Apoptosis of hepatocytes; activation of the apoptotic pathways by the tumor necrosis factor-alpha receptor of Fas may trig-

ger the cascade of intercellular caspases, which results in programmed cell death; and (5) Bile duct injury; toxic metabolites excreted in bile may cause injury to the bile duct epithelium<sup>[41]</sup>. In cultured rat hepatocytes, the hydrophobic bile acid glycochenodeoxycholate at pathophysiologically relevant concentrations (20-100 mmol/L) induces apoptosis, as documented by cell shrinkage, nuclear condensation and lobulation, caspase activation, DNA fragmentation, and phosphatidylserine externalization. Thus, bile acids provide a valuable model to dissect the mechanisms of liver cell apoptosis and the role of apoptosis in liver injury from endogenous toxicants. Apoptosis occurs by one of two pathways: (1) a death receptor pathway; and (2) the mitochondrial pathway.

The main pathogenic mechanisms responsible for functional and organic damage caused by solvents are: inflammation, dysfunction of cytochrome P450, mitochondrial dysfunction and oxidative stress.

## INFLAMMATION

Inflammation plays an important role in classical chemical toxicities<sup>[42,43]</sup>. Hepatic non-parenchymal cells, the Kupffer, sinusoidal endothelial, and fat-storing or Ito (stellate) cells, and recruited leukocytes, i.e., monocytes and neutrophils, contribute to the pathogenesis of hepatic toxicity.

Proinflammatory cytokines, chemokines, reactive oxygen and nitrogen species, that promote oxidative stress in the damage induced by toxic substances, are produced by Kupffer cells and neutrophils.

In response to a direct action of the chemical, the Kupffer cells are activated resulting in the production of proinflammatory cytokines such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)  $\alpha$ , and chemokine receptor chemokines.

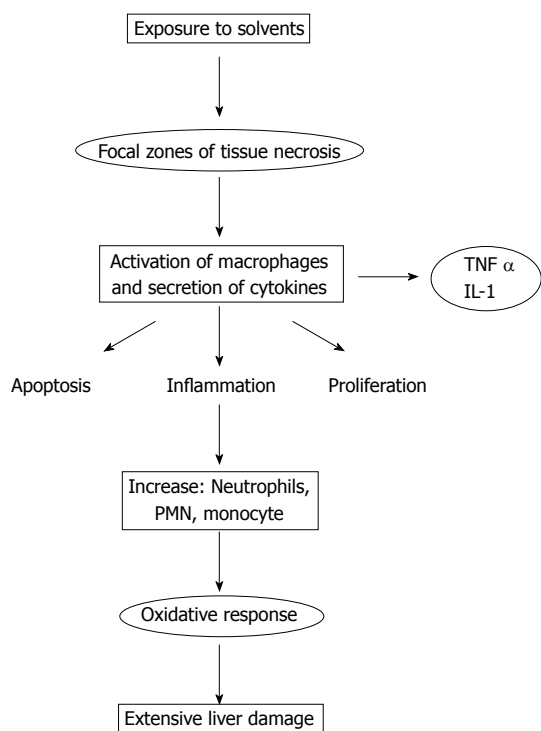
Each of these factors can upregulate expression of  $\beta$ -2 integrin [cluster of differentiation (CD)11b/CD18] and prime neutrophils for reactive oxygen species (ROS) formation. C5a also stimulates Kupffer cells to release ROS.

The aforementioned cytokines can regulate genes for the production of factors that induce and/or promote apoptosis, or that stimulate the proliferation of hepatocytes.

These cytokines can mediate many pathological effects, including inflammatory cell infiltrates, lipogenesis, fibrogenesis and cholestasis<sup>[44]</sup>.

For example, TNF  $\alpha$ /IL-6 induction occurs within minutes following CCl<sub>4</sub> exposure and is responsible for the activation of nuclear transcription factors including AP-1, NF $\kappa$ B, and STAT 3<sup>[45,46]</sup>, which regulate genes involved in cell growth. Liver toxicity induced by solvents is presented schematically in Figure 1.

In addition, cytokines activate the expression of adhesion molecules on endothelial cells and hepatocytes. If primed neutrophils receive a chemotactic signal from the parenchyma, they will transmigrate and adhere to hepatocytes. This leads to the final activation of neutrophil with degranulation (protease release) and adherence-dependent



**Figure 1** Hypothetical role of inflammation in chemical-induced hepatotoxicity. TNF: Tumor necrosis factor; IL: Interleukin; PMN: Prime neutrophils.

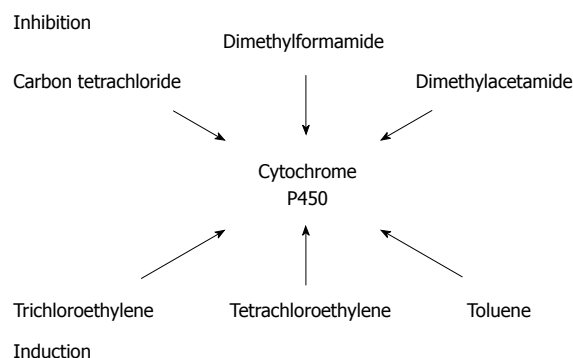
oxidant stress, which causes cell necrosis. Mediators generated during cell injury, such as lipid peroxidation products (LPO) and chemokines, become chemotactic signals for further neutrophil activation and transmigration<sup>[47]</sup>.

Therefore, both neutrophils and Kupffer cells, directly or through activation of a complement, are activated by solvents and drugs toxicity, tissue trauma, ischemia-reperfusion, sepsis, and other pathophysiological events. In particular, Kupffer cells release cytotoxic mediators, such as proinflammatory mediators, and reactive oxygen species, such as cytokines and chemokines. Functionally, complement factors (e.g., C5a) and cytokines prime and activate neutrophils to promote their recruitment into the hepatic vasculature. Chemotactically-stimulated neutrophils extravasate and adhere to parenchymal cells. Through the release of reactive oxygen and proteases, they induce necrotic cell death adhesion molecules on neutrophils ( $\beta$ -2 integrins, especially CD11b/CD18) and ICAM-1 on endothelial cells and hepatocytes, which are essential for neutrophil margination, extravasation, and oxidant production.

Cytokines can induce hepatic adhesion molecule and chemokine formation, which in turn is modulated by oxidant stress<sup>[48]</sup>.

## CYTOCHROME P450

Cytochrome P450 (P450 or CYP) plays an important role in the biotransformation of many endogenous compounds and xenobiotics. The most common enzyme system for oxidation of xenobiotics is cytochrome P450,



**Figure 2** Effects of solvents on cytochrome P450.

also known as CYP450 monooxygenase, hydroxylase or oxidase<sup>[49,50]</sup>.

The liver is the major source of P450, although it is expressed in various extrahepatic tissues like the kidney<sup>[51]</sup>.

P450 isoform CYP2E1 is the most abundant isoform in the human liver and therefore is responsible for the metabolism of a wide variety of exogenous and endogenous substrates<sup>[52,53]</sup>. CYP2E1 dependent ethanol metabolism produces oxidative stress through generation of ROS, a possible mechanism by which solvents are hepatotoxic<sup>[54,55]</sup>.

The cyt p450 is a membrane protein for the disposal of xenobiotics. It is mainly associated with the smooth membranes of the ER of liver cells, while the enzymes for changes to endogenous substances (prostaglandins, cholesterol, *etc.*) are mainly associated with mitochondria.

For example, toxic substances that are known to be substrates of cytochrome P450 enzymes include acetaminophen, cyclophosphamide, doxorubicin and diclofenac.

The detoxification process *via* enzymatic systems, principally the glutathione and cytochrome P450, is different genetically and therefore plays a significant role in the way some individuals detoxify solvents differently from others. It has been shown that all conditions resulting in reduced activity of cytochrome P450, reduced the ability to detoxify solvents and, alternatively, increase the percentage of fat in the liver (e.g., malnutrition). In the study of toxic hepatitis there is a need to evaluate the potential inhibition and inductions of some of the substances involved, and assess the potential interaction with the myriad of drugs that are substrates of CYP<sup>[56,57]</sup> (Figure 2).

The metabolite responsible for the liver damaging effect of carbon tetrachloride is a C Chloride III which is formed from carbon tetrachloride<sup>[31-58]</sup>.

The studies by Brady *et al.*<sup>[59]</sup> and Lindros *et al.*<sup>[60]</sup> have shown that the enzymes involved in the bioactivation of carbon tetrachloride are cytochrome P-450, localized in the liver endoplasmic reticulum.

## MITOCHONDRIAL DYSFUNCTION

Various endogenous and exogenous substances impair mitochondrial  $\beta$ -oxidation to cause micro-vesicular

steatosis through oxidative stress and damage to mitochondrial proteins, lipids, and DNA. In humans, these oxidative lesions cause mitochondrial DNA (mtDNA) deletions<sup>[61]</sup>.

In normal mitochondria, enzymes involved in the import and  $\beta$ -oxidation of fatty acids or the tricarboxylic acid cycle are encoded by nuclear DNA<sup>[62]</sup>. Import polypeptides and enzymes involved in the  $\beta$ -oxidation of long-chain fatty acids are in the inner membrane, while those involved in the  $\beta$ -oxidation of medium- and short-chain fatty acids or the tricarboxylic acid cycle are in the matrix, together with mtDNA<sup>[62]</sup>.

mtDNA is a circular, double-stranded molecule. Each cell contains many copies of this DNA, as there are several mtDNA copies in a single mitochondrion and many mitochondria per cell<sup>[62,63]</sup>. MtDNA is extremely sensitive to oxidative damage owing to its proximity to the inner membrane (the main cellular source of ROS), the absence of protective histones, and incomplete repair mechanisms in mitochondria<sup>[62-64]</sup>.

Many solvents (cationic and amphiphilic) are able to concentrate in mitochondria as a result of the mitochondrial membrane potential<sup>[65]</sup>. Accumulation of these solvents within liver mitochondria inhibits fatty acid  $\beta$ -oxidation (causing steatosis) and electron transfer along the respiratory chain<sup>[65]</sup>. Overly reduced respiratory chain intermediates react with oxygen to form the superoxide anion. ROS oxidize fat deposits<sup>[65]</sup>. Similarly, in alcohol abuse, increased ROS formation causes extensive peroxidation of fat deposits and frequent steatohepatitis<sup>[66]</sup>.

## OXIDATIVE STRESS

The oxidative damage caused by free radicals is thought to be a basic mechanism underlying many pathological conditions, including hepatotoxicity by solvents.

Oxidative stress develops when there is an imbalance between the pro-oxidant and antioxidant ratio, leading to the generation of ROS. Environmental contaminants such as solvents, herbicides, and insecticides are known to modulate antioxidant defensive systems and cause oxidative damage in organisms by ROS production<sup>[67,68]</sup>.

Oxidative damage accumulates more in mitochondria than in the rest of the cells because electrons continually leak from the respiratory chain to form damaging ROS (Smith, R., 1999).

ROS, such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), superoxide anion O<sub>2</sub><sup>-</sup>, and hydroxyl radical (OH•) at supranormal levels, can react with biological macromolecules potentially leading to enzyme inactivation, LPO, DNA damage and cell death, but at low concentrations their effects are less pronounced<sup>[69]</sup>.

These free radicals are capable of damaging many cellular components such as DNA, proteins and lipids<sup>[70]</sup>.

## CLINIC OF TOXIC HEPATITIS

Toxic effects on the liver have been studied as early as

1887 and it was determined that there must be a change in the rate of the metabolism of these compound in order to create toxic products, otherwise toxicity will not occur. This chain of events is obligatory from a pharmacokinetic point of view for the majority of solvents<sup>[7-9]</sup>.

There are a few clinical features associated with occupational liver disease including fatigue, appetite loss, arthralgia, hypertransaminasemia, hypergamma glutamyl transferase (GT), and splenomegaly. As there are many causes of liver injury, it is essential to exclude other aetiologies. Other aetiologies include viral hepatitis, biliary diseases, alcohol abuse, and non-alcoholic fatty liver disease.

Clinical presentation of occupational liver disease may be acute/subacute or chronic but is often insidious. Since some of the solvents may cause chronic health effects, it may take decades to study and document such events. Some hepatotoxins are capable of causing malignancy. The most famous example is vinyl chloride which was once thought to be safe and was used for many years until it was found to cause liver tumors.

Signs and symptoms of toxic hepatitis occurring may include: jaundice, itching, and abdominal pain in the upper right portion of the abdomen, fatigue, loss of appetite, nausea and vomiting, rash, weight loss, and dark or tea-color urine.

In acute toxic hepatitis the patient's condition is similar to viral hepatitis and rapidly deteriorates, resulting in marked liver dysfunction, encephalopathy and coagulopathy. The features of toxic hepatitis are: apoptosis of hepatocytes, ischemic liver injury, sepsis, and cholestasis. Hepatocyte apoptosis and necrosis, when massive, result in fulminant hepatic failure<sup>[71]</sup>.

Acute exposure and toxicity has been associated with liver necrosis, and liver steatosis, and chronic exposure has been associated with liver cirrhosis. The mechanism of injury is most likely the result of metabolic changes by the liver.

Liver damage in the form of hepatomegaly, jaundice, and elevation in levels of several hepatic transaminases and bilirubin has also been reported.

Orthotopic liver transplantation (OLT) has improved the survival of these patients (49% undergo OLT), yet 37% die while awaiting OLT.

Steatosis (fat accumulation in the liver) is important in order to look at the effect of solvents, which are known to be toxic to the liver. Steatosis is the result of anomalous transport of lipids and, as a consequence, accumulation of lipids in the liver. For that reason, exposure to hepatotoxic solvents is clinically associated with liver steatosis, among others, and is a good clinical marker of solvent hepatotoxicity (having ruled out other factors).

After steatosis, necrosis is the second most common effect of liver damage as a result of hepatotoxic solvents. It is the result of destruction of the cell architecture, as well as damage of the biochemical pathways.

Chronic effects on the liver in long-term occupational exposure to low levels of organic solvents remain undetermined.

Indeed, epidemiological studies in this field were difficult for several reasons.

These include: (1) the cause of chronic liver injury can be attributed to a number of factors and not only isolated in occupational exposure; and (2) failure to control chronic liver damage among workers because of vague symptoms and signs, and lack of specificity and sensitivity of conventional liver enzyme tests<sup>[72]</sup>.

Many cases of liver cirrhosis with no known aetiology raise the suspicion that some may be of occupational origin.

## DIAGNOSIS OF TOXIC HEPATITIS

### Laboratory

Liver damage can be of two types: hepatocellular damage (death of liver cells), in which alanine aminotransferase and aspartate aminotransferase are altered; and cholestatic damage (bile stasis) with an increase of parameters such as alkaline phosphatase and  $\gamma$ -GT. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities are routinely used as clinical endpoints indicative of hepatotoxicity. Toxic hepatic damage includes necrosis and fatty degeneration.

The liver is one of the target organs for toxins, thus biological effects monitoring is required medical surveillance of workers exposed to hepatotoxin. It is also important to evaluate platelet count abnormalities and serum bilirubin in patients.

The evaluation of plasma enzyme showed the advantage of being in the past been well tested in clinical practice, but the main disadvantage is that the enzymes are not organ specific and this can cause occasional diagnostic problems in clinical practice<sup>[73,74]</sup>.

Alanine aminotransferase is considered to be liver-specific in the rat<sup>[75]</sup>. Aspartate aminotransferase activity is high in the rat liver, along with the kidney, pancreas and erythrocytes<sup>[76]</sup>, which means that elevated serum AST is indicative of tissue and cellular damage, but is not specific for hepatotoxicity.

As a general rule, clinically significant liver injury is often defined as ALT > 3 times the upper limit of normal (ULN)<sup>[77]</sup>.

A factor that may portend a worse prognosis than the elevation of transaminases liver alone is associated with the increase with aminotransferase<sup>[78,79]</sup>.

Serum levels of  $\gamma$ -GT have been recognized as a marker of hepatobiliary disease. Conditions that could cause increases in  $\gamma$ -GT levels are different; hepatotoxic agents and other non-hepatic factors such as renal, pulmonary, and myogenic (including cardiac) disorders. Indeed  $\gamma$ -GT is not merely a sensitive marker for liver and bile disorders, but it may also serve as a risk marker for a multiplicity of other chronic diseases; the metabolic syndrome (namely, obesity, hypertension, lipid metabolism, and in particular type 2 diabetes) for example<sup>[80,81]</sup>.

The measurement of total bile acids in serum may be a more sensitive indicator of hepatic function and has the advantage of being organ specific. Measurement of bile

acids has not been standard clinical practice, however, and interpretation of high results might present difficulties.

Bile acids are synthesized from cholesterol in the liver, and secreted to the duodenum *via* the bile duct<sup>[82]</sup>.

Secreted bile acids are reabsorbed almost completely into the intestine and returned to the liver (Hofman, 2007). Thanks to efficient enterohepatic circulation of bile acids, less than 10% of intestinal bile acids are eliminated in the feces. The secretion of bile acids into the systemic circulation is small. The blood concentration of bile acids is less than 10  $\mu$ mol/L under normal conditions, but is increased in the case of a disorder of the liver or biliary tract<sup>[82]</sup>. Hepatotoxicity implicated from occupational and drug exposure has also been associated with elevations in total bile acid concentrations. Retention of bile constituents within the hepatocyte during cholestasis is associated with hepatocyte apoptosis.

In cholestatic disease, endogenously generated bile acids produce hepatocellular apoptosis by stimulating Fas translocation from the cytoplasm to the plasma membrane, where self-aggregation occurs, to trigger apoptosis. In cholestasis, secretion is impaired, resulting in elevated concentrations<sup>[83]</sup> of toxic bile acids (TBA) within hepatocytes. At pathophysiologic concentrations, TBA trigger translocation of intracellular Fas bearing vesicles to the plasma membrane where they self-aggregate in the absence of ligand. Activated Fas receptor complexes on the plasma membrane then cause caspase 8 activation and an apoptotic cascade.

Evaluation of the concentration of bile acids is not widely used as a routine screening test. Recently, a simpler and more rapid method is the direct enzymatic assay of urine sulfated bile acids (USBA)<sup>[84]</sup>.

Mild forms of toxic hepatitis may not cause any symptoms and may be detected only by blood tests. Histological samples obtained from workers exposed to solvents that had only mild biochemical abnormalities have shown prominent fatty change, or steatosis, with degrees of inflammation and fibrosis, which suggest that parenchymal changes may be an early feature of solvent induced liver injury.

### Diagnostic imaging

The diagnosis of toxic hepatitis improves with the use of imaging techniques (ultrasound, contrast enhanced ultrasonography, computed tomography, magnetic resonance imaging). In most cases the findings are not characteristic, but history and laboratory investigations allow us to make a diagnostic hypothesis. Indeed, they are essential in determining the clinical-pathological example for the evaluation of steatosis<sup>[85]</sup>.

Routine evaluation includes ultrasound (US) and contrast enhanced ultrasound (CEUS), as reliable methods of first instance. Assessment may include, where necessary, computed tomography (CT) scans, magnetic resonance imaging (MRI) and liver biopsy.

Presently, the most widely used method for assessment of fatty liver is abdominal ultrasound, as it has sev-



eral advantages. It is not invasive, is readily available, and provides reliable information.

CT and MRI techniques are sensitive for the evaluation of steatosis, but are more expensive and less readily available<sup>[86]</sup>.

However, it is necessary to clarify that recent studies have shown that abdominal ultrasound is not accurate in cases of chronic liver disease with fibrosis. In the latter case, it is important to confirm the steatosis and hepatic fibrosis assessment CT<sup>[87]</sup>.

Toxic hepatitis is characterized by different degrees of steatosis and fibrosis, which can lead to cirrhosis.

Various degrees of steatosis can be defined as: (1) "light steatosis", presence of slight "bright liver" and no deep attenuation; (2) "moderate steatosis", presence of mild "bright liver" and with deep attenuation; and (3) "severe steatosis", presence of diffusely severe "bright liver" and deep attenuation without visibility of the diaphragm.

Chronic toxic hepatitis can progress to cirrhosis and liver failure. Early diagnosis of cirrhosis is important to prevent the development of severe liver failure. The diagnosis of cirrhosis of the liver requires a histological demonstration for the evaluation of abnormal nodules of regeneration and fibrosis. Therefore, liver biopsy is still considered the gold standard instrument. However, liver biopsy is limited by a number of disadvantages, such as availability of expert practitioners, cost, invasiveness of the procedure, and risk of complications (bleeding, pneumothorax, pain perforation and bile peritonitis)<sup>[88,89]</sup>.

## CONCLUSION

Many occupational activities can cause abnormalities in liver function tests without any symptoms suggestive of liver disease.

In patients with abnormalities in liver function tests without an obvious cause, a careful history including not only drugs, but also herbal remedies, should first be obtained.

History taking should also include occupational activity, exposition time, alcohol consumption and underlying chronic liver disease.

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## Simple and reproducible hepatectomy in the mouse using the clip technique

Tomohide Hori, Norifumi Ohashi, Feng Chen, Ann-Marie T Baine, Lindsay B Gardner, Toshiyuki Hata, Shinji Uemoto, Justin H Nguyen

Tomohide Hori, Feng Chen, Ann-Marie T Baine, Lindsay B Gardner, Department of Neuroscience, Mayo Clinic in Florida, Jacksonville, FL 32224, United States

Norifumi Ohashi, Gastroenterological Surgery, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550, Japan  
Tomohide Hori, Toshiyuki Hata, Shinji Uemoto, Division of Hepato-Biliary-Pancreatic and Transplant Surgery, Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

Justin H Nguyen, Division of Transplant Surgery, Department of Transplantation, Mayo Clinic in Florida, Jacksonville, FL 32224, United States

**Author contributions:** Hori T, Ohashi N and Hata T performed the research; Hori T analyzed the data and wrote the paper; Chen F, Baine AMT and Gardner LB supported this research; Nguyen JH provided the originality of this technique; Uemoto S and Nguyen JH supervised the research.

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**Correspondence to:** Tomohide Hori, PhD, MD, Department of Neuroscience, Mayo Clinic in Florida, 4500 San Pablo Road, Jacksonville, FL 32224, United States. [hori.tomohide@mayo.edu](mailto:hori.tomohide@mayo.edu)

Telephone: +1-904-9532449 Fax: +1-904-9537117

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**RESULTS:** According to anatomical results, models with 75%, 80%, and 90% hepatectomy produced massive hepatectomy. Learning curves and operative times were most optimal with the clip technique. Each hepatectomy performed using the clip technique produced a reasonable survival curve, and there were no differences in histopathological findings between the suture and clip techniques.

**CONCLUSION:** Massive hepatectomy by the clip technique is simple and can provide reliable and relevant data.

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**Key words:** Hepatectomy; Animal model; Clip; Microsurgery; Surgical technique

**Peer reviewers:** Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450 Tenjin-cho, Kodaira, Tokyo 187-8510, Japan; Misha Luyer, MD, PhD, Department of Surgery, Orbis Medical Centre, Postbus 5500, 6130 MB, Sittard, The Netherlands

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### Abstract

**AIM:** To investigate the reliability of massive hepatectomy models by using clip techniques.

**METHODS:** We analyzed anatomical findings in 100 mice following massive hepatectomy induced by liver reduction > 70%. The impact of various factors in the different models was also analyzed, including learning curves, operative time, survival curves, and histopathological findings.

### INTRODUCTION

New insights into mechanisms in the hepatology field have been established from experimental animal models. The rodent hepatectomy model is mainly employed to examine liver regeneration, liver failure, and tumor metastasis, and the 70% hepatectomy by *en bloc* ligation of the lobes is well established in the rat<sup>[1,2]</sup>. However, mouse

models allow for the use of gene-altered or knockout strains and for laboratory assays, due to the development of specific agents and antibodies<sup>[3]</sup>. At present, there are a number of murine hepatectomy models<sup>[4-10]</sup>, of which the 70% hepatectomy (termed 2/3 partial hepatectomy) is most common. The introduction of microsurgical techniques has also enabled high rates of successful surgery, especially in individualized dissection and ligation of vessels<sup>[6,11,12]</sup>. However, murine hepatectomy remains challenging because of the delicate nature of the liver, the lack of intravenous access, and the risk of hemorrhage<sup>[9]</sup>, resulting in high rates of mortality and morbidity<sup>[5,13]</sup>. In a previous paper, the rate of complications was reported as approximately 30%<sup>[6,14,15]</sup>. Currently, innovative methods for the hepatectomy model have been documented, such as the ligation method<sup>[7,9,14,15]</sup> and clip technique<sup>[5]</sup>.

Despite the widespread use of the 70% hepatectomy<sup>[4-7]</sup>, alternative hepatectomy models with resected volumes > 70% (so-called, “massive hepatectomy”) are required to provide more clinically relevant experiments on liver regeneration and hepatic failure. Nikfarjam *et al.*<sup>[5]</sup> reported benefits of the hemostatic clip in hepatectomy models with 37% and 70% of resected volumes in the mouse. Herein, we describe detailed surgical procedures of our institutional hepatectomy models with > 70% resection using suture hemostatic clip in the mouse and compare various factors between suture and clip techniques. Then, we discuss the usefulness of this simple and reproducible technique in hepatectomy models of > 70% resection.

## MATERIALS AND METHODS

### Animals

Inbred C57BL/6 mice (male, 10-20 wk of age, approximately 25 g body weight) were used (C57BL/6NHsd; Harlan Laboratories, Indianapolis, IN, United States). All mice were maintained under specific pathogen-free conditions. All experimental protocols were approved by IACUC at Mayo Clinic (Protocol No. 33307 and 24907).

### Hepatectomy by suture technique

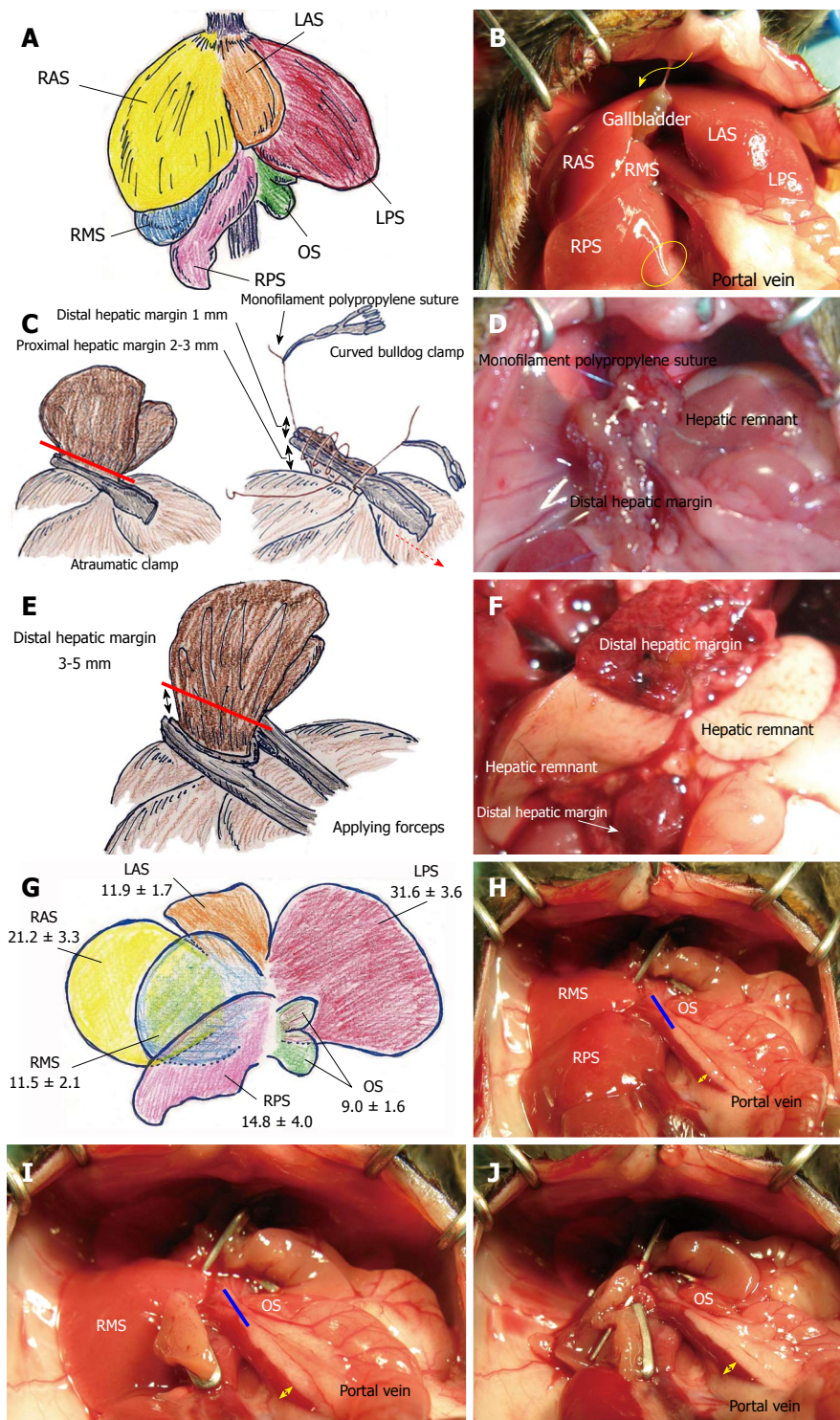
General anesthesia was provided with an anesthesia system (VetEquip Inc., Pleasanton, CA, United States). Inhalational anesthesia was induced and maintained by isoflurane (Isoflurane USP, 250 mL; Webster Veterinary, Sterling, MA, United States). Isoflurane with oxygen flow at 5 L/min was used in the induction phase, and was reduced to 0.5-2.0 L/min in the maintenance phase. Body weight was measured after anesthesia. The abdominal wall was shaved and prepped with povidone-iodine. A transverse incision was performed and hemostasis was obtained using an electrocautery scalpel of bipolar type (Bipolar forceps, Martin ME 102 Electrosurgical unit and Bipolar Accessory Set for ME 102; Harvard Apparatus, Holliston, MA, United States) and that of monopolar type (Bovie, low temperature cautery kit; Aaron Medi-

cal, Clearwater, FL, United States). Mice that received only laparotomy served as a control group in the study. The gastrointestinal tract was moistened with warm saline, covered with gauze, and positioned to the outside of the left abdominal cavity. The liver was handled delicately with cotton-tipped applicators (Hardwood Products Company, Guilford, ME, United States). A surgical microscope at 5-10 × magnification (Surgical Scope M680, Type 10445496; Leica Microsystems Inc., Bannockburn, IL, United States) was used. Even though high magnification (12.5-20 × magnification) is not required, a sufficient light source was indispensable. We used fine-tipped and delicate scalpels which are suitable for ultra-microsurgery, i.e. structures including nerve or vessels < 0.5 mm in diameter<sup>[16]</sup>. The murine liver was divided into three lobes (right, left, and caudate lobes) and arranged into six segments<sup>[4-6,14,17]</sup>: right anterior segment (RAS), right middle segment (RMS), right posterior segment (RPS), left anterior segment (LAS), left posterior segment (LPS), and omental segment (OS) (Figure 1A and B). The falciform and triangular ligaments were sharply cut. The infra-hepatic vena cava runs into the RMS and RPS. The infra-hepatic inferior vena cava was skeletonized. The LPS was freed from the diaphragm and OS. The LAS and LPS were mobilized off the hepatic hilum and gastroduodenal tract.

We did not use Pringle's maneuver during the operation. Hepatectomy by suture technique requires a proximal hepatic margin for suture bait. An atraumatic clamp (Micro Vascular Clip, RS-6470 or RS-6472; Roboz Surgical Instrument Co., Gaithersburg, MD, United States) was placed on the hepatic segment, and the optimal proximal hepatic margin was 2-3 mm from the division of each segment. The clamped segment was sharply cut with a 1 mm distal hepatic margin (Figure 1C). Monofilament polypropylene sutures (7-0 Prolene, BV-1, 8304H-X, or 8-0 Prolene, BV130-5, 8732H; Ethicon, Inc., Somerville, NJ, United States) were bilaterally placed without involvement of a clamp. These bilateral sutures were ligated, and each suture was grasped with a curved bulldog clamp (Cooley Bulldog clamps, curved, serrated, MB586R or MB587R; V.Mueller, Aesculap Inc., Center Valley, PA, United States). The liver was sutured by a loose continuous suture that initially involved the clamp, and the last suture reached the right side. The clamp was removed and the suture was tightened and ligated with the stay suture (Figure 1D). The presence of bleeding points was carefully checked using a cotton swab. An additional suture was made with a monofilament nylon suture (10-0 Ethilon, BV130-3, 2820G; Ethicon, Inc.) for well-focused hemostasis if required. Microfibrillar collagen (Avitene; C.R. Bard, Inc., Murray Hill, NJ, United States) was used in hemostasis.

The intraperitoneal cavity and organs were washed with warm saline. The peritoneum and fascia were closed with a continuous suture using absorbable thread (5-0 Coated Vicryl Plus; Ethicon, Inc.). The skin layer was closed separately using the same method.





**Figure 1** Hepatic segments and hepatectomy by suture or clip techniques. A: The murine liver is divided into six segments (RAS, RMS, RPS, LAS, LPS, and OS); B: The gallbladder is detected between the RAS and LAS; C: Atraumatic clamp was placed on hepatic segment, with optimal proximal hepatic margin of 2-3 mm from division of each segment. Clamped segment was sharply cut with 1 mm distal hepatic margin (red line). Sutures not involving the clamp were placed bilaterally. Bilateral sutures were ligated, and each suture was grasped with a curved bulldog clamp. By using suture of left-side ligation, cut surface of liver was sutured from left side to right side with loose continuous suture that involved a clamp, and the last suture reached the right side; D: Clamp was removed (dotted red arrow) and hepatic margin was immediately treated by tightening the continuous suture. The thread of tightened continuous suture was ligated with stay suture of right side without over-tightening. Continuous suture was made from right side to left side. The last suture was ligated with stay suture of left side without over-tightening; E: The relevant segment was cut with a 3-5 mm distal hepatic margin (red line); F: Ischemic change of distal hepatic margin at 24 h after 80% hepatectomy was shown. Although liver remnants after massive hepatectomy showed color change due to vacuolization, distal hepatic margins clearly showed necrotic changes. Color change between liver remnant and distal hepatic margin was more enhanced at autopsy; G: Percentages of total volume of each segment were  $21.2 \pm 3.3\%$  for RAS,  $11.5 \pm 2.1\%$  for RMS,  $14.8 \pm 4.0\%$  for RPS,  $11.9 \pm 1.7\%$  for LAS,  $31.6 \pm 3.6\%$  for LPS, and  $9.0 \pm 1.6\%$  for OS; H: Traditional 2/3 hepatectomy of RMS + RPS + OS ( $35.4 \pm 4.0\%$ ) is shown. Additional resection of OS (blue line) will make a 75% hepatectomy (RMS + RPS,  $26.4 \pm 3.8\%$ ); I: Hepatic remnant in 80% hepatectomy was RMS + OS ( $20.6 \pm 2.6\%$ ). Additional resection of OS (blue line) will make an RMS-remnant 90% hepatectomy (RMS,  $14.8 \pm 4.0\%$ ); J: OS-remnant 90% hepatectomy is shown (OS,  $9.0 \pm 1.6\%$ ). Dilatation of portal vein due to portal hypertension is confirmed in reverse proportion to volume of hepatic remnant (yellow arrows). LAS: Left anterior segment; LPS: Left posterior segment; OS: Omental segment; RAS: Right anterior segment; RMS: Right middle segment; RPS: Right posterior segment.



### Hepatectomy by hemostatic clip technique

The preparation and mobilization of each liver segment was similarly performed as described above. M-sized hemostatic clips were used (Horizon Ligation System; Teleflex Medical, Durham, NC, United States). The hepatic segment was retracted using tissue forceps when the clip was applied. A hemostatic clip was applied near the point of division of each segment (Figure 1E), and the proximal hepatic margin was approximately 1 mm from a segmental division. The segment was then cut with a distal hepatic margin of 3–5 mm. An additional suture for complete hemostasis was not typically required. Even during surgery, color change of the clipped segment due to ischemia was confirmed, and the color change between remnant liver and distal hepatic margin was observed at autopsy (Figure 1F).

### Post-operative care

A warmer (RightTemp, RTHS-SM; Kent Scientific Co., Torrington, CT, United States) was used to maintain body temperature immediately after surgery, and a heating pad (E12107; Sunbeam, Gainesville, FL, United States) was used to warm the cage until mice completely recovered from anesthesia and surgery. Each mouse was kept separately. Postoperative observation was performed every 2 h for the first 12 h after surgery, and thereafter every 4 h. An analgesic agent (0.1 µg/g body weight, i.m.; buprenorphine 100 µg/mL; Cerilliant, Round Rock, TX, United States) was routinely given intramuscularly every 12 h for 5 d after surgery. Antibiotics (30 µg/g body weight, i.p.; Cephalexin Hydrate; MP Biomedicals, Cleveland, OH, United States) was administered every 12 h for 24 h after surgery. For each mouse, 1 mL of lactate Ringer's solution (Lactated Ringer's Injection USP; B. Braun Medical Inc., Irvine, CA, United States) was routinely administered every 6 h for 24 h.

### Assessments and data analysis

We weighed each segment in a total of 100 mice, and segmental percentages were calculated as segmental weight (g)/whole liver weight (g) for each mouse. The extent of the hepatectomy is based on the segmental volumes. Of note, the gallbladder can be easily detected in the mouse between the RAS and the LAS (Figure 1B), while the rat does not have a gallbladder.

The learning curve for surgery is important for reliability of the hepatectomy model. To compare hepatectomy models using the suture technique *vs* the clip technique, we examined learning curves and operative time.

Rare complications of outflow block or biliary obstruction due to surgical clips were previously reported<sup>[18,19]</sup>. To confirm that the surgical issues were due to the clip itself, it was proposed that the clip may secondarily obstruct outflow or biliary ducts near the proximal hepatic margin or hepatic remnant. At first, we checked histopathological findings of the hepatic remnant near the proximal hepatic margin and the distal hepatic margin in the hepatectomy model by suture and clip techniques.

Focal and/or patchy necrosis is an important histopathological finding after hepatectomy<sup>[4,20]</sup>. To calculate the rate of the appearance of focal and/or patchy necrosis in hepatectomy models by the suture and clip techniques, we examined ten cases for each technique on the same day. Histopathological analyses and calculations were performed at 6 h after hepatectomy. These examinations were repeated three times.

In this study, data are presented as mean  $\pm$  SD. Student's *t* test was used for the comparisons of unpaired variables between the two groups. A survival curve was made by the Kaplan-Meier method, and the log-rank test was used for the comparisons of survival rates between two groups. Statistical calculations were performed using StatView Version 5.0 (SAS, Cary, NC, United States). A *P* value  $< 0.05$  was considered statistically significant.

## RESULTS

### Measurement of the weight of each segment

The percentages of total volume for each of the segments were 21.2%  $\pm$  3.3% for RAS, 11.5%  $\pm$  2.1% for RMS, 14.8%  $\pm$  4.0% for RPS, 11.9%  $\pm$  1.7% for LAS, 31.6%  $\pm$  3.6% for LPS, and 9.0%  $\pm$  1.6% for OS (Figure 1G).

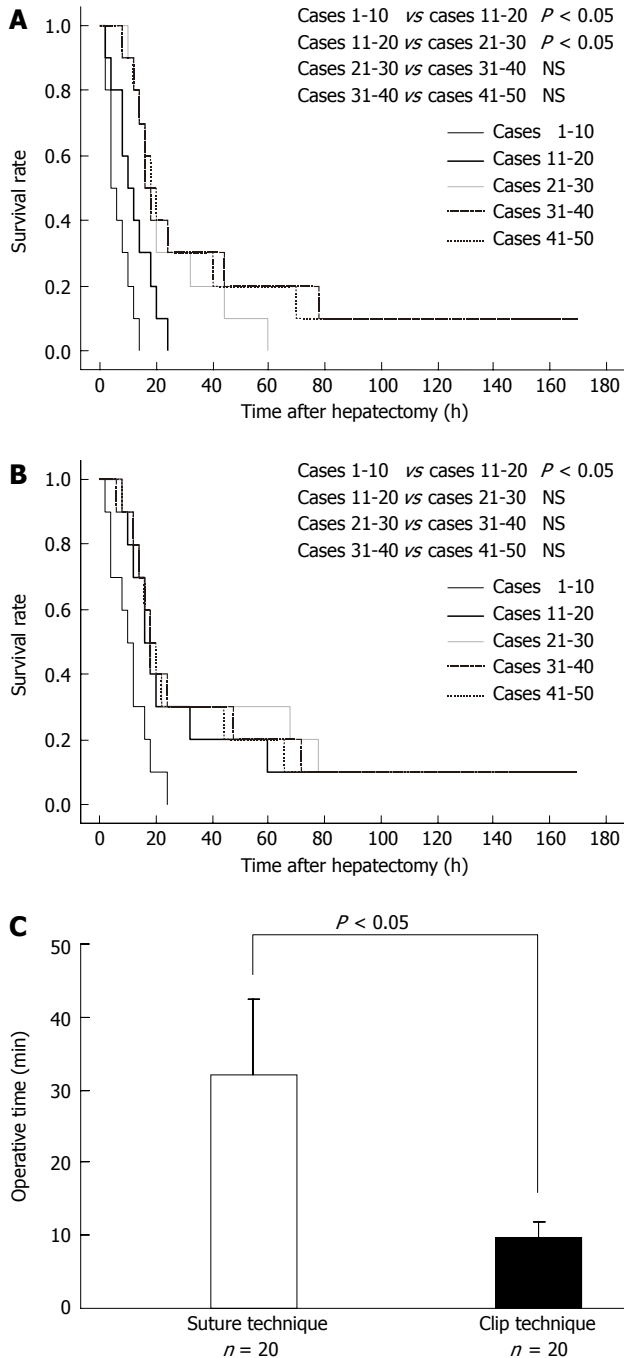
### Hepatectomy models with > 70% resection in the mouse

Based on our results in the measurement of segmental weights, we calculated the liver volume of resection and the hepatic remnant. The hepatic remnant in the 75% hepatectomy was RMS + RPS (26.4%  $\pm$  3.8%); while for traditional 2/3 hepatectomy the remnant was RMS + RPS + OS (35.4%  $\pm$  4.0%) (Figure 1H). Hepatic remnant in the 80% hepatectomy was RMS + OS (20.6%  $\pm$  2.6%) (Figure 1I). We had two surgical options for 90% hepatectomy by sparing either OS (9.0%  $\pm$  1.6%) or RMS (11.5%  $\pm$  2.1%) (Figure 1J). Portal venous dilatation inversely proportional to the volume of the hepatic remnant was observed.

### Learning curves

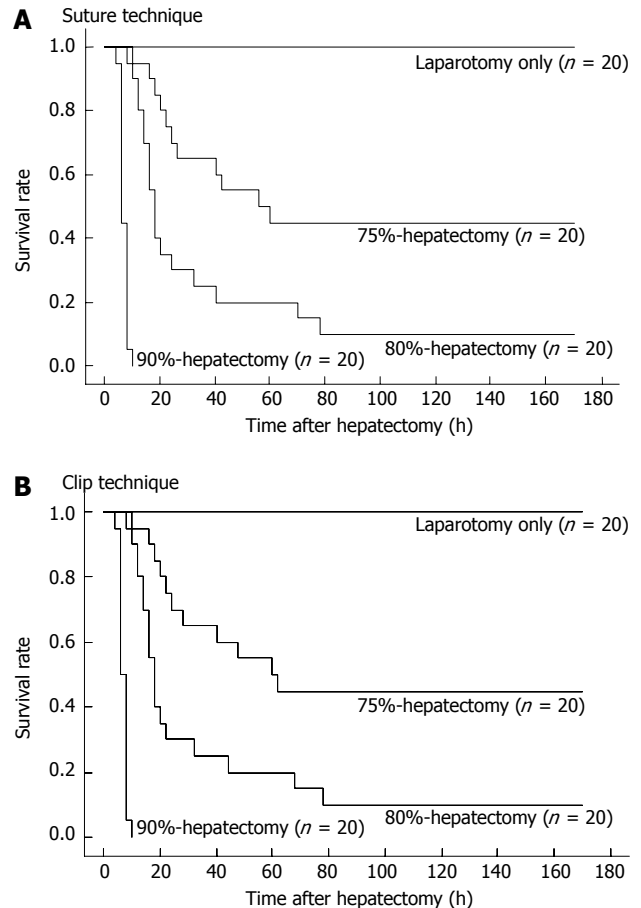
For each technique, ten cases were performed on the same day as one unit for training. The required units that showed no statistical differences in survival curves after hepatectomy with those at the next unit were analyzed. The survival curves of hepatectomy after the training period were compared between the two techniques.

Survival curves of each batch of 10 cases at early term in training in the 80% hepatectomy by the suture technique, and statistical results, are shown in Figure 2A. The *P* values between the first 10 cases and the second 10 cases, between the second 10 cases and the third 10 cases, between the third 10 cases and the fourth 10 cases, and between the fourth 10 cases and the fifth 10 cases were 0.0445, 0.0471, 0.4790, and 0.8941, respectively. The first and second units showed statistical differences in comparison with the next unit. Survival curves of each batch of 10 cases at early term in training in the 80% hepatectomy



**Figure 2** Survival outcomes in 80% hepatectomy after initial training. A: In 80% hepatectomy by suture technique, survival curves of each 10 cases at early term in training are shown; B: In 80% hepatectomy by the clip technique, survival curves of each 10 cases at early term in training are shown; C: In each technique, operative time was investigated in 20 cases of 80% hepatectomy. These data were corrected by experienced surgeons. Operative times of 80% hepatectomy by suture and clip techniques were significantly different ( $P < 0.0001$ ). NS: Not significant.

by the clip technique, and statistical results, are shown in Figure 2B. The  $P$  values between the first 10 cases and the second 10 cases, between the second 10 cases and the third 10 cases, between the third 10 cases and the fourth 10 cases, and between the fourth 10 cases and the fifth 10 cases were 0.0363, 0.6854, 0.9127, and 0.9007, respectively. Only the first unit showed statistical differences in



**Figure 3** Actual survival curves after each hepatectomy with > 70% resection. A: Actual survival curves of each hepatectomy by suture technique; B: Actual survival curves of each hepatectomy by clip technique. NS: Not significant.

comparison with the second unit.

In the 80% hepatectomy, the fifth batch of 10 cases (i.e., 10 cases after the experience of 40 cases) in the suture technique and the fourth batch of 10 cases (i.e., 10 cases after the experience of 30 cases) in the clip technique showed similar survival curves ( $P = 0.8516$ ), even though 30 cases in the suture technique and 20 cases in the clip techniques seemed to be enough statistically. Our institution has similar results in the 90% hepatectomy model.

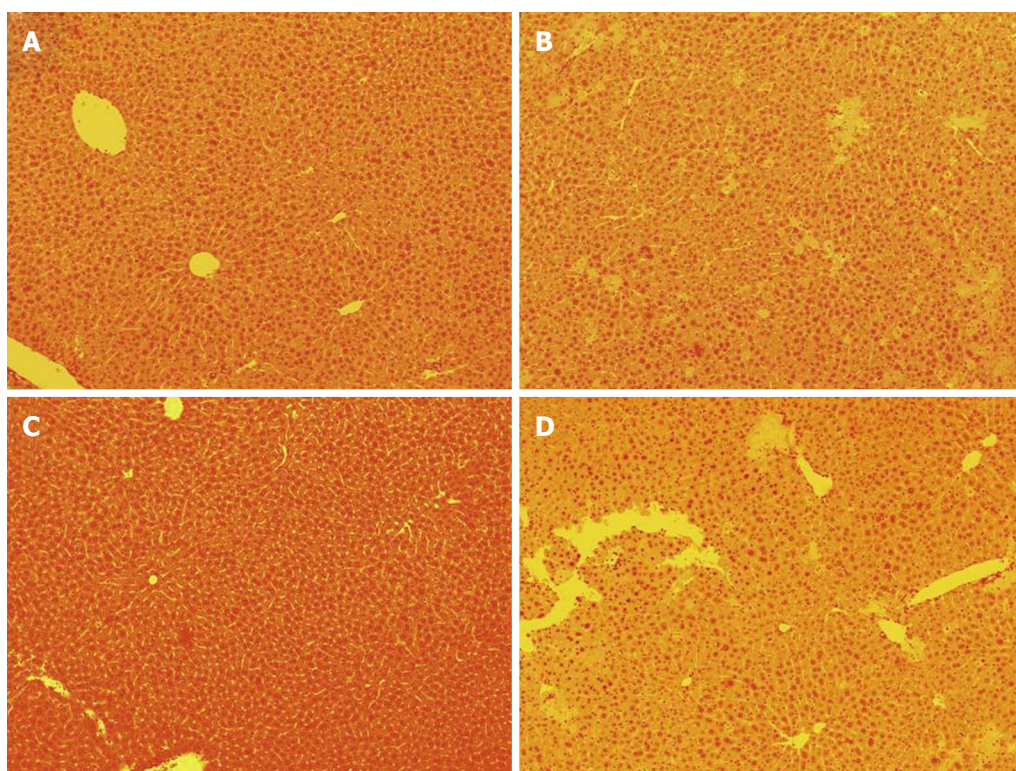
### Operative time

Operative time data were also collected from experienced surgeons in 20 cases of 80% hepatectomy for each technique. Even with experienced surgeons, operative times for hepatectomy using the suture technique and the clip technique were significantly different ( $P < 0.0001$ ) (Figure 2C). Operative times were clearly shortened in the clip techniques.

### Survival curves after hepatectomy with > 70% resection

Actual survival curves of each hepatectomy are shown in Figure 3A and B. Twenty cases were followed for each hepatectomy by suture (Figure 3A) or clip technique (Figure 3B).

In the 90% hepatectomy, either the OS or RMS was



**Figure 4** Histopathological findings of hepatic remnant near proximal hepatic margin and distal hepatic margins. Findings at 6 h after 80% hepatectomy are shown by hematoxylin and eosin staining at 100 × magnification. A: No findings suggested obstructions in hepatic remnant near proximal hepatic margin of hepatectomy by suture technique; B: In distal hepatic margin of hepatectomy by suture technique, severe ischemia and massive necrosis are confirmed; C: No findings suggested obstructions by hemostatic clip itself in clip-touched hepatic remnant near proximal hepatic margin of hepatectomy by clip technique; D: In distal hepatic margin of hepatectomy by clip technique, severe ischemia and massive necrosis are confirmed.

left as the remnant. Although not shown, we observed no significant differences in survival curves between the OS-remnant and the RMS-remnant following the 90% hepatectomy using suture and clip techniques ( $P = 0.3784$  and  $0.3588$ , respectively).

In the 90% hepatectomy, although not shown, there were no significant differences in survival curves after hepatectomy by suture technique between the OS-remnant ( $n = 10$ ) and the RMS-remnant ( $n = 10$ ) ( $P = 0.3784$ ). Similarly, there were no significant differences in survival curves after hepatectomy by clip technique between the OS-remnant ( $n = 10$ ) and the RMS-remnant ( $n = 10$ ) ( $P = 0.3588$ ).

#### **Histopathological findings of hepatic remnant and distal hepatic margin**

Histopathological findings of the hepatic remnant near the proximal hepatic margin at 6 h after 80% hepatectomy by suture technique are shown in Figure 4A. No findings suggested obstructions by the suture, except for the changes consistent with hepatectomy. Histopathological findings of the distal hepatic margin showed severe ischemic and massive necroses at 6 h after 80% hepatectomy by the suture technique (Figure 4B).

Histopathological findings of the clip-touched hepatic remnant near the proximal hepatic margin at 6 h after 80% hepatectomy by the clip technique are shown in Figure 4C. Again, no findings suggested obstructions

by the hemostatic clip. Histopathological findings of the distal hepatic margin at 6 h after 80% hepatectomy by clip technique are shown in Figure 4D, showing severe ischemic and massive necroses as expected.

#### **Focal and/or patchy necrosis after hepatectomy**

Focal and patchy necrosis is usually observed as early as one hour after hepatectomy, as reported by others<sup>[20,21]</sup>. In our studies, we observed similar occurrence of focal necrosis following all types of massive hepatectomy. At 6 h after 70%, 80%, and 90% hepatectomies, there was no difference in the rate of appearance of focal and/or patchy necrosis between the suture technique and the clip technique ( $P = 0.6202$ ; data not shown).

## **DISCUSSION**

A number of different survival curves have been previously reported following hepatectomy with > 70% resection in the mouse. Differences in study design and institutional methodology, as well as various complications, may explain these discrepancies<sup>[4]</sup>. As such, we recommend that survival curves should be checked beforehand according to institutional methodology and study design in each laboratory. In our institution we have two types of 90% hepatectomy models available, while other models of approximately 90% hepatectomy exist<sup>[1,7]</sup>. Anatomical analysis of each institution's animal strain is crucial for de-



velopment of reliable models. All mice that receive 90% hepatectomy eventually die after surgery, although 90% hepatectomy has some advantages, especially in studies of liver failure and hepatic surgery. Inderbitzin *et al*<sup>[8]</sup> reported that the OS exhibits different behaviors according to the degree of liver-volume, and will not work in small-volume hepatectomy. Currently, our massive hepatectomy models employed the OS in only the 80% hepatectomy model, because we have a surgical option in the 90% hepatectomy model. In our experience, the OS of some mice exhibit few branches from veins, except for the portal vein, because swelling due to portal hypertension was often mild even after 90% hepatectomy, and color change was often not enhanced after ligation of the portal venous trunk. Overall, we consider that RMS-remnant 90% hepatectomy will provide more relevant data from the viewpoint of strict effects (i.e., liver damage) due to portal hypertension.

The outcomes of murine hepatectomy can vary greatly if surgical procedures are not performed properly. It is difficult to strictly estimate the stability of surgery, because the learning term may be still not enough, even if statistical analysis showed no differences. Based on our experience, we speculate that approximately 40 cases are required in hepatectomy by the suture technique for initial achievement of reliable survival curves, and that approximately 50 or more cases are required to start the study. By contrast, approximately 30 or 40 cases seem to be enough in hepatectomy by the clip technique for reliable samples. Our results of operative time for hepatectomy clearly indicated that the procedures in the suture technique were more complicated than those in the clip method. Overall, we suggest that the clip technique is advantageous even in hepatectomy models with > 70% resection because of its simplicity.

The hepatocyte is the first cell to start the proliferation reaction after hepatectomy<sup>[4]</sup>. Although histopathological injury can be confirmed from several hours after hepatectomy, some important signals for liver regeneration start in the later phase. As such, a suitable hepatectomy model must be selected according to experimental purpose. Jin *et al*<sup>[22]</sup> reported no focal and/or patchy necrosis even after hepatectomy models with 70% and approximately 90% resections, although this necrosis is an important finding after hepatectomy<sup>[4,20]</sup>. These histopathological findings are confirmed several hours after hepatectomy<sup>[4,20]</sup>. However, in our study a few mice that received 80% or 75% hepatectomy did not demonstrate necrosis, while all mice that received 90% hepatectomy showed necrosis in the early postoperative period. These discrepancies may be consistent with survival curves because a few mice that received 80% or 75% hepatectomy survived long term. These survivors can be used to provide liver samples with long-term observation after hepatectomy with > 70% resection and are useful for many studies that require long recovery times after hepatectomy with > 70% resection. Based on our results of survival curves and histopathological findings in comparison with suture technique, clip method seems to provide reliable

and relevant data, even in murine hepatectomy models with > 70% resection.

Focal and patchy necroses are also observed as secondary findings after biliary stasis and/or congestion by outflow obstruction. Some surgical problems, including inflow and outflow obstructions, massive bleeding, and bile stasis, will invalidate results. All mice move well after complete recovery, and it is possible that the clip may slide following aggressive movements after recovery from anesthesia and surgery, although our results showed similar phenomena between the clip and suture techniques. During surgery, the hepatic margin may be changed according to clip size. Large-sized clips require more proximal hepatic margin for flow safety and more distal hepatic margin for complete hemostasis. Although we often treat the RAS and LAS in an *en bloc* manner using an ML-sized clip, only one of the smallest feasible clips to one segment should be used to prevent unexpected surgical issues. Surgeons should also note any unexpected findings after hepatectomy procedures. A dilated infra-hepatic inferior vena cava is a sign of disturbed flow of the inferior vena cava, because clipping of the RAS, LAS and LPS may cause a twist of the supra-hepatic inferior vena cava, while clipping of the RMS and RPS may cause stenosis of the hepatic vena cava. Segmental congestion and partial color change in the liver also suggest outflow and/or inflow obstruction. During autopsy, unexpected color changes of the liver, massive coagula, and bile leakage are informative for unstable postoperative course. To produce reliable samples, mice that indicate unexpected signs during surgery or autopsy should not be used to take samples.

In conclusion, our clip technique for murine hepatectomy models with > 70% resection (i.e., 75%, 80%, and 90% hepatectomy models) is simple and requires only basic surgical skills. Moreover, this technique provides reproducible results in comparison with the suture technique.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

New insights into mechanisms in the hepatology field have been established from experimental animal models. Reliable models for massive hepatectomy in the mouse are required for experimental liver research.

### Research frontiers

Despite the widespread use of the 70% hepatectomy, alternative hepatectomy models with resected volumes > 70% (so-called, "massive hepatectomy") are required to provide more clinically relevant experiments on liver regeneration and hepatic failure.

### Innovations and breakthroughs

In 2004, Nikfarjam *et al* reported benefits of the hemostatic clip in hepatectomy models with 37% and 70% of resected volumes in the mouse. Herein, the



authors describe detailed surgical procedures of our institutional hepatectomy models with > 70% resection using suture hemostatic clips in the mouse and compare various factors between suture and clip techniques. The clip technique for murine hepatectomy models with > 70% resection is simple and requires only basic surgical skills.

### Applications

The clip technique provides reproducible results in comparison with suture technique, even for massive hepatectomy in the mouse. This technique has an advantage in the simplicity of surgical procedures.

### Peer review

The authors presented mouse experimental models for hepatectomy exceeding 70%. They evaluated two techniques of hepatectomy, which is suture technique and clip technique. They concluded that clip technique was superior to suture technique. The manuscript was well organized and well written.

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## Regional lymphadenectomy for gallbladder cancer: Rational extent, technical details, and patient outcomes

Yoshio Shirai, Toshifumi Wakai, Jun Sakata, Katsuyoshi Hatakeyama

Yoshio Shirai, Toshifumi Wakai, Jun Sakata, Katsuyoshi Hatakeyama, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata City 951-8510, Japan

**Author contributions:** Shirai Y conceived the study and drafted the manuscript; Wakai T performed chart review and follow-up of the study cohort; Sakata J helped to draft the manuscript and performed statistical analyses; Hatakeyama K was responsible for the whole study and participated in its coordination; all authors read and approved the final manuscript.

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Correspondence to: Yoshio Shirai, MD, PhD, Division of Digestive and General Surgery, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Chuoku, Niigata City 951-8510, Japan. [shiray@med.niigata-u.ac.jp](mailto:shiray@med.niigata-u.ac.jp)  
 Telephone: +81-25-2272228 Fax: +81-25-2270779

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### Abstract

**AIM:** To define the rational extent of regional lymphadenectomy for gallbladder cancer and to clarify its effect on long-term survival.

**METHODS:** A total of 152 patients with gallbladder cancer who underwent a minimum of "extended" portal lymph node dissection (defined as *en bloc* removal of the first- and second-echelon nodes) from 1982 to 2010 were retrospectively analyzed. Based on previous studies, regional lymph nodes of the gallbladder were divided into first-echelon nodes (cystic duct or pericholedochal nodes), second-echelon nodes (node groups posterosuperior to the head of the pancreas or around the hepatic vessels), and more distant nodes.

**RESULTS:** Among the 152 patients (total of 3352 lymph nodes retrieved, median of 19 per patient), 79 patients (52%) had 356 positive nodes. Among node-

positive patients, the prevalence of nodal metastasis was highest in the pericholedochal (54%) and cystic duct (38%) nodes, followed by the second-echelon node groups (29% to 19%), while more distant node groups were only rarely (5% or less) involved. Disease-specific survival after R0 resection differed according to the nodal status ( $P < 0.001$ ): most node-negative patients achieved long-term survival (median, not reached; 5-year survival, 80%), whereas among node-positive patients, 22 survived for more than 5 years (median, 37 mo; 5-year survival, 43%).

**CONCLUSION:** The rational extent of lymphadenectomy for gallbladder cancer should include the first- and second-echelon nodes. A considerable proportion of node-positive patients benefit from such aggressive lymphadenectomy.

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**Key words:** Gallbladder neoplasms; Lymphatic metastasis; Lymph node excision; Prognosis; Radical surgery

**Peer reviewers:** Kazuaki Takabe, MD, PhD, Surgical Oncology, Virginia Commonwealth University, West Hospital 7-402, 1200 East Broad Street, Richmond, VA 23298-0011, United States; Toru Ishikawa, MD, PhD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, 280-7 Teraji, Niigata City 950-1104, Japan

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### INTRODUCTION

Oncological resection for gastrointestinal cancers con-

ventionally involves *en bloc* resection of the primary tumor and the regional lymphatic basin. However, in cases of gallbladder cancer, most surgeons have developed a negative attitude toward regional lymphadenectomy because of reported poor outcomes after radical resection for node-positive disease<sup>[1-3]</sup>. In contrast, some surgeons including us advocate aggressive lymphadenectomy<sup>[6-10]</sup>. Thus, the role of regional lymphadenectomy in treating gallbladder cancer remains controversial.

The National Comprehensive Cancer Network (NCCN) guidelines<sup>[11]</sup> recommend portal lymph node dissection for pathologic T1b (pT1b) or more advanced gallbladder cancer, where the extent of lymphadenectomy is described as “porta hepatis, gastrohepatic ligament, retroduodenal.” The guidelines also state, “Patients with nodal disease outside this area are unable to undergo resection”<sup>[11]</sup>. In contrast, some Japanese and Western groups advocate more extensive lymphadenectomy including some peripancreatic (head only) node groups<sup>[6,12-14]</sup>. Thus, the extent of regional lymphadenectomy for gallbladder cancer remains non-standardized worldwide.

Previously we explored the routes and directions of gallbladder lymphatic drainage in a dye injection study<sup>[6]</sup>; lymph flow originating from the gallbladder descends around the bile duct, and *via* the first-echelon nodes (cystic duct or pericholedochal nodes) into the second-echelon nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries, before finally flowing into the paraaortic nodes<sup>[6]</sup>. Thereafter, we adopted *en bloc* removal of the first- and second-echelon nodes (designated as “extended” portal lymph node dissection) as a standard procedure for pT2 or more advanced gallbladder cancer<sup>[6,10,15]</sup>.

This retrospective study sought to define a rational extent of regional lymphadenectomy for gallbladder cancer and to clarify outcomes after such a procedure in 152 patients subjected to radical resection. The study goal was to establish the role of regional lymphadenectomy in treating gallbladder cancer.

## MATERIALS AND METHODS

### Patient population

From May 1982 through December 2010, a total of 173 consecutive Japanese patients with gallbladder cancer underwent a radical resection at the Niigata University Medical and Dental Hospital. A radical resection was defined as removing both the primary tumor and the regional lymph nodes of the gallbladder. Cancer arising in the cystic duct was included as gallbladder cancer<sup>[16]</sup>. We excluded 13 patients due to an invasive primary malignancy in other organs and 8 patients who did not undergo removal of the second-echelon nodes, leaving 152 patients in this study. They included 96 women and 56 men ranging in age from 37 years to 86 years (median, 68 years).

### Radical resection procedures

A variety of radical resection procedures were performed

**Table 1** Radical resection procedures for 152 patients with gallbladder cancer

Procedure	No. of patients
Extended cholecystectomy (n = 93)	
C + WR + BD + N <sup>1</sup>	54
C + WR + N	21
C <sup>2</sup> + BD + N	12
C <sup>2</sup> + N	6
More extensive resection (n = 59)	
C + ERH + BD + N	27
C + Central hepatectomy <sup>3</sup> + BD + N	3
C + Extended left hepatectomy <sup>4</sup> + BD + N	1
C + Right trisectionectomy + BD + N	1
C + WR + PD + N	15
C + ERH + PD + N	7
C + ERH + PPPD + N	3
C <sup>2</sup> + PD + N	2

<sup>1</sup>Designated as “extended” radical cholecystectomy (modified Glenn operation) at our department since 1982; <sup>2</sup>Cholecystectomy with full-thickness dissection: cholecystectomy combined with removal of the cystic plate; <sup>3</sup>Resection of Couinaud segments IV, V, and VIII; <sup>4</sup>Left hemihepatectomy extended to an inferior portion of the right anterior section. C: Cholecystectomy; WR: Wedge resection of the gallbladder fossa; BD: Bile duct resection; N: Regional lymphadenectomy; ERH: Extended right hepatectomy (right hemihepatectomy extended to an inferior portion of Couinaud segment 4); PD: Whipple pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

for gallbladder cancer in the patients analyzed; the choice of procedure for each patient depended on the extent of tumor spread (Table 1). An “extended” radical cholecystectomy, which was instituted at our department in 1982<sup>[6,10,15]</sup>, was the most common operation used among our study cohort; it involved a cholecystectomy, wedge resection of the gallbladder fossa with a rim of non-neoplastic liver tissue (about 2 cm in thickness or more), resection of a suprapancreatic segment of the extrahepatic bile duct (bile duct resection), and “extended” portal lymph node dissection in an *en bloc* fashion. This “extended” radical cholecystectomy is a modification of the “radical cholecystectomy” (Glenn operation) as proposed by Glenn *et al*<sup>[17,18]</sup> in 1954; we now also call it the “modified Glenn operation”<sup>[15]</sup>.

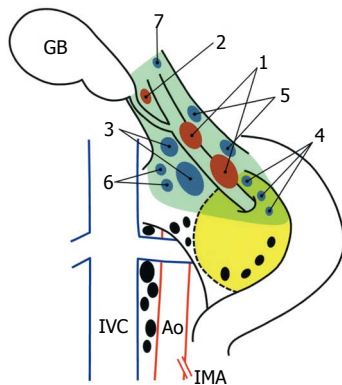
Some patients with early-stage disease, comorbid disease(s), or advanced age had a less aggressive resection, by omitting the bile duct resection and/or wedge hepatectomy (Table 1). Although pT1 tumors do not warrant radical resection<sup>[19]</sup>, 14 patients with pT1 tumors underwent a radical resection because pT2 or more advanced tumor was not ruled out preoperatively. In contrast, patients with late-stage disease often required more extensive resections such as major hepatectomy (removal of two sections or more extended hepatectomy), pancreaticoduodenectomy (Whipple procedure or pylorus-preserving procedure), or combined major hepatectomy and pancreaticoduodenectomy (Table 1)<sup>[12,20]</sup>.

Despite an aggressive attitude toward lymphadenectomy, we maintain a rather modest attitude toward hepatectomy for gallbladder cancer, and consequently, the most common hepatectomy procedure in our series was

Table 2 Topographical distribution of 3352 lymph nodes evaluated in 152 patients with gallbladder cancer

Node group	No. of patients with node group evaluated	No. of lymph nodes evaluated		No. of patients with positive nodes	No. of positive nodes	
		Range per patient (median)	Total		Range per patient (median)	Total
First-echelon node groups						
Pericholedochal <sup>1</sup>	152	0-9 (2)	410	43	1-9 (1)	75
Cystic duct	152	0-2 (1)	109	30	1-2 (1)	33
Second-echelon node groups						
Retroportal	152	0-9 (3)	458	23	1-5 (2)	47
Posterior superior pancreaticoduodenal <sup>2</sup>	150	0-9 (2)	341	20	1-7 (1)	37
Hepatic artery	148	0-12 (3)	536	20	1-10 (1)	50
Right celiac <sup>3</sup>	128	0-8 (2)	320	15	1-4 (1)	28
Hilar (porta hepatis)	121	0-5 (0)	37	0	NA	0
More distant node groups						
Superior mesenteric	50	0-14 (2)	171	4	1-5 (2)	10
Posterior inferior pancreaticoduodenal	38	0-7 (1)	56	3	1-5 (2)	8
Anterior superior pancreaticoduodenal	29	0-5 (0)	19	1	1-1 (1)	1
Anterior inferior pancreaticoduodenal	27	0-4 (0)	15	2	1-3 (2)	4
Perigastric	52	0-23 (2)	205	4	1-1 (1)	4
Para-aortic node groups	93 <sup>4</sup>	1-28 (6)	675	15	1-16 (2)	59

<sup>1</sup>Also referred to as retroduodenal nodes in the NCCN guidelines<sup>[11]</sup>; <sup>2</sup>Also referred to as posterosuperior pancreaticoduodenal nodes in our previous study<sup>[6]</sup> or superior retropancreaticoduodenal nodes by Uesaka *et al*<sup>[22]</sup>; <sup>3</sup>Also referred to as posterior common hepatic nodes by Uesaka *et al*<sup>[22]</sup>; <sup>4</sup>Includes 55 who underwent a para-aortic lymph node dissection and 38 who underwent a sampling of para-aortic nodes for staging of the disease.



**Figure 1** Topographical distribution of the regional lymph nodes of the gallbladder, shown after a full Kocher maneuver. Solid ellipses represent individual lymph nodes: first-echelon nodes (red), second-echelon nodes (blue), and more distant nodes (black). The yellow painted area represents the head of the pancreas, and the dashed line indicates the border of the uncinate process of the pancreas. Arabic numerals indicate each of the first- and second-echelon node groups: (1) pericholedochal, nodes along the common bile duct; (2) cystic duct, node(s) along the cystic duct; (3) retroportal, nodes posterior to the portal vein and cephalad to the uncinate process; (4) posterior superior pancreaticoduodenal, nodes on the posterosuperior aspect of the head of the pancreas; (5) hepatic artery, nodes along the common or proper hepatic artery; (6) right celiac, nodes located right of the celiac axis and posterior to the common hepatic artery; and (7) hilar, nodes within the porta hepatis. The pale green area indicates the extent of a typical "extended" portal lymph node dissection (*en bloc* removal of the first- and second-echelon node groups) in the study department. GB: Gallbladder; IVC: Inferior vena cava; Ao: Aorta; IMA: Inferior mesenteric artery.

(nonanatomic) wedge resection of the gallbladder fossa. However, cases of deep hepatic invasion through the gallbladder fossa and/or invasion of the right portal pedicle (i.e., extension into the triangle of Calot) required a right hemihepatectomy extended to an inferior part of Couin-

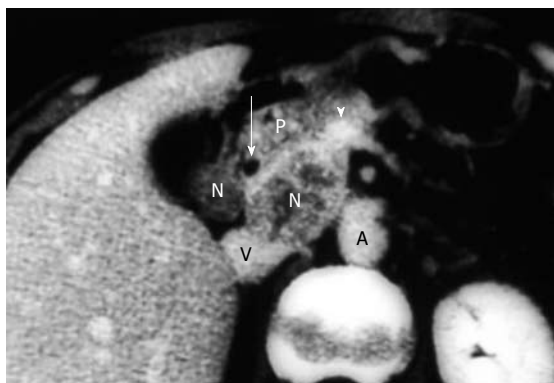
aud segment 4 (designated as extended right hepatectomy in this study) for tumor clearance. Five patients underwent one hepatectomy procedures (Table 1).

There were also 25 patients in this study who underwent a combined resection of contiguous tissues comprising the transverse colon ( $n = 12$ ), portal vein ( $n = 9$ ), duodenum ( $n = 5$ ), stomach ( $n = 2$ ), and inferior vena cava ( $n = 2$ ). Of the 152 patients, 127 underwent an initial radical resection and 25 underwent a radical second resection after a prior simple cholecystectomy for presumed benign disease.

### Regional lymph nodes of the gallbladder

According to earlier studies<sup>[6,8,15,21,22]</sup>, we classified the regional lymph nodes of the gallbladder into three categories as detailed in Table 2: first-echelon nodes, second-echelon nodes, and more distant nodes. The topographical distribution of the first- and second-echelon node groups is illustrated in Figure 1. Briefly, the first-echelon nodes are located along the cystic duct or the common bile duct, and the second-echelon nodes are located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries. Although the hilar (porta hepatis) nodes were not regarded as significant regional nodes<sup>[6,21,22]</sup>, we categorized them as second-echelon nodes for convenience (Table 2) because these nodes are located within the hepatoduodenal ligament, and thus are usually harvested during regional lymphadenectomy<sup>[11]</sup>. Perigastric nodes are not regarded as regional, but they were analyzed in this study since they were included in the Whipple procedure collection. Para-aortic lymph nodes were considered the terminal station for the regional lymphatic system of the gallbladder<sup>[6,21,22]</sup>.





**Figure 2** Computed tomography revealing enlarged peripancreatic nodes with heterogeneous contrast enhancement (N). Note that the posterior superior pancreaticoduodenal node (N, left) and the retroportal node (N, right) are adhered to the head of the pancreas (P). The latter node shifts the portal vein (arrowhead) in a right-upper direction. Histological examination confirmed metastases in 10 regional nodes, some of which had invaded the pancreatic parenchyma. This patient remains well with no evidence of disease at 15 years after a pancreaticoduodenectomy combined with wedge resection of the gallbladder fossa. The arrow indicates common bile duct; V: Inferior vena cava; A: Aorta.

### Regional lymphadenectomy procedures for gallbladder cancer

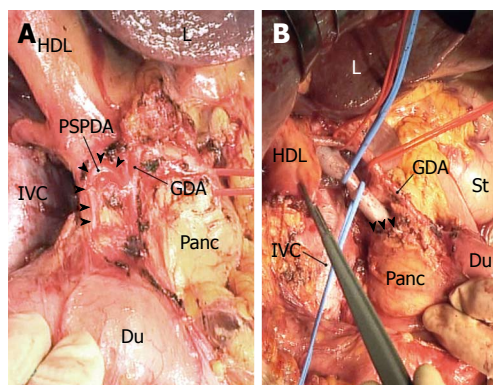
In the current study, regional lymphadenectomy for gallbladder cancer was divided into two categories according to the extent of nodal dissection: “extended” portal lymph node dissection and “peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy”<sup>[15]</sup>. The former was defined as *en bloc* removal of the first- and second-echelon node groups (without pancreaticoduodenectomy), while the latter was defined as *en bloc* removal of more distant node groups with pancreaticoduodenectomy in addition to the first- and second-echelon node groups. The extent of an “extended” portal lymphadenectomy is illustrated in Figure 1.

Of all the study patients, 125 underwent an extended portal lymph node dissection (without pancreaticoduodenectomy), while the remaining 27 patients underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy (Table 1). In addition, 55 of the total number of patients who showed suspected (or confirmed) regional nodal disease also underwent a para-aortic (mainly interaortocaval) lymph node dissection, provided that they were fit enough.

It should be noted that despite our consistent policy of aggressive lymphadenectomy, the degree of regional lymphadenectomy varied somewhat in individual patients at the discretion of the individual surgeons, primarily due to the current study spanning a long period of time. For example, elderly patients or those with comorbid diseases tended to undergo a less aggressive lymphadenectomy.

### Details of “extended” portal lymph node dissection

The following describes the technical details of our “extended” portal lymph node dissection (combined with both wedge hepatectomy of the gallbladder fossa and bile



**Figure 3** Photographs taken during an “extended” portal lymph node dissection. A: The posterior superior pancreaticoduodenal artery (PSPDA; arrowheads) is shown following dissection of the posterior superior pancreaticoduodenal nodes. The common hepatic artery is secured with the red loop; B: The superior border of the uncinate process (arrowheads) is exposed, ensuring that dissection of the retroportal nodes is complete. The common bile duct was already transected at the level of PSPDA. The blue loops secure the portal vein, whereas the red loops secure the hepatic arteries. A forceps is picking up the node-bearing adipose tissues dissected *en bloc*. L: Liver; HDL: Hepatoduodenal ligament; GDA: Gastroduodenal artery; IVC: Inferior vena cava; Panc: Head of the pancreas; Du: Duodenum; St: Stomach.

duct resection) for gallbladder cancer. After laparotomy, scrutinizing distant metastases by meticulous inspection and palpation is mandatory. Any suspicious nodules in the liver or on the peritoneal surface should be excised and submitted to frozen section examination, while any plan of radical resection should be abandoned if distant metastases are confirmed histologically. If no distant metastases are found, a full Kocher maneuver should be conducted (Figure 1) to enable the peripancreatic nodal status to be appropriately assessed. We favor extended portal lymphadenectomy in the absence of clinically evident peripancreatic (head only) nodal disease, while pancreaticoduodenectomy is often required for peripancreatic (head only) nodal disease adherent to or invading the pancreatic parenchyma (Figure 2)<sup>[15]</sup>.

“Extended” portal lymph node dissection usually starts by dissecting the posterior superior pancreaticoduodenal nodes, where a layer of node-bearing adipose tissue is carefully peeled from the posterosuperior surface of the head of the pancreas. Several superior duodenal vessels are then divided to expose the superior aspect of the head of the pancreas. After the lesser omentum is incised, the common hepatic artery is secured with tape and skeletonized with dissecting nodes around the artery; the hepatic-gastroduodenal artery junction is also exposed. The posterior superior pancreaticoduodenal artery (PSPDA), usually the first branch of the gastroduodenal artery, which traverses the common bile duct, can now be identified (Figure 3A). This ensures that dissection of the posterior superior pancreaticoduodenal nodes is complete. We often sacrifice PSPDA, because its tiny branches often cause unexpected bleeding due to injury during the nodal dissection. Transection of the common bile duct, just above or at the level of PSPDA, ensues.

The peritoneum covering the hepatoduodenal ligament is incised longitudinally along the proper hepatic artery, which is then skeletonized. The right hepatic artery is also skeletonized by dividing its ductal branch. The portal vein is then secured with tape and skeletonized by dissecting the retroportal nodes, which are then dissected from the superior border of the uncinate process. Next, the right celiac nodes are dissected from the right of the celiac artery. At this stage, the whole node-bearing tissues dissected *en bloc* are freed from the hepatic vessels and the head of the pancreas (Figure 3B); exposure of the superior border of the uncinate process ensures that dissection of the retroportal nodes has been completed (Figure 3B). A wedge hepatectomy of the gallbladder fossa ensues. Following parenchymal division, the cystic plate is exposed as a dense fibrous plate connecting with the portal pedicle of the right hemiliver, and then is divided at its base. The adipose tissue within the triangle of Calot, underneath the cystic plate, contains a cystic node(s), and is dissected downward by dividing the cystic artery at its origin. The extended portal lymph node dissection is now complete. Finally, the common hepatic duct is transected just below the confluence of the right and left hepatic ducts, and the surgical specimen including the gallbladder, gallbladder fossa, bile duct, and node-bearing tissues is harvested *en bloc*.

### Retrieval of lymph nodes from fresh surgical specimens

Immediately after resection, the surgeon(s) retrieved lymph nodes from the node-bearing adipose tissues of the fresh surgical specimen, which were then divided by the surgeon(s) into individual node groups according to their locations.

### Pathological examination

Pathological findings were documented using the American Joint Committee on Cancer (AJCC) cancer staging manual (7th edition)<sup>[16]</sup>. The primary tumor was pT1 in 14 patients, pT2 in 60, pT3 in 54, and pT4 in 24. Resection margin status was judged as no residual tumor (R0) or microscopic/macrosopic residual tumor (R1-2). The lymph nodes retrieved were examined histologically for metastases using a representative 3- $\mu$ m thick section cut from each node.

The number of positive lymph nodes as well as the total lymph node count (TLNC) was recorded for each patient. Here, TLNC represented the sum of regional and paraaortic (if any) nodes evaluated in the patient; the number of positive lymph nodes represented the sum of positive regional and paraaortic (if any) nodes in the patient.

### Patient follow-up after resection

Of 152 patients, 6 died during their hospital stay for the definitive resection, giving an in-hospital mortality rate of 4%. Patients discharged to home were followed regularly in outpatient clinics every 1-6 mo for at least five years, with a median follow-up time of 142 mo (range, 0.5 mo

to 351 mo). Adjuvant chemotherapy was administered to 53 patients at the discretion of the individual surgeons.

### Statistical analysis

Medical records and survival data were obtained for all 152 patients. Continuous variables were compared with the Mann-Whitney *U* test. Only deaths from tumor recurrence were treated as failure cases in the analysis of disease-specific survival (DSS), whereas those from other causes were recorded as censored cases. The survival time in each patient was defined as the interval between the date of definitive resection and the date of last follow-up or death. Survival curves were constructed using the Kaplan-Meier method, and differences in survival were evaluated with the log rank test. The IBM SPSS Statistics 19 software (IBM Japan, Inc., Tokyo, Japan) was used for all statistical evaluations. All tests were two-tailed and  $P < 0.05$  was used to indicate statistical significance.

## RESULTS

### Topographical distribution of lymph nodes harvested by regional lymphadenectomy

A total of 3352 lymph nodes taken from the 152 study patients were evaluated. TLNC ranged from 3 to 78 (median, 19) per patient and varied according to the type of regional lymphadenectomy: 27 patients who underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy had a greater TLNC (median, 36; range, 7 to 78) compared with 125 patients who underwent an extended portal lymph node dissection (median, 17; range, 3 to 53) ( $P < 0.001$ ).

The topographical distribution of the analyzed lymph nodes included 519 first-echelon nodes and 1692 second-echelon nodes (Table 2). There were significantly more second-echelon nodes per patient (median, 10; range, 2 to 29) than first-echelon nodes (median, 3; range, 0 to 10) ( $P < 0.001$ ).

### Topographical distribution of positive lymph nodes

Of the 152 study patients, 79 (52%) had a total of 356 positive lymph nodes. The number of positive nodes per patient ranged from 1 to 41 (median, 2). None of the 14 patients with pT1 tumor had nodal disease, whereas 79 (57%) of 138 patients with pT2 or more advanced tumor had nodal disease.

The topographical distribution of all positive lymph nodes is shown in Table 2. Among the 79 node-positive patients, the prevalence of nodal disease was highest in the pericholedochal (43 of 79; 54%) or cystic duct (30 of 79; 38%) node group, followed by the retroportal (29%), posterior superior pancreaticoduodenal (25%), hepatic artery (25%), and right celiac (19%) node groups. The hilar nodes were not involved in any of our patients. The prevalence of nodal disease was 5% or less in more distant node groups. Of note, paraaortic nodal involvement was found in 15 (19%) patients.

Of 27 patients with a single positive node, 20 (74%)

**Table 3** Site of nodal involvement in 27 patients with a single positive node

Node group involved	No. of patients
Cystic duct	11
Pericholedochal	9
Posterior superior pancreaticoduodenal	3
Retroportal	2
Hepatic artery	1
Para-aortic	1

**Table 4** Five-year survivors with nodal disease according to type of regional lymphadenectomy

No. of positive nodes	No. of 5-year survivors		
	Extended portal LND	Peripancreatic (head only) LND with PD	Total
1	12	1	13
2-3	4	1	5
≥ 4	1	3	4

LND: Lymph node dissection; PD: Pancreaticoduodenectomy (Whipple procedure or pylorus-preserving procedure).

had nodal disease in either the pericholedochal or cystic duct node group, suggesting that initial nodal involvement occurred primarily in these node groups (Table 3).

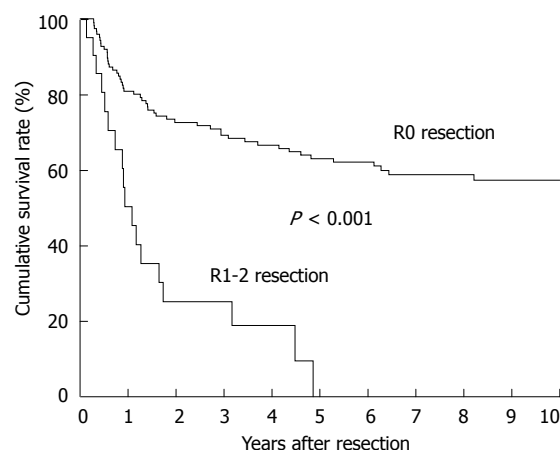
### Route of lymphatic spread

Analysis of the topographical distribution of positive lymph nodes (Tables 2 and 3) can be used to derive the route of lymphatic spread from gallbladder cancer. In our study patients, gallbladder cancer primarily spread to the first-echelon nodes, then to the second-echelon nodes (other than the hilar nodes), and finally to the para-aortic nodes, while other distant node groups were only rarely involved.

### Long-term outcome after regional lymphadenectomy

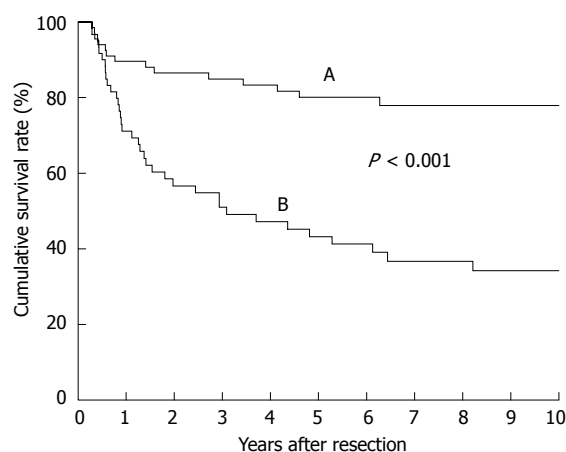
For all 152 patients, DSS was significantly worse in 22 patients undergoing an R1-2 resection than in 130 patients undergoing an R0 resection ( $P < 0.001$ ; Figure 4); all of the patients undergoing an R1-2 resection died of recurrence within 5 years of resection. This indicates that an R0 resection is a prerequisite for long-term survival after radical resection.

We then focused on a subgroup of 130 patients who had undergone an R0 resection for survival analysis; they comprised 68 node-negative and 62 node-positive patients. DSS after R0 resection differed according to the nodal status ( $P < 0.001$ ; Figure 5), with most node-negative patients achieving long-term survival (median survival time, not reached; 5-year survival rate, 80%). Of the 62 node-positive patients, 22 survived for more than 5 years after an R0 resection, 37 expired within 5 years, and the remaining 3 had survived within 5 years at the time of last follow-up (median survival time, 37 mo; 5-year survival rate, 43%) (Figure 5). These findings suggested that regional lymphadenectomy could achieve



No. of patients at risk												
R0 resection	130	100	87	81	76	69	59	46	41	39	34	
R1-2 resection	22	10	5	4	2	0	0	0	0	0	0	

**Figure 4** Kaplan-Meier disease-specific survival estimates stratified for residual tumor (R) status in all 152 patients. The median survival was not reached with a 5-year survival rate of 63% in patients undergoing an R0 resection, whereas it was 13 mo with a 5-year survival rate of 0% in patients undergoing an R1-2 resection.



No. of patients at risk												
A	68	60	56	54	52	47	41	32	27	26	23	
B	62	40	31	27	24	22	18	14	14	13	11	

**Figure 5** Kaplan-Meier disease-specific survival estimates stratified for nodal status in 130 patients who underwent an R0 resection. A, without nodal disease; B, with nodal disease.

an acceptable rate of long-term survival even in patients with nodal metastasis, provided that an R0 resection is feasible.

### Five-year survival with node-positive disease

The 22 patients with node-positive disease who survived for more than 5 years (Figure 5) comprised 13 with a single positive node, 5 with 2-3 positive nodes, and 4 with ≥ 4 positive nodes (Table 4). Of the 5-year survivors with ≥ 4 positive nodes, three underwent a peripancreatic (head only) lymph node dissection with pancreaticoduodenectomy for marked peripancreatic

nodal disease (Figure 2), suggesting that the addition of pancreaticoduodenectomy is effective for selected patients with peripancreatic nodal disease.

## DISCUSSION

The high propensity for lymphatic spread in gallbladder cancer<sup>[6,8,10,15,23]</sup> renders adequate lymphadenectomy indispensable for improving patient outcomes after resection. However, what constitutes adequate lymph node dissection for these patients remains unresolved and prompted the current study. Since the 1980s, we have routinely harvested at least the first- and second-echelon nodes (Figure 1) during curative-intent resection for pT2 or more advanced gallbladder cancer. As a result, and to the best of our knowledge, we report herein the largest single-institutional series of 5-year survivors (22 patients) with nodal disease ever analyzed. The study findings also indicated that a considerable proportion of node-positive patients could benefit from regional lymphadenectomy, providing that an R0 resection is feasible, and would seem to justify continuing our policy of aggressive lymphadenectomy for gallbladder cancer.

In this study cohort, initial nodal involvement occurred primarily in the cystic duct or pericholedochal nodes (Table 3). Although these node groups are widely accepted as first-echelon nodes of the gallbladder, further possible routes of lymphatic spread have been poorly defined<sup>[6,21-23]</sup>. In 1991, an autopsy study by Ito and colleagues<sup>[21]</sup> implicated the cholecystoretropancreatic pathway, which descends along the common bile duct into the retroportal nodes, as the main lymphatic pathway of the gallbladder. Soon after, we identified the same pathway in a dye injection study and showed the second-echelon nodes located posterosuperior to the head of the pancreas or around the hepatic vessels; the dye solution finally drained into the interaortocaval nodes near the left renal vein<sup>[6]</sup>. In 1996, Uesaka *et al*<sup>[22]</sup> reported similar findings using vital staining. These studies therefore uniformly confirmed that lymph from the gallbladder first flows in a hepatofugal direction around the common bile duct and into the first-echelon nodes, before reaching the second-echelon nodes (other than the hilar nodes), and finally the paraaortic nodes<sup>[6,21,22]</sup>. In addition, the prevalence of nodal disease in our series was high in both the first-echelon and second-echelon node groups (other than the hilar nodes), while the other node groups were only rarely involved (Table 2). Thus, the rational extent of regional lymphadenectomy for gallbladder cancer should include at least the first- and second-echelon node groups as defined in the current study (Figure 1).

Despite a number of 5-year survivors with nodal disease reported in the Japanese literature<sup>[8,10,12,13]</sup>, such survivors are exceptionally rare in the Western literature<sup>[1,2,5,24]</sup>. Since the first proposal by Glenn *et al*<sup>[17]</sup> in 1954, portal lymph node dissection has been regarded as the standard lymphadenectomy procedure for localized gallbladder cancer throughout the world<sup>[15]</sup>. However, the scope of

portal lymph node dissection differs considerably among institutions. In 2005, Dixon *et al*<sup>[24]</sup> from the University of Toronto described “a complete portal lymph node dissection, with thorough skeletonization of the portal structures, down to and including the suprapyloric lymph node overlying the hepatic-gastroduodenal artery junction,” while Ito and colleagues from the Memorial Sloan-Kettering Cancer Center (MSKCC) in 2011<sup>[5]</sup> reported a portal lymphadenectomy including “the lymph nodes in the hepatoduodenal ligament and those along with the common hepatic artery.” The NCCN guidelines<sup>[11]</sup> described the extent of portal lymphadenectomy as “porta hepatis, gastrohepatic ligament, retroduodenal.” From the above descriptions, we thus assume that the portal lymphadenectomy performed in North America may leave behind some of the second-echelon nodes, particularly the retroportal, posterior superior pancreaticoduodenal, and right celiac node groups, which were frequently involved in the current series (Table 2). This may explain, in part, why portal lymph node dissection only rarely achieves 5-year survival in cases of node-positive gallbladder cancer<sup>[1,2,5,24]</sup>.

Although the AJCC staging manual (6th edition)<sup>[25]</sup> recommended “analysis of a minimum of three lymph nodes” for accurate staging of gallbladder cancer, a recent population-based study by Coburn *et al*<sup>[26]</sup> disclosed that among patients in the United States with resectable T1-T3 disease, only 5.3% had retrieval of three or more lymph nodes. In addition, a recent report from MSKCC of 122 patients who underwent a portal lymph node dissection cited a median TLNC of only 3<sup>[5]</sup>. In contrast, TLNC was much greater (median, 19) in the current series. Insufficient lymph node retrieval with portal lymphadenectomy may also explain, in part, the poor survival of node-positive patients in the Western literature<sup>[5,26]</sup>.

Thorough dissection of the second-echelon nodes is challenging even in the hands of expert hepatobiliary surgeons. In particular, complete removal of the posterior superior pancreaticoduodenal, retroportal, and right celiac node groups mandates a meticulous technique. As described in the Materials and Methods section, there are some measures that can be taken to facilitate adequate dissection of the second-echelon nodes: first, a full Kocher maneuver (Figure 1) is essential for assessing the peripancreatic nodal status; second, identification of PSPDA (Figure 3A) ensures that dissection of the posterior superior pancreaticoduodenal nodes has been completed; and third, exposure of the superior border of the uncinate process (Figure 3B) ensures complete dissection of the retroportal nodes.

In our institution, we prefer to perform bile duct resection in fit patients with pT2 or more advanced gallbladder cancer; indeed, 66 (71%) of the 93 patients who underwent an extended cholecystectomy had a bile duct resection (Table 1). Although the presence of ductal involvement is an absolute indication for such an approach, controversy exists regarding bile duct resection for tumors without clinically evident ductal involvement<sup>[27]</sup>. Our rationale for bile duct resection for tumors with no evident ductal in-



involvement is to facilitate regional lymphadenectomy, to remove the pericholedochal lymphatic vessels and nodes simultaneously, and to remove the possible presence of microscopic ductal (periductal) involvement as suggested by Shimizu *et al*<sup>[28]</sup>. Another justification is to avoid the occurrence of ischemic biliary stricture after aggressive periductal nodal dissection<sup>[29]</sup>. The suprapancreatic segment of the extrahepatic bile duct gets its arterial blood supply mainly from ductal branches of both PSPDA ("retroduodenal artery" by Northover *et al*<sup>[30]</sup>) and the right hepatic artery<sup>[30]</sup>. As described in the Materials and Methods section, during our extended portal lymph node dissection, the PSPDA (Figure 3A) was often sacrificed with division of the ductal branch of the right hepatic artery. In addition, skeletonization of the bile duct may inadvertently injure the periductal arterial plexus with "the 3 o'clock and 9 o'clock arteries"<sup>[30]</sup>. Thus, we believe that simultaneous bile duct resection is a safer option if such aggressive periductal nodal dissection is required<sup>[29]</sup>.

Indications for pancreaticoduodenectomy for gallbladder cancer include direct invasion of the pancreaticoduodenal region and evident peripancreatic (head only) nodal disease (Figure 2)<sup>[10,12,15]</sup>. While the former indication is widely accepted, it seems that most Western surgeons hesitate to undertake this procedure for the purpose of lymph node dissection because they believe that peripancreatic nodal disease is beyond the scope of resection<sup>[1,11,16,27]</sup>. Shirai *et al*<sup>[12]</sup> in 1997 and Sasaki *et al*<sup>[13]</sup> in 2006 independently reported the effectiveness of pancreaticoduodenectomy for selected cases of peripancreatic nodal disease. This was also suggested by the current study. In 2002, Doty *et al*<sup>[14]</sup> also suggested that the addition of pancreaticoduodenectomy could result in an R0 resection by removing extensive peripancreatic nodal disease in a select group of patients. Western surgeons should therefore be more open than ever to performing pancreaticoduodenectomy for gallbladder cancer.

The main limitations of the current study revolved around the retrospective nature of the analysis and considerable variability in the degree of nodal dissection among individual patients. However, the unique nature of this study has more clearly defined the role of regional lymphadenectomy for gallbladder cancer than earlier studies, although the survival of node-positive patients remains unsatisfactory (Figure 5). Since 2009, we have therefore routinely administered adjuvant chemotherapy (using gemcitabine and/or S-1 for 6-12 mo) to patients with nodal disease (especially those with multiple positive nodes) to improve survival. In addition, we now use "extended" portal lymphadenectomy for both gallbladder and bile duct cancers, with the latter also showing some success (unpublished data). Thus, it seems that "extended" portal lymph node dissection is applicable to a wide range of biliary tract malignancies.

In conclusion, gallbladder cancer first spreads to the first-echelon nodes (cystic duct or pericholedochal nodes), then to the second-echelon nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries, and finally to the paraaortic nodes. The

rational extent of regional lymphadenectomy for pT2 or more advanced tumors should include the first- and second-echelon node groups. Such aggressive lymphadenectomy can achieve an acceptable rate of long-term survival even in patients with nodal metastasis, provided that a potentially curative (R0) resection is feasible. The addition of pancreaticoduodenectomy may also be beneficial in selected patients with peripancreatic (head only) nodal disease. This study confirmed that regional lymphadenectomy plays a key role in radical surgery for gallbladder cancer.

## COMMENTS

### Background

The extent of regional lymphadenectomy for gallbladder cancer has not been standardized worldwide. Since 1982, the authors have consistently adopted an aggressive lymphadenectomy strategy. What constitutes adequate lymph node dissection for gallbladder cancer remains unresolved and prompted the current study.

### Research frontiers

The study aims to define the rational extent of regional lymphadenectomy for gallbladder cancer and to clarify its effect on long-term survival.

### Innovations and breakthroughs

The authors clearly define the rational extent of regional lymphadenectomy for pT2 or more advanced gallbladder cancer as at least the first- and second-echelon node groups. They also demonstrate that such aggressive lymphadenectomy can achieve an acceptable rate of long-term survival even in node-positive patients.

### Applications

The authors imply that "extended" portal lymph node dissection is applicable to a wide range of biliary tract malignancies (both gallbladder and bile duct cancers).

### Terminology

First-echelon nodes mean lymph nodes located along the cystic duct or the common bile duct; second-echelon nodes mean lymph nodes located posterosuperior to the head of the pancreas or around the portal vein/hepatic arteries; "extended" portal lymph node dissection mean *en bloc* removal of the first- and second-echelon node groups.

### Peer review

This paper provides detailed description of their surgical technique for gallbladder cancer, "extended" portal lymph node dissection, of which this group has been conducting over the years. Documentation of detailed surgical technique is important not only as a material and method of a study, but even more as a tool to compare studies, and as guidance to the next generation of surgeons. They boast the largest single-institutional number of 5-year survivors with nodal disease (22 cases), suggesting the effectiveness of such aggressive lymphadenectomy.

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## Globulin-platelet model predicts minimal fibrosis and cirrhosis in chronic hepatitis B virus infected patients

Xu-Dong Liu, Jian-Lin Wu, Jian Liang, Tao Zhang, Qing-Shou Sheng

Xu-Dong Liu, Jian Liang, Tao Zhang, Qing-Shou Sheng, Department of Liver Diseases, Ruikang Hospital of Guangxi Traditional Chinese Medicine University, Nanning 530011, Guangxi Zhuang Autonomous Region, China

Jian-Lin Wu, Department of Infectious Disease, the First Affiliated Hospital of Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China

Author contributions: Liu XD designed the research; Liu XD, Wu JL, Liang J, Zhang T and Sheng QS collected the data and Liu XD and Wu JL analyzed data and wrote the paper.

Correspondence to: Dr. Xu-Dong Liu, Department of Liver Diseases, Ruikang Hospital of Guangxi Traditional Chinese Medicine University, 10 Huadong Road, Nanning 530011, Guangxi Zhuang Autonomous Region, China. [lxdlhx@163.com](mailto:lxdlhx@163.com)

Telephone: +86-771-2188071 Fax: +86-771-2411156

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### Abstract

**AIM:** To establish a simple model consisting of the routine laboratory variables to predict both minimal fibrosis and cirrhosis in chronic hepatitis B virus (HBV)-infected patients.

**METHODS:** We retrospectively investigated 114 chronic HBV-infected patients who underwent liver biopsy in two different hospitals. Thirteen parameters were analyzed by step-wise regression analysis and correlation analysis. A new fibrosis index [globulin/platelet (GP) model] was developed, including globulin (GLOB) and platelet count (PLT). GP model = GLOB (g/mL)  $\times$  100/PLT ( $\times 10^9/L$ ). We evaluated the receiver operating characteristics analysis used to predict minimal fibrosis and compared six other available models.

**RESULTS:** Thirteen clinical biochemical and hematological variables [sex, age, PLT, alanine aminotransferase, aspartate aminotransferase (AST), albumin, GLOB, total bilirubin (T.bil), direct bilirubin (D.bil), glutamyl-

transferase, alkaline phosphatase, HBV DNA and prothrombin time (PT)] were analyzed according to three stages of liver fibrosis (F0-F1, F2-F3 and F4). Bivariate Spearman's rank correlation analysis showed that six variables, including age, PLT, T.bil, D.bil, GLOB and PT, were correlated with the three fibrosis stages (FS). Correlation coefficients were 0.23, -0.412, 0.208, 0.220, 0.314 and 0.212; and  $P$  value was 0.014,  $< 0.001$ , 0.026, 0.018, 0.001 and 0.024, respectively. Univariate analysis revealed that only PLT and GLOB were significantly different in the three FS (PLT:  $F = 11.772$ ,  $P < 0.001$ ; GLOB:  $F = 6.612$ ,  $P = 0.002$ ). Step-wise multiple regression analysis showed that PLT and GLOB were also independently correlated with FS ( $R^2 = 0.237$ ). By Spearman's rank correlation analysis, GP model was significantly correlated with the three FS ( $r = 0.466$ ,  $P < 0.001$ ). The median values in F0-F1, F2-F3 and F4 were 1.461, 1.720 and 2.634. Compared with the six available models (fibrosis index, AST-platelet ratio, FIB-4, fibrosis-cirrhosis index and age-AST model and age-PLT ratio), GP model showed a highest correlation coefficient. The sensitivity and positive predictive value at a cutoff value  $< 1.68$  for predicting minimal fibrosis F0-F1 were 72.4% and 71.2%, respectively. The specificity and negative predictive value at a cutoff value  $< 2.53$  for the prediction of cirrhosis were 84.5% and 96.7%. The area under the curve (AUC) of GP model for predicting minimal fibrosis and cirrhosis was 0.762 [95% confidence interval (CI): 0.676-0.848] and 0.781 (95% CI: 0.638-0.924). Although the differences were not statistically significant between GP model and the other models ( $P$  all  $> 0.05$ ), the AUC of GP model was the largest among the seven models.

**CONCLUSION:** By establishing a simple model using available laboratory variables, chronic HBV-infected patients with minimal fibrosis and cirrhosis can be diagnosed accurately, and the clinical application of this model may reduce the need for liver biopsy in HBV-infected patients.

**Key words:** Globulin; Platelet; Globulin/platelet model; Liver fibrosis; Noninvasive fibrosis biomarker; Chronic hepatitis B virus

**Peer reviewers:** Yasuji Arase, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2Toranomonminato-ku, Tokyo 105-8470, Japan; Ming-Lung Yu, MD, PhD, Professor, Division of Hepatology, Department of Medicine, Kaohsiung Medical University Hospital, 100 Tzyou 1st Rd, Kaohsiung 807, Taiwan, China

Liu XD, Wu JL, Liang J, Zhang T, Sheng QS. Globulin-platelet model predicts minimal fibrosis and cirrhosis in chronic hepatitis B virus infected patients. *World J Gastroenterol* 2012; 18(22): 2784-2792 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2784.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2784>

## INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a global public health problem and it is estimated that approximately 350 000 000 people are infected with this virus in the world. More often, it may progress into liver cirrhosis and hepatocellular carcinoma (HCC). Although anti-virus therapy has reduced greatly the risk of cirrhosis and HCC, some positive hepatitis B surface antigen (HBsAg) carriers with less HBV-DNA may eventually develop cirrhosis and HCC<sup>[1,2]</sup>. Liver biopsy is not common. Patients with more than a 2-fold increase in aminotransferase levels were regarded as having severe liver necroinflammation. However, liver fibrosis was difficult to evaluate by noninvasive methods.

Liver fibrosis is known as the major problem causing morbidity and mortality in chronic HBV-infected patients. Once diagnosed, fibrosis should be treated as early as possible by appropriate methods. Although liver biopsy is being used as the gold standard in diagnosing the degree of fibrosis, not many patients are willing to undergo a liver biopsy because of its invasiveness and there might be inter- and intra-observer variability in the evaluation of specimens obtained<sup>[3]</sup>, and a risk of complications (even if it is low) causing discomfort<sup>[4,5]</sup>.

Some noninvasive methods are expected and used in patients with hepatitis C or B virus infection. The best results were obtained with liver stiffness measurement by means of transient elastography (TE) (FibroScan), or with FibroTest-ActiTest (Biopredictive, Labcorp)<sup>[4]</sup> and Fibrospect II (Prometheus)<sup>[5]</sup>. However, all these noninvasive methods are expensive and/or require equipments not widely used. Therefore, it is necessary to screen for simpler and cheaper methods for the examination of hepatic fibrosis. Poynard *et al*<sup>[6-10]</sup> investigated the correlations between the serum aminotransferases level, age, hyaluronic acid level, collagen level, platelet count and different fibrosis stages (FS), but did not draw clear con-

clusions. Several scoring systems like age-platelet count (PLT) ratio (AP index), aspartate aminotransferase (AST)-platelet ratio (APRI), age-AST model, fibrosis index (FI), FIB-4 and fibrosis-cirrhosis index (FCI), using different thresholds, have been proposed to detect presence or absence of fibrosis or cirrhosis in patients infected with hepatitis C virus (HCV) or HBV<sup>[11-18]</sup>. HBV-infected patients are prone to fibrosis; however, there have been few studies on the relationship between noninvasive fibrosis biomarker and liver biopsy. For this purpose, in this study we developed a new noninvasive serum model by assessing several clinicopathological features. We also compared and evaluated the diagnostic accuracy of this noninvasive model consisting of the variables of FI, APRI, FIB-4, FCI, age-AST model and AP index.

## MATERIALS AND METHODS

### Patients

This is a retrospective cross-sectional study and was carried out from March 2008 to March 2011. Screening of patients was conducted at the Department of Liver Disease (Ruikang Hospital of Guangxi Traditional Chinese Medical University, Nanning, China) and Department of Infectious Disease (The First Affiliated Hospital of Guangxi Medical University, Nanning, China). A total of 114 patients were enrolled to the study (male/female 91/23; mean age  $38.32 \pm 11.36$  years, range 15-67 years). Diagnosis of chronic HBV-infected patients was established based on the presence of positive results of surface antigen ( $> 0.5$  ng/mL) and/or e antigen ( $> 0.05$  NCU/mL) lasting more than six months. Clinical, biochemical and hematologic data were recorded from each patient at the time of liver biopsy. Patients with the following conditions were excluded: presence of other causes of liver disease such as hepatitis A/C/E virus infection, HCC, prior nucleoside medication, prior interferon therapy, fatty liver disease, alcohol intake  $> 30$  g/wk (female) and  $60$  g/wk (male), insufficient liver tissue for staging of fibrosis, clinical or ultrasonographic evidence of cirrhosis.

### Histological staging

Ultrasonographic-guided liver biopsy was performed according to a standardized protocol. The liver biopsy procedure, its advantages and possible adverse effects were explained to the patients. Informed consent was obtained from the patients about the possible transmission of HBV infection. Specimens of 15-20 mm liver tissues were fixed, paraffin-embedded, stained with hematoxylin-eosin and Masson's trichrome. A minimum of six portal tracts was required for diagnosis. Liver biopsy was evaluated with or without knowing the history of the patients. Five fibrosis degrees of histological staging were defined according to "The program of prevention and cure for viral hepatitis", generally used in hospitals of China<sup>[19]</sup>, as F0 (no fibrosis), F1 (mild fibrosis without septa), F2 (moderate fibrosis with few septa), F3 (severe fibrosis



**Table 1** Characteristics of the study population

Features	mean $\pm$ SD	Minimum	Maximum
Gender (male/female)	91/23		
HBV DNA (copies/mL)			
(< 1000/ $\geq$ 1000)	44/70		
(< 10 <sup>3</sup> /10 <sup>3</sup> -10 <sup>5</sup> / $\geq$ 10 <sup>5</sup> )	44/32/38		
E antigen (+/-)	52/62		
Age (yr)	38.32 $\pm$ 11.36	15	67
PLT ( $\times 10^9$ /L)	174.44 $\pm$ 62.68	50.6	334
T.Bil (g/dL)	0.88 $\pm$ 0.49	0.27	3.14
D.Bil (g/dL)	0.29 $\pm$ 0.23	0.08	1.39
ALT (IU/L)	69.32 $\pm$ 116.40	10.00	983
AST (IU/L)	48.60 $\pm$ 64.56	13	594
ALB (g/dL)	4.15 $\pm$ 0.43	2.85	5.26
GLOB (g/dL)	2.98 $\pm$ 0.52	1.75	4.47
ALP (IU/L)	80.10 $\pm$ 27.38	24	154
GGT (IU/L)	59.46 $\pm$ 66.92	5	430
PT (s)	12.01 $\pm$ 1.58	9.3	18.1

HBV: Hepatitis B virus; GLOB: Globulin; PLT: Platelet count; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; T.bil: Total bilirubin; D.bil: Direct bilirubin; ALB: Albumin; GGT: Glutamyltransferase; ALP: Alkaline phosphatase; PT: Prothrombin time.

with numerous septa without cirrhosis) and F4 (cirrhosis). In our study, we simplified the FS as F0-F1 (minimal fibrosis), F2-F3 (advanced fibrosis) and F4 (cirrhosis).

### Blood tests

All tests were performed in the patients after a fasting period of 12 h. The routine liver function tests included alanine aminotransferase (ALT), AST, total bilirubin (T.bil), direct bilirubin (D.bil), albumin, globulin (GLOB), glutamyltransferase (GGT), alkaline phosphatase (ALP), PLT, HBV-DNA and prothrombin time (PT). Serum tests were performed using an automatic biochemistry analyzer. The viral load was measured by real-time polymerase chain reaction, with a detection limit of 1000 copies/mL. All biochemical tests and their scores were evaluated without knowledge of liver biopsy results. Thirteen clinical, biochemical and hematological variables were used for the analysis: sex, age, PLT, ALT, AST, albumin, GLOB, T.bil, D.bil, GGT, ALP, HBV DNA and PT.

### Statistical analysis

The data was analyzed using statistical package SPSS version 13.5 for Windows. A *P* value of 0.05 was considered statistically significant. All data was presented as mean values or number of patients. Spearman's rank correlation was used to assess the significant correlation between variables and liver FS. The Student *t* test or variance analysis was used to compare arithmetic means and parameters, while  $\chi^2$  test was used to compare categorical data.

A predictive model, named Globulin/platelet (GP) model, was constructed by modeling the values of the independent variables and their coefficient of regression. The diagnostic value of the model was assessed by calculating the areas under the receiver operating characteristic (ROC) curves. An area under the curve (AUC) of 1.0 rep-

resents an ideal test, whereas 0.5 indicates a test of no diagnostic value. The diagnostic accuracy was calculated by sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV).

The best cutoff points were selected according to the Youden index from the ROC curve to identify the presence and absence of minimal fibrosis or cirrhosis. For this purpose, we selected cutoff points with a 95% certainty, thus assuming a 5% false negative result which is acceptable clinically.

### Comparison of available noninvasive biomarkers to evaluate patient's liver biopsy data

Serum samples and data of the characteristics of the patients and liver specimens were collected from each patient for further biochemical analysis. All patients were evaluated for FI, APRI, FIB-4, FCI, age-AST model and AP index. Superiority of GP model was compared with the other selected fibrosis models, and ROC curves and correlation analysis were employed to predict minimal fibrosis or cirrhosis, and deduce the diagnostic accuracy.

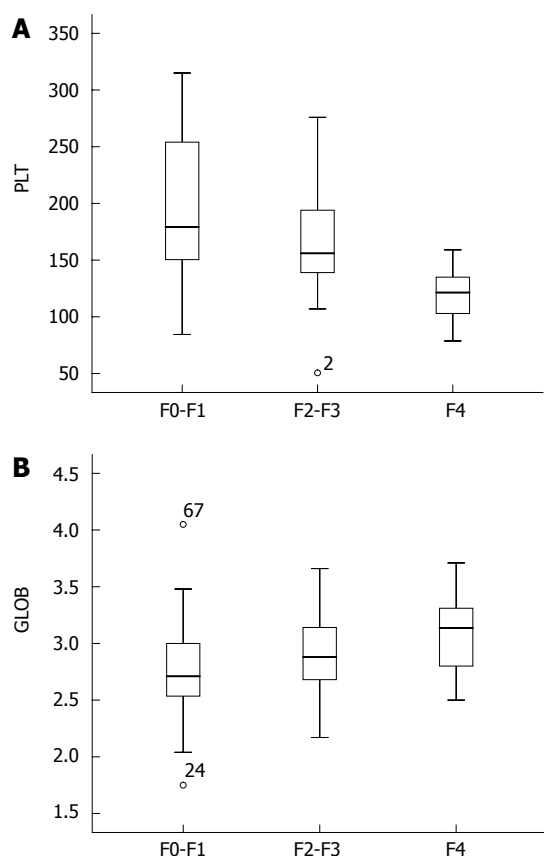
## RESULTS

### Patient data

The demographic and clinical outcomes of the 114 patients with HBV infection are shown in Table 1. The evaluation of inflammatory grade yielded mild chronic hepatitis in 44 patients, moderate chronic hepatitis in 64 patients and severe chronic hepatitis in 6 patients. And 22 patients had liver fibrosis at stage F0, 34 at F1, 28 at F2, 18 at F3, and 12 at F4. Among the patients with HBV DNA < 1000 copies/mL, there were 20 cases of mild chronic hepatitis, 24 moderate chronic hepatitis, and none had severe chronic hepatitis, while among those with HBV DNA > 1000 copies/mL, there were 24 cases of mild chronic hepatitis, 40 moderate chronic hepatitis, and six severe chronic hepatitis. The latter showed a higher inflammatory grade than the former ( $\chi^2 = 37.487$ ,  $P < 0.001$ ). Among the E antigen positive (e+) patients, 12 at stage F0, 15 at F1, 11 at F2, 7 at F3, and 7 at F4. Among E antigen negative (e-) patients, 10 at stage F0, 19 at F1, 17 at F2, 11 at F3 and 5 at F4. There was no significant difference between e+ and e- patients in FS ( $\chi^2 = 2.301$ ,  $P = 0.681$ ).

### Correlation between clinical findings and three FS

Three levels of liver fibrosis (F0-F1, F2-F3 and F4) were analyzed. Thirteen demographical, hematological, and biochemical variables were studied. HBV DNA was divided into two groups ( $\geq 1000$  copies/mL and < 1000 copies/mL) or three groups (< 1000 copies/mL,  $\geq 1000$  copies/mL and < 10<sup>5</sup> copies/mL,  $\geq 10^5$  copies/mL) and gender was described as categorical data. Of the 13 variables studied by Bivariate Spearman's rank correlation analysis, 6 variables (age, PLT, T.bil, D.bil, GLOB and PT) were correlated significantly with the three FS (cor-



**Figure 1** Scores of platelet count (A) and globulin (B) in three fibrosis stages (F0-F1, F2-F3 and F4). The top and bottom of each box are the 25% and the 75% centiles. The line through the box is the median, and the error bars are the 5th and 95th centiles. GLOB: Globulin; PLT: Platelet count.

relation coefficients were 0.23, -0.412, 0.208, 0.220, 0.314 and 0.212;  $P$  value 0.014, < 0.001, 0.026, 0.018, 0.001 and 0.024).

### Selection of variables and construction of a model for predicting FS

Univariate analysis revealed that only PLT and GLOB of the 13 variables were independent predictive factors and significantly different in FS (PLT:  $F = 11.772$ ,  $P < 0.001$ , GLOB:  $F = 6.612$ ,  $P = 0.002$ ). There was no difference among the other variables ( $P$  all > 0.05). These biochemical markers can also be helpful in staging the liver fibrosis. Figure 1 shows the box plots of the two markers with liver histological stages. It is clear from Figure 1 that as the fibrosis progressed, GLOB level increased, while PLT gradually decreased with fibrosis progression. However, it was interesting to note that GLOB and PLT both were in the normal limits, making the diagnosis of fibrosis difficult by using single biochemical markers.

To amplify the opposite relationship between the stage of fibrosis and the two markers, GLOB and PLT, based on their significance not only by the rank correlation analysis but also by univariate analysis, we developed a new fibrosis model named GP model in HBV infection for predicting cirrhosis and minimal fibrosis. It can be represented as GP model = GLOB (g/dL)  $\times$  100/PLT ( $\times 10^9/L$ ).

The GP model distribution for the patients in the respective FS is represented in Figure 2. The median values for GP model in F0-F1, F2-F3 and F4 patients were 1.461, 1.720 and 2.634, respectively. GP model was correlated significantly with the liver FS ( $r = 0.466$ ,  $P < 0.001$ ). The diagnostic values of GP model to predict F0-F1 and F4 patients were evaluated by the AUC (Figure 3).

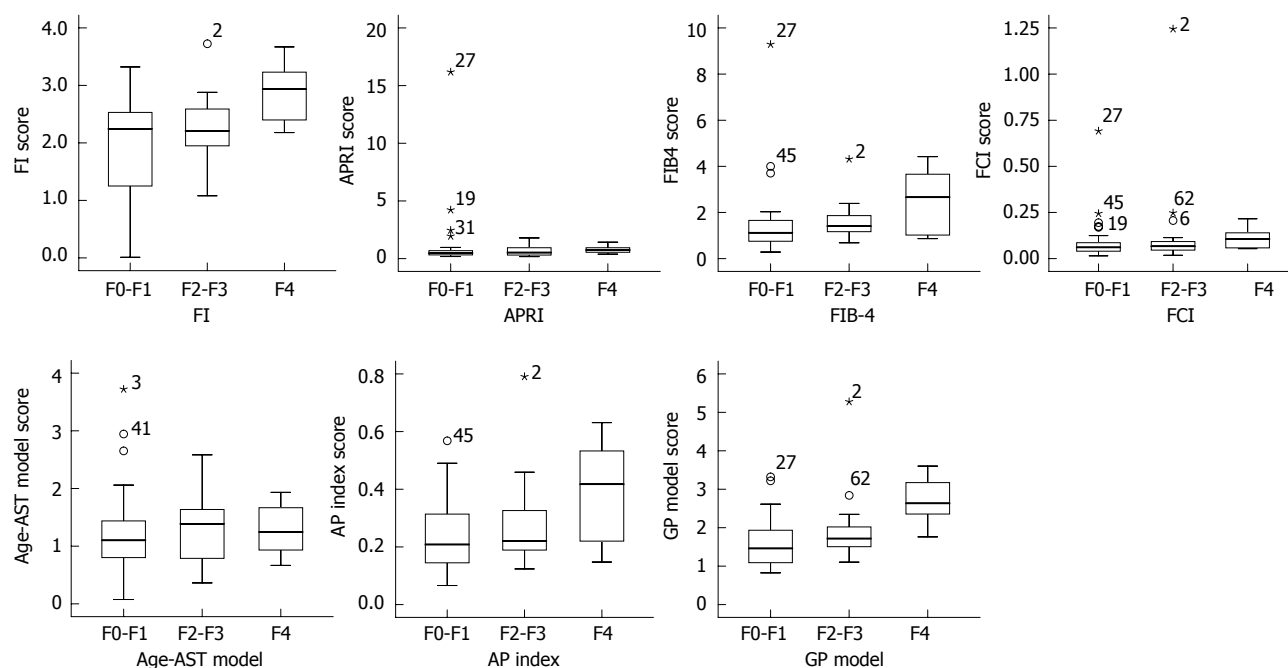
Step-wise multiple regression analysis, which was used to formulate a suitable multivariable model for the correlation between 11 count variables and the three FS, F0-F1, F2-F3 and F4, revealed that PLT and GLOB were also independently correlated with histological FS,  $R^2 = 0.237$ . The contribution of PLT and GLOB to  $R^2$  was 0.197 and 0.04, respectively (data not shown). The final multiple regression model incorporating both PLT and GLOB was: FS =  $1.683 - 0.008 \times \text{PLT} (\times 10^9/L) + 0.485 \times \text{GLOB} (\text{g/dL})$ . We simplified it as: FS =  $1.7 - 0.01 \times \text{PLT} (\times 10^9/L) + 0.5 \times \text{GLOB} (\text{g/dL})$ .

The median values for FS model in F0-F1, F2-F3 and F4 patients were 1.218, 1.480 and 2.085, respectively. FS model significantly correlated with the liver FS (Spearman's rank correlation coefficient,  $r = 0.47$ ,  $P < 0.001$ , data not shown). The diagnostic values of FS to differentiate F0-F1 and F4 patients were assessed using the ROC. Interestingly, AUC was very close between FS model and GP model in F0-F1 (0.760 *vs* 0.762) and F4 (0.781 *vs* 0.783), and in complete coincident sensitivity and specificity for the detection of minimal fibrosis and cirrhosis (data not shown). GP model was simpler than FS model in term of calculation. Therefore, we used GP model to express our findings.

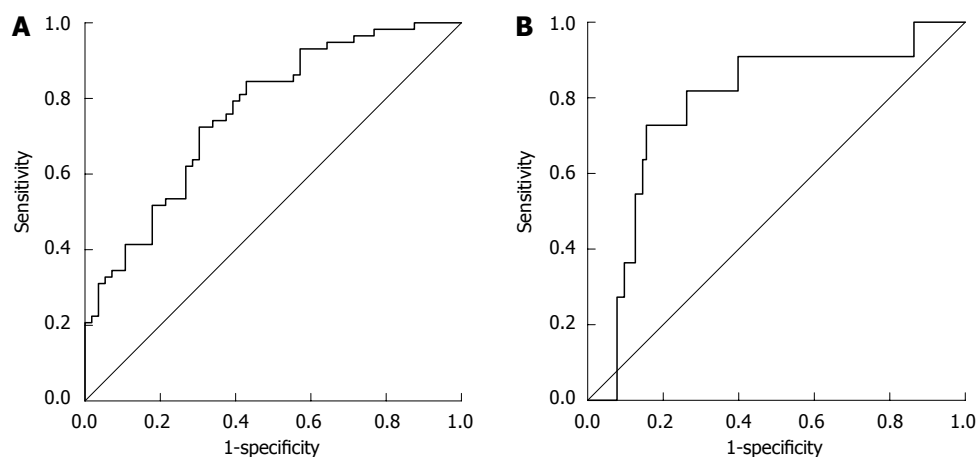
### Comparison of GP model with FI, APRI, FIB-4, FCI, age-AST model and AP index

The scores of six indexes (FI, APRI, FIB-4, FCI, age-AST model and AP index) were calculated by the formula shown in Table 2. The relationship between the histological severity of fibrosis (F0-F1, F2-F3 and F4) and six models is shown in Figure 2 and Table 3. There was a significant correlation between F0-F1, F2-F3 and F4 and serum indexes of FIB-4, AP index and GP model ( $P < 0.05$ ), but not in FI, APRI, FCI, age-PLT model ( $P > 0.05$ ). A gradual increase at the level of AP index, FIB-4, GP model was observed in different FS. GP model showed the strongest correlation with the three FS ( $r = 0.441$ ,  $P < 0.001$ ).

The predictive value of the seven noninvasive models for predicting minimal fibrosis (F0-F1) is summarized in Table 4. APRI, FIB-4 and FCI had a high PPV, specificity, but the sensitivity and NPV were low. AP index had a high sensitivity and NPV, but specificity and PPV were low. GP model had not only high PPV and specificity, but also high NPV and sensitivity. AUC of age-AST model was close to 0.5, showing little value for prediction of minimal fibrosis. We compared the AUC of GP model with the other five noninvasive models using the 95% confidence interval (CI) and the standard error (SE) of the mean, and found that although the differences were not statistically



**Figure 2** Box plot of fibrosis index, aspartate aminotransferase-platelet ratio, FIB-4, fibrosis-cirrhosis index, age-aspartate aminotransferase model, age-platelet count ratio and globulin/platelet model in relation to F0-F1, F2-F3 and F4 fibrosis stage. The box represents the interquartile range. The whiskers indicate the highest and lowest values, and the asterisks represent outliers. The line across the box indicates the median value. FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; AP index: Age-platelet count ratio.



**Figure 3** Receiver operating characteristic curves generated by globulin/platelet model for prediction of F0-F1 (A) and F4 (B). The area under the curve of GP model for the discrimination between minimal fibrosis (F0-F1) and significant fibrosis (F2, F3, F4), and between non-cirrhosis (F0, F1, F2, F3) and cirrhosis (F4) was 0.732 [confidence interval (CI): 0.642-0.823] and 0.738 (CI: 0.612-0.864). Using a cutoff value of < 1.68, GP model had a sensitivity of 72.4%, a positive predictive value (PPV) of 71.2%, a specificity of 69.6%, and a negative predictive value (NPV) of 70.8% for the prediction of F0-F1. For the prediction of cirrhosis (F4) at a cutoff value of > 2.53, GP model had a sensitivity of 72.7%, a PPV of 33.4%, a specificity of 84.5%, and a NPV of 96.7%.

significant, the GP model seemed to have a best predictive value (largest AUC) ( $P$  all > 0.05) (Table 4).

The predictive value of GP model for cirrhosis (F4) is shown in Table 5. All of the models had very good NPV (> 96%), also high sensitivity (> 70%). The AUC of age-AST model was less than 0.5, which cannot be used to predict cirrhosis. We compared the AUC of GP model with the other five noninvasive models as well using the 95% CI and the SE, and found that AUC of GP model was not significantly high ( $P$  all > 0.05) (Table 5).

## DISCUSSION

Although only a small number of patients with chronic inactive HBV infection develop advanced liver disease<sup>[20-22]</sup>, the risk of HCC is higher for HBV infected patients than for those without HBV infection<sup>[23]</sup>. The cirrhosis in HBsAg carriers often progress insidiously. Chu *et al.*<sup>[24]</sup> concluded that the so-called inactive carrier state cannot be considered generally as an innocuous, persistent condition with good prognosis, suggesting that regular follow-

**Table 2** Noninvasive simple fibrosis models composed of routine clinical and laboratory parameters

Fibrosis test	Calculation
FI	$8.0-0.01 \times \text{PLT} (\times 10^9/\text{L}) - \text{ALB} (\text{g/dL})$
APRI	$\text{AST} (\text{IU/L}) \times 100 / \text{PLT} (\times 10^9/\text{L})$
FIB-4	$\text{Age} (\text{yr}) \times \text{AST} (\text{IU/L}) / \text{PLT} (\times 10^9/\text{L}) \times \text{ALT} (\text{IU/L})^{1/2}$
FCI	$\text{ALP} (\text{IU/L}) \times \text{T.bil} (\text{g/dL}) / \text{ALB} (\text{g/dL}) / \text{PLT} (\times 10^9/\text{L})$
Age-AST model	$\text{Age} (\text{yr}) / \text{AST} (\text{IU/L})$
AP index	$\text{Age} (\text{yr}) / \text{PLT} (\times 10^9/\text{L})$

PLT: Platelet count ; ALB: Albumin; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; T.bil: Total bilirubin; AP index: Age-PLT ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; FCI: Fibrosis-cirrhosis index.

**Table 3** Correlation analysis of seven models and different fibrosis stages (F0-F1, F2-F3 and F4)

F0-F1, F2-F3 and F4 fibrosis stages	Bivariate Spearman's rank correlation coefficient	P value
FI	0.231	0.06
APRI	0.146	0.237
FIB-4	0.307	0.012
FCI	0.159	0.20
Age-AST model	0.132	0.286
AP index	0.246	0.045
GP model	0.441	0.000

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index.

**Table 4** Validity of age-platelet count ratio, aspartate aminotransferase-platelet ratio, age-aspartate aminotransferase model, fibrosis index, FIB-4 and fibrosis-cirrhosis index for prediction of minimal fibrosis (F0-F1) and comparison with globulin/platelet model

Fibrosis test	Cutoff value	Specificity %	Sensitivity %	PPV %	NPV %	AUC (95% CI)	Standard error	P value (vs GP model)
FI	1.58	41.1	89.7	61.2	79.4	0.695 (0.600-0.791)	0.049	> 0.05
APRI	0.85	87.5	41.4	77.4	59.0	0.635 (0.533-0.738)	0.052	< 0.05
FIB-4	1.7	85.7	51.7	78.9	63.1	0.720 (0.627-0.813)	0.047	> 0.05
FCI	0.17	80.4	55.2	74.5	63.4	0.692 (0.594-0.790)	0.050	> 0.05
Age-AST model	-	19.6	94.8	55.0	78.4	0.524 (0.417-0.632)	0.055	< 0.001
AP index	0.17	46.4	89.7	63.4	81.3	0.726 (0.634-0.818)	0.047	> 0.05
GP model	1.68	69.6	72.4	71.2	70.8	0.762 (0.676-0.848)	0.044	

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; PPV: Positive predictive values; NPV: Negative predictive values; AUC: Area under the curve; CI: Confidence interval.

**Table 5** Validity of age-platelet count ratio, aspartate aminotransferase-platelet ratio, age-aspartate aminotransferase model, fibrosis index, FIB-4, and fibrosis-cirrhosis index for prediction of cirrhosis (F4) and comparison with globulin/platelet model

Fibrosis test	Cutoff value	Specificity %	Sensitivity %	PPV %	NPV %	AUC (95% CI)	Standard error	P value (vs GP model)
FI	2.16	53.4	81.8	15.8	96.4	0.717 (0.569-0.865)	0.075	> 0.05
APRI	0.77	72.8	72.7	22.2	96.1	0.703 (0.584-0.822)	0.061	> 0.05
FIB-4	2.29	89.3	72.7	42.0	96.8	0.768 (0.050-0.931)	0.083	> 0.05
FCI	0.10	68.0	81.8	21.4	97.2	0.738 (0.612-0.864)	0.064	> 0.05
Age-AST model	-	-	-	-	-	0.486 (0.365-0.608)	0.062	-
AP index	0.32	74.8	72.7	23.6	96.2	0.735 (0.575-0.895)	0.082	> 0.05
GP model	2.53	84.5	72.7	33.4	96.7	0.781 (0.638-0.924)	0.073	

AP index: Age-platelet count ratio; AST: Aspartate aminotransferase; APRI: AST-platelet ratio; FI: Fibrosis index; GP: Globulin/platelet; FCI: Fibrosis-cirrhosis index; PPV: Positive predictive values; NPV: Negative predictive values; AUC: Area under the curve; CI: Confidence interval.

up is necessary. Active HBV infection more often caused significant fibrosis and cirrhosis than inactive HBV infection. We attempted to find a simple and common index to evaluate their fibrosis in active and inactive HBV infections. We retrospectively studied the pathological changes about fibrosis in patients with HBV infection from two liver disease centers. We found that the HBV DNA with a higher inflammation grade, was not significantly different among different FS. We also found no significant difference between e+ and e- populations in FS. A study<sup>[25]</sup> recruited patients with HBV DNA < 10<sup>4</sup> copies/mL, and found the mean liver stiffness in inactive HBsAg carriers was 5.6 ± 2.1 kPa, significantly higher than in normal

subjects. In 16.4% (23) of inactive carriers, liver stiffness exceeded 7 kPa (the cutoff for fibrosis F2-F4); and in patients with undetectable viral loads (with a detection limit of 51 copies/mL for HBV DNA), the mean liver stiffness was significantly lower than in those with detectable DNA (< 10<sup>4</sup> copies/mL). Assessing the noninvasive liver stiffness in inactive HBsAg carriers by transient elastography, Fattovich *et al*<sup>[26]</sup> reported that HBeAg-negative chronic hepatitis patients had a higher risk for progression to cirrhosis (8-10 per 100 person years) than HBeAg-positive chronic hepatitis patients. This may reflect the duration of infection, and a late phase in the natural history of the disease, as opposed to *de novo* infec-



tion with a variant not producing HBeAg. Another study found in chronic HBV carriers with clinical normal liver function tests, inflammation grade and FS had no correlation with the level of HBV DNA or the state of HBeAg positivity<sup>[27]</sup>. Our study with a detection limit of 1000 copies HBV DNA/mL, enrolled all of HBV-infected patients, including some patients with active inflammation. This may be the reason for the inconsistency with other studies<sup>[28]</sup>.

Many studies on prediction of significant fibrosis (F2-F4) and cirrhosis (F4) in patients with HBV infection have been published in the past few years, such as Fibroscan and FibroTest-ActiTest, galactose and methacetin breath tests, TE, fibrotest, cirrhosis discriminant score, AST/ALT ratio, APRI, FIB-4 and AP index<sup>[11,29,33]</sup>. However, some of the tests need expensive instruments and some are somewhat difficult to use in clinical practice, since these assay utilizes less common biochemical markers such as  $\alpha$ 2-macroglobulin, haptoglobin, and apolipoprotein A1, and also requires use of a special computer program to perform calculations.

In our study, we attempted to develop a single model using routinely available laboratory test results to predict minimal fibrosis and cirrhosis in treatment-naïve patients with HBV infection. We found by Step-wise multiple regression analysis and correlation analysis, that PLT and GLOB were significantly correlated with different FS. Other variables such as T.bil, D.bil, PT, or GGT may play a role in the discrimination function and have been found useful in patients with minimal fibrosis or cirrhosis. However, compared with PLT and GLOB, other variables were correlated with the histological FS at a much smaller coefficient of determination. Wai *et al.*<sup>[16]</sup> also suggested that two variables can be practically used as a prediction index, and in our model we used PLT and GLOB because of their convenience of application in general practice. Nevertheless, the value of these two parameters was proposed to evaluate minimal fibrosis and cirrhosis. The concept in prediction of minimal fibrosis by a ratio of two important variables is not new. These findings echoed results from many previous studies. The value of PLT as a marker of liver fibrosis has already been reported<sup>[6,16,34-37]</sup>. Some studies showed that GLOB was correlated with FS and was a predictor of either significant fibrosis or cirrhosis<sup>[28,38]</sup>.

GP model was simple to use and accurate in predicting both minimal fibrosis and cirrhosis in HBV-infected patients (Figure 3 and Tables 3-5). To evaluate its predictive value, we compared GP model with the other available models, because the variables used in these models were also involved in our collected data. GP model showed the highest correlation coefficient with FS. Comparison of GP model with the other six models in AUC, GP model showed the highest value than other models, although there was no significant difference. Using values below the lower cut-off level (1.68), a presence of minimal fibrosis could be predicted in 71% of patients. Similarly, using values below the higher cut-off level (2.53),

a prediction of non-cirrhosis could be made in 96% of patients.

There exist some limitations in our study. Our study is based on the data from liver biopsy, which is considered as the gold standard for assessing hepatic fibrosis, but sampling error as well as intra- and inter-observer variability can complicate the correlations between histology and noninvasive markers of hepatic fibrosis. Arase *et al.*<sup>[39]</sup> found that some patients with a nodular liver surface at laparoscopy were not diagnosed as having liver cirrhosis when only histological samples were used; HBV-positive patients with a nodular liver surface have a tendency of sampling error compared with the HCV-positive patients. On the other hand, there was overlap among patients with different stages of fibrosis. Thus, the value of GP model for the prediction of fibrosis in individual patients with HBV infection must be confirmed in prospective studies. However, we are not yet able to get enough patient data for verification of GP model in a new cohort. The data of patients were derived from two different hospitals, and the sample size is still small. Whether the model can reflect the change of FS in treatment process awaits further studies.

In conclusion, We established a simple model using available laboratory variables. Minimal fibrosis and cirrhosis can be diagnosed accurately using this model, thus reducing the need for liver biopsy in chronic HBV-infected patients.

## COMMENTS

### Background

Liver fibrosis is known as the major condition causing morbidity and mortality in chronic hepatitis B virus (HBV)-infection patients. Liver biopsy (LB) as an invasive method is used as the gold standard in diagnosing the degree of fibrosis, but because there is a risk of complications causing discomfort, not many patients are willing to undergo a LB. HBV-infected patients are prone to fibrosis, but there have been few studies about the relationship between noninvasive fibrosis biomarker and liver LB among these patients.

### Research frontiers

Some noninvasive methods were expected and used in patients with hepatitis C or B virus infection. Simpler and cheaper methods for the prediction of hepatic fibrosis are being studied. Several scoring systems have been proposed, such as age-platelet count (PLT) ratio (AP index), aspartate aminotransferase (AST)-platelet ratio, age-AST model, fibrosis index, FIB-4, and fibrosis-cirrhosis index, using different thresholds to predict presence or absence of fibrosis or cirrhosis in patients infected with hepatitis C virus or HBV.

### Innovations and breakthroughs

This study developed a new noninvasive serum model named Globulin/platelet (GP) model, including globulin and PLT, by assessing several clinicopathological features. Comparing with six available models, GP model showed highest correlation coefficient. The sensitivity and positive predictive value at a cutoff value < 1.68 for predicting minimal fibrosis F0-F1 were 72.4% and 71.2%. The specialty and negative predictive value at a cutoff value < 2.53 for the prediction of cirrhosis were 84.5% and 96.7%. The area under the curve of GP model for predicting minimal fibrosis and cirrhosis were 0.762 and 0.781, respectively, which is the largest among the seven models.

### Applications

The simple model developed in this study using readily available laboratory results can identify chronic HBV-infected patients with minimal fibrosis and cirrhosis with a high degree of accuracy, and it seems more efficient than frequently used models. This model may decrease the need for liver biopsy in

HBV-infected patients.

### Peer review

This study might be interesting and useful for the readers regarding the daily management of patients with viral hepatitis. Application of this model may decrease the need for liver biopsy in HBV infection cases.

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## Transepithelial leak in Barrett's esophagus patients: The role of proton pump inhibitors

Christopher Farrell, Melissa Morgan, Owen Tully, Kevin Wolov, Keith Kearney, Benjamin Ngo, Giancarlo Mercogliano, James J Thornton, Mary Carmen Valenzano, James M Mullin

Christopher Farrell, Melissa Morgan, Owen Tully, Kevin Wolov, Keith Kearney, Benjamin Ngo, Giancarlo Mercogliano, James J Thornton, Division of Gastroenterology, Lankenau Hospital, Wynnewood, PA 19096, United States

Mary Carmen Valenzano, James M Mullin, Lankenau Institute for Medical Research, Wynnewood, PA 19096, United States

**Author contributions:** Farrell C and Mullin JM jointly wrote this paper; Farrell C, Morgan M, Tully O, Wolov K, Kearney K, Ngo B, Mercogliano G and Thornton JJ were responsible for patient recruitment, informed consent and exclusions; Farrell C and Mullin J formulated study protocols and obtained The Main Line Hospitals Institutional Review Board approval; Valenzano MC prepared sucrose permeability test kits, measured urine sucrose concentrations and scheduled all patient testing.

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**Correspondence to:** James M Mullin, PhD, Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood, PA 19096, United States. [mullinj@mlhs.org](mailto:mullinj@mlhs.org)

Telephone: +1-484-4762703 Fax: +1-484-4762205

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### Abstract

**AIM:** To determine if the observed paracellular sucrose leak in Barrett's esophagus patients is due to their proton pump inhibitor (PPI) use.

**METHODS:** The *in vivo* sucrose permeability test was administered to healthy controls, to Barrett's patients and to non-Barrett's patients on continuous PPI therapy. Degree of leak was tested for correlation with presence of Barrett's, use of PPIs, and length of Barrett's segment and duration of PPI use.

**RESULTS:** Barrett's patients manifested a near 3-fold greater, upper gastrointestinal sucrose leak than healthy controls. A decrease of sucrose leak was observed in Barrett's patients who ceased PPI use for 7 d.

Although initial introduction of PPI use (in a PPI-naïve population) results in dramatic increase in sucrose leak, long-term, continuous PPI use manifested a slow spontaneous decline in leak. The sucrose leak observed in Barrett's patients showed no correlation to the amount of Barrett's tissue present in the esophagus.

**CONCLUSION:** Although future research is needed to determine the degree of paracellular leak in actual Barrett's mucosa, the relatively high degree of leak observed with *in vivo* sucrose permeability measurement of Barrett's patients reflects their PPI use and not their Barrett's tissue *per se*.

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**Key words:** Barrett's esophagus; Sucrose; Tight junction; Paracellular; Omeprazole; Proton pump inhibitor; Transepithelial

**Peer reviewers:** Dr. Ahmet Tekin, Department of General Surgery, IMC Hospital, Istiklal Cad no:198, Mersin 33100, Turkey; Julian Abrams, MD, MS, Assistant Professor of Clinical Medicine, Division of Digestive and Liver Diseases, Columbia University Medical Center, 622 W 168th Street, PH 20-303, New York, NY 10032, United States

Farrell C, Morgan M, Tully O, Wolov K, Kearney K, Ngo B, Mercogliano G, Thornton JJ, Valenzano MC, Mullin JM. Transepithelial leak in Barrett's esophagus patients: The role of proton pump inhibitors. *World J Gastroenterol* 2012; 18(22): 2793-2797 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2793.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2793>

### INTRODUCTION

The sucrose permeability test is designed to measure paracellular (non-transcellular) leak of sucrose from the lumen



of the upper gastrointestinal (GI) tract into the vasculature because sucrose is markedly hydrophilic and lacks any significant affinity for any carbohydrate transport proteins on the cell surface<sup>[1]</sup>. Moreover sucrose is destroyed enzymatically in the duodenum, and ceases to exist as a disaccharide after the early-mid portion of the small bowel. Sucrose leak would therefore be detected only while the probe (sucrose) still exists chemically in the upper GI lumen. This limits potential sites of leak to the esophageal, gastric and early intestinal mucosa. However, considerations of surface area and contact time (after oral consumption of the sucrose) argue that the sucrose permeability test results reflect primarily the gastroduodenal mucosa and less so the esophageal mucosa which has the smallest surface area (no villi or folds-except in Barrett's) and the shortest contact time.

Still, Barrett's patients are believed not to have a histological mucosal defect other than in the esophagus. Therefore when in previous work our group published that patients with a known diagnosis of Barrett's esophagus (BE) manifested a greater magnitude of transepithelial sucrose leak across the upper gastrointestinal tract than that seen in healthy control subjects, the strong difference was ascribed to the presence of Barrett's metaplasia in these patients<sup>[2]</sup>. In other words, a transepithelial leak was presumed to exist in the Barrett's metaplasia.

In this current work we revisited that conclusion by asking whether considerations other than the simple presence of Barrett's metaplasia were the reason for the increased leak observed in BE patients. Aside from the presence of Barrett's tissue, the foremost distinction about BE patients is their chronic, long-term use of acid suppression medications, most notably proton pump inhibitors (PPIs). We therefore posed the question of whether the leak we observed in BE patients was traceable to their regular use of PPI medications or to the presence of Barrett's metaplasia. Results indicated that the observed leak is due to PPI use by these patients.

## MATERIALS AND METHODS

### Study population

Patients with a prior known history of Barrett's esophagus or healthy controls with no current or history of upper GI disease were recruited by a gastroenterologist in a tertiary care teaching hospital bordering the suburbs of Philadelphia, PA. Diagnosis of BE was made by endoscopic exam and only after biopsies were documented to possess goblet cell metaplasia. All enrolled subjects gave informed consent and the study was approved by the Main Line Hospitals Institutional Review Board committee.

Test subjects were recruited without regard to gender or ethnicity. Exclusion criteria were: diabetes mellitus, steroid use, prior gastric or esophageal surgery, age < 18 years, current GI bleeding, weight loss, intractable nausea and vomiting or renal insufficiency. PPI use comprised omeprazole, esomeprazole, lansoprazole, pantoprazole and rabeprazole.

### Sucrose permeability testing

All test subjects consumed in their homes a chilled solution of 100 g of sucrose in 200 cc of water containing 5 g of a citric acid-based flavoring agent at bedtime. An 8 h (overnight) urine sample was collected in a container with 5 mL of 10% thymol in isopropanol and mixed. For patients undergoing upper endoscopic examination, the sucrose permeability test was performed either before the procedure or at least two weeks later to avoid potential effects of endoscope trauma on epithelial barrier tissue. The total urine volume was measured and recorded. The concentration of sucrose in the urine sample was then measured by an enzymatic/spectrophotometric assay after prior desalting of the urine sample by anion and cation exchange resins<sup>[3]</sup>. The total amount of sucrose in the urine in mg was determined by multiplying the urine volume in mL by the sucrose concentration in mg/mL. This equates to the amount of sucrose which leaked out of the upper GI lumen.

Test subjects were instructed to refrain from solid food for at least 2 h prior to the sucrose permeability test and 8 h after testing. Specific foods were however not prohibited. Test subjects were also instructed to refrain from alcohol or non-steroidal anti-inflammatory medications for 24 h prior to testing and for 8 h after testing. Brushing teeth or flossing was proscribed before testing and until at least 20 min after testing.

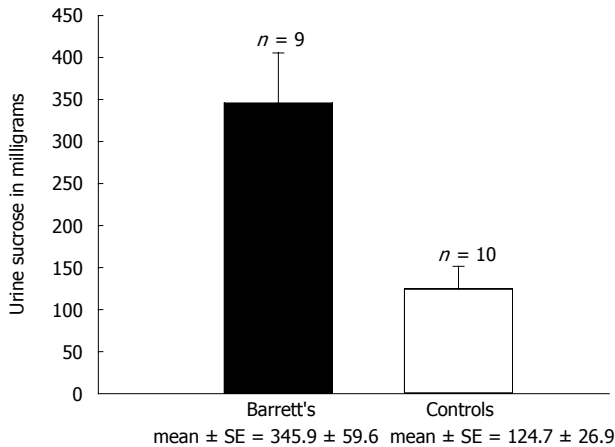
### Statistical analysis

Data are reported throughout as the mean  $\pm$  SE. Experimental and control groups are compared throughout by unpaired Student's *t* tests, with statistical significance being ascribed when  $P < 0.05$ .

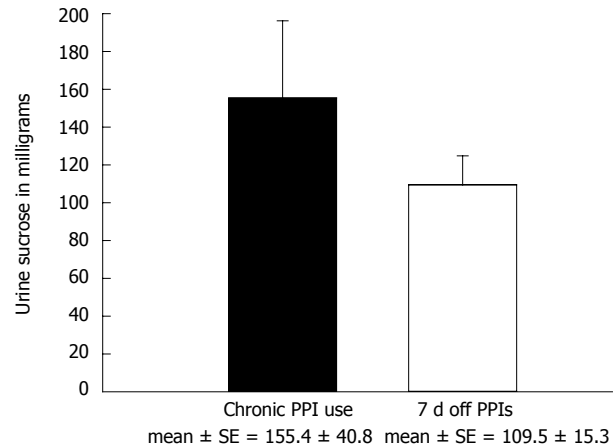
## RESULTS

Sucrose permeability testing was conducted on 19 patients, 9 carrying a diagnosis of BE and 10 being healthy controls. The 9 BE patients were on PPI therapy at the time of their testing while the healthy controls were receiving no acid-suppressive medication. As was shown in previous studies from our group<sup>[2]</sup>, a significantly greater sucrose leak was observed in the BE patients ( $345.9 \pm 59.6$  mg) compared to the healthy controls ( $124.7 \pm 26.9$  mg) (Figure 1). This difference was statistically significant with a  $P < 0.003$ . Following this confirmation of an increased transepithelial sucrose leak in BE patients, the etiology of the leak (as a result of BE itself or of PPI use) was pursued.

First, a second group of BE patients was studied to evaluate the effect of cessation of their PPI therapy on their observed sucrose leak. Thirty-eight BE patients underwent sucrose permeability testing while on a continuous PPI regimen. They then performed a second sucrose permeability test after having stopped their PPIs for 7 d, in order to look for any change in observed leak. Patients were allowed to consume antacid medications during this period but not PPIs or H-2 receptor antagonists. Any patients reporting very difficult reflux symptoms



**Figure 1** Results of sucrose permeability studies showing a significant difference in sucrose leak in patients with Barrett's esophagus (and taking proton pump inhibitors) compared to healthy controls. Data represents the mean ± SE.  $P < 0.003$  (Student's *t* test). SE: Standard error.



**Figure 2** Sucrose leak among Barrett's esophagus patients after discontinuation of proton pump inhibitors for 7 d. Data represents the mean ± SE.  $P = 0.28$  ( $n = 38$ ). SE: Standard error; PPI: Proton pump inhibitor.

were allowed to leave the study. The mean sucrose leak decreased by approximately 30% from  $155.4 \pm 40.8$  mg to  $109.5 \pm 15.3$  mg after PPI medications were temporarily stopped for this 7 d period (Figure 2). These findings were however not statistically significant, with a  $P = 0.283$  (paired Student's *t*-test). As before, a high SE was found for patients on PPI therapy, indicating a considerable amount of variability. The sucrose leak observed in the BE patients after their PPIs were discontinued for 7 d decreased to nearly the same level observed in Figure 1 for healthy controls not taking PPIs.

Since BE exists in a wide range of segment lengths, and a (passive diffusion) transepithelial leak due specifically to Barrett's metaplasia should correlate with the surface area of the Barrett's metaplasia, we investigated whether sucrose leak in the Barrett's patients correlated with length of segment of the Barrett's tissue. Endoscopically, characteristic findings of reddish/velvety Barrett's mucosa are defined as short segment BE if  $< 3$  cm and long segment BE if  $\geq 3$  cm in length. Data from 49 BE patients were examined. Greater leak was not observed in patients with long-segment Barrett's. Mean leak was actually greater in short-segment Barrett's ( $209$  vs  $148.9$ ). Analysis of sucrose leak between the two segment groups showed similar medians of  $130.5 \pm 40.4$  mg (short) and  $121.0 \pm 21.4$  mg (long) ( $P = 0.36$ ) with no statistically significant difference between them.

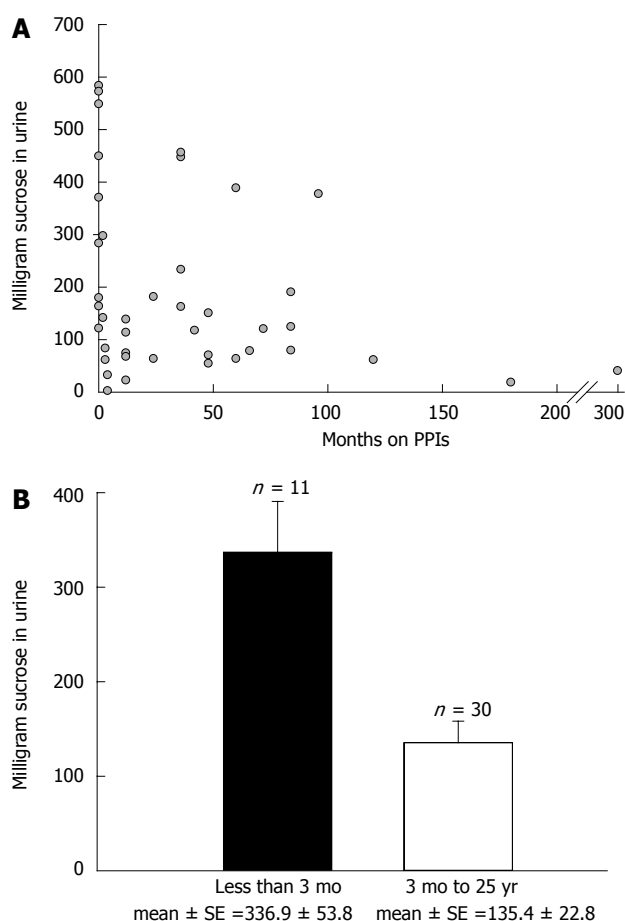
Due to the magnitude of sucrose leak variability that we observed among patients (especially among patients on PPIs), other potential confounding sources of variability were explored. First, the issue of potential effect of duration of PPI use on observed transepithelial sucrose leak was investigated. In a non-BE population taking PPIs on a regular, long-term basis (gastroesophageal reflux disease patients not known to have Barrett's), the sucrose leak was found to vary as a function of the duration of PPI use. Specifically, the magnitude of leak correlated inversely with the length of time on PPI medi-

cations (Figure 3A). A statistically significant difference in leak was observed in patients on PPIs for  $< 3$  mo ( $336.9 \pm 53.8$  mg) in comparison to those on PPIs for 3 mo to 25 years ( $135.4 \pm 22.8$  mg) ( $P < 0.0003$ ; Figure 3B). The PPI-induced leak thus appeared to exhibit tachyphylaxis, and this would lead to wider observed variation in leak data if one did not correct for duration of PPI therapy.

Discontinuation of long-term PPI use followed by resumption of PPIs did not result in re-induction of leak. In a separate group of 14 patients on long-term PPI therapy, PPI use was discontinued for 7 d, and was then reinstituted. Sucrose permeability testing was again utilized to assess upper gastrointestinal leak 7 d after resumption of PPI use. A significant leak did not re-occur in these patients who had previously been on long-term PPI treatment (Table 1). This is in sharp contrast to patients taking PPIs for the first time, where induced sucrose leak is substantial<sup>[3]</sup>.

## DISCUSSION

When our clinical studies with Barrett's patients were begun in 2005, we did not anticipate increased barrier leak being induced by PPIs. In fact, the opposite was expected due to PPIs' documented ability to allow for mucosal (both gastric and esophageal) healing from inflammation and micro-ulceration by means of suppression of acid secretion and subsequent elevation of the pH of gastric luminal contents. Therefore when we first noted a transmucosal leak in BE patients we ascribed the observed leak to the presence of Barrett's metaplasia, as BE patients typically do not have other upper GI pathology<sup>[2]</sup>. When we later observed that Barrett's patients' transmucosal sucrose leak did not correlate with the length of the Barrett's segment and the surface area of the Barrett's metaplasia, we began to question if the source of the leak was in fact the metaplasia. We realized that PPI medications were the other common characteristic-in addition to the metaplasia itself-of all the Barrett's patients studied.



**Figure 3** Proton pump inhibitor-induced transepithelial leak as a function of time. A: Scatter plot illustrating a decrease in sucrose leak in reflux disease patients on proton pump inhibitors (PPIs) over time with each data point representing the sucrose leak of one patient ( $n = 41$ ); B: Statistically significant decrease in sucrose leak between patients on PPIs for  $< 3$  mo vs 3 mo to 25 years. Data represents the mean  $\pm$  SE.  $P = 0.003$ . SE: Standard error.

We then began a series of clinical studies to ask whether PPIs in fact could induce upper GI leak in healthy controls free of upper GI disease, and found that PPIs indeed have this effect<sup>[3]</sup>. We further explored that phenomenon in an animal model (Sprague Dawley rat), observing that exposure of rat gastric corpus to omeprazole can lead to an immediate increase in transmucosal permeability which is bidirectional, concentration-dependent and size-specific<sup>[4-6]</sup>. This work confirmed the earlier findings by Hopkins *et al*<sup>[7]</sup> that omeprazole induced leak to <sup>14</sup>C-mannitol in rat gastric corpus. These previous findings together with our current observation (that the sucrose leak seen in Barrett's patients decreases when their PPI medications are discontinued) suggests that PPI use, not the presence of BE *per se*, is the cause of the upper GI leak seen in BE patients.

Note however that this still does not address whether Barrett's epithelium is or is not paracellularly leaky. It simply means that the clinical, *in vivo*, sucrose permeability test which is employed here is reflecting the effects of PPIs on upper GI barrier function. To determine whether Barrett's metaplasia is leaky will likely require Ussing chamber-type permeability studies with actual Barrett's

**Table 1** Sucrose leak in Barrett's patients as a function of proton pump inhibitor use

	On PPIs	7 d off PPIs	7 d after PPI resumption
Median	87.8	101.9	63.0
mean $\pm$ SE	116.6 $\pm$ 16.5	142.7 $\pm$ 34.2	101.5 $\pm$ 21.0

Sucrose leak in Barrett's esophagus patients ( $n = 14$ ) on proton pump inhibitors (PPIs), following 7 d off PPIs, and after the reintroduction of PPIs for 7 d. The acute leak phenomenon observed in previous studies in PPI naïve patients is not seen with resumption of PPIs after 7 d. A statistical significance in sucrose leak is not demonstrated among the different groups (on PPIs vs off PPIs  $P = 0.497$ , off PPIs vs back on PPIs  $P = 0.323$ , on PPIs vs back on PPIs  $P = 0.574$ ). SE: Standard error.

tissue. This could be difficult to perform since the major source of Barrett's tissue that is large enough in surface area for typical Ussing studies is esophagectomy surgery for adenocarcinoma. Here, adjacent Barrett's tissue may be available but it can be considerably modified/eliminated by radiation and chemotherapy prior to surgery, which is now a current, common practice in esophageal adenocarcinoma management. This is therefore clearly a situation where Ussing studies using Barrett's biopsy tissue (readily available through upper endoscopic screening) would be highly useful<sup>[8-10]</sup>.

An unanswered question from our earlier study on sucrose leak in Barrett's patients is that not only was sucrose leak in Barrett's patients significantly greater than sucrose leak in healthy controls, but it was also greater than the leak measured in patients with chronic gastroesophageal reflux disease (GERD)<sup>[1]</sup>. As both GERD and Barrett's patient groups would be receiving PPI therapy, it is unclear why leak in the Barrett's group would be quantitatively and significantly different if PPIs were the cause of leak in both patient groups. One possible explanation is that our patient population happened to be too small in the earlier study to get a fully accurate representation, and that sucrose permeability testing can carry intrinsically high variability, being affected by certain dietary constituents as well as certain over-the-counter drugs such as aspirin<sup>[11-13]</sup>.

The sucrose leak that we observed in the Barrett's cohort, or any of our patient groups taking PPIs, was not only high in absolute value but was associated with a large variance and high standard error of the mean. This led us to examine whether the PPI-induced leak was stable over time, since the length of the duration on PPI therapy was a frequent variable in our studies. As shown in Figure 3, the effect of PPIs on leak is indeed not stable over time, but in fact decreases over long-term PPI use. In contrast, a similar tachyphylactic aspect to PPI activity has not been observed for PPI-inhibition of acid secretion<sup>[14]</sup>. The inability of PPIs to induce a major transmucosal, molecular leak ( $> 200$  mg of sucrose in the described test) after long-term PPI use has been interrupted for 7 full days, suggests that long-term PPI use has caused modification of intracellular signaling pathways and/or cell/tissue structural aspects that mitigate against reintroduction of leak.

In summary, sucrose leak observed in Barrett's esophagus patients appears to be due to the use of PPIs by these patients, not due to their Barrett's metaplasia. This PPI-induced upper GI leak appears to diminish during long-term PPI therapy. After long-term use of PPIs, leak is difficult to re-induce even after short interruption of PPI therapy. Finally, variability associated with sucrose permeability testing in a clinical population may necessitate use of relatively large patient groups to support one's conclusions.

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## COMMENTS

### Background

In earlier work, the research group has shown that Barrett's esophagus (BE) patients manifest a barrier leak to the paracellular probe, sucrose, in their upper gastrointestinal tract. This phenomenon could be due to a molecular-level leak in the Barrett's metaplasia, or to some other aspect of the Barrett's patient cohort. This study was an attempt to determine if the common medication of all BE patients, proton pump inhibitors (PPIs), is responsible for the phenomenon.

### Innovations and breakthroughs

The phenomenon of PPI-induced gastric barrier leak is certainly an unexpected side-effect of PPI therapy. The mechanism for the phenomenon remains unknown. More importantly, the clinical implications of the phenomenon are as yet poorly understood.

### Applications

If one adopts a stance that a PPI-induced gastric barrier leak is clinically benign, it is possible that the phenomenon could be useful in drug delivery. However a greater understanding of the characteristics of the leak are needed (what can actually permeate through the leak), as well as a full realization of the clinical implications (if any). These could range from localized inflammation to altered kinetics of uptake of other (oral) medications into the bloodstream.

### Terminology

Barrier function refers here to the ability of the gastric mucosa to separate the stomach luminal compartment from interstitial fluid and bloodstream. Barrier leak can result from either injured/dying/detaching epithelia or altered (and leaky) epithelial tight junctions.

### Peer review

This study found that BE manifested a greater magnitude of transepithelial sucrose leak across the upper gastrointestinal tract than that seen in healthy con-

trol subjects, previously. In the present study, they determined if the observed paracellular sucrose leak in BE patients is due to their proton pump inhibitors use. This subject is a new topic in the research area and may contribute to the literature.

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## Plasma microRNA profiles distinguish lethal injury in acetaminophen toxicity: A research study

Jeanine Ward, Shashi Bala, Jan Petrasek, Gyongyi Szabo

Jeanine Ward, Department of Emergency Medicine, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, MA 01655, United States

Shashi Bala, Jan Petrasek, Gyongyi Szabo, Division of Gastroenterology, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01655, United States

**Author contributions:** Ward J and Szabo G contributed to the research concept, experimental design, data analysis and drafting of the manuscript; Bala S carried out the data analyses and drafting of the manuscript; Petrasek J contributed to the experimental design and performed the statistical analysis; all authors read and approved the final manuscript.

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**Correspondence to:** Gyongyi Szabo, MD, PhD, Professor, Vice Chair for Research, Division of Gastroenterology, Department of Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01655, United States. [gyongyi.szabo@umassmed.edu](mailto:gyongyi.szabo@umassmed.edu)

Telephone: +1-508-8565275 Fax: +1-508-8564770

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### Abstract

**AIM:** To investigate plasma microRNA (miRNA) profiles indicative of hepatotoxicity in the setting of lethal acetaminophen (APAP) toxicity in mice.

**METHODS:** Using plasma from APAP poisoned mice, either lethally (500 mg/kg) or sublethally (150 mg/kg) dosed, we screened commercially available murine microRNA libraries (SABiosciences, Qiagen Sciences, MD) to evaluate for unique miRNA profiles between these two dosing parameters.

**RESULTS:** We distinguished numerous, unique plasma miRNAs both up- and downregulated in lethally compared to sublethally dosed mice. Of note, many of the greatest up- and downregulated miRNAs, namely

574-5p, 466g, 466f-3p, 375, 29c, and 148a, have been shown to be associated with asthma in prior studies. Interestingly, a relationship between APAP and asthma has been previously well described in the literature, with an as yet unknown mechanism of pathology. There was a statistically significant increase in alanine aminotransferase levels in the lethal compared to sublethal APAP dosing groups at the 12 h time point ( $P < 0.001$ ). There was 90% mortality in the lethally compared to sublethally dosed mice at the 48 h time point ( $P = 0.011$ ).

**CONCLUSION:** We identified unique plasma miRNAs both up- and downregulated in APAP poisoning which are correlated to asthma development.

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**Key words:** Plasma microRNA; Hepatotoxicity; Acetaminophen; Drug-induced liver injury; Alanine aminotransferase

**Peer reviewer:** Oliver Grundmann, PhD, Clinical Assistant Professor, Department of Medicinal Chemistry, College of Pharmacy, University of Florida, 1600 SW Archer RD, Room P6-20, Gainesville, FL 32610-0484, United States

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### INTRODUCTION

Acetaminophen (APAP) continues to be an important cause of acute liver failure in the developed world, second only to infectious etiologies worldwide<sup>[1]</sup>. It is also the most common cause of death due to analgesic ingestion in the United States<sup>[2]</sup>. Numerous emergency department

patients in the setting of acetaminophen overdose are admitted to hospital for N-acetylcysteine (NAC) treatment. This necessary treatment modality places these patients at risk for health-care associated disease. It also places likely unnecessary additional financial strain on an already burdened healthcare system. In addition, the estimated United States cost of treating intentional acetaminophen overdose is \$86.9 million per year<sup>[3]</sup>. However, in over 30 years of research it is still unclear how the exact mechanism of acetaminophen toxicity occurs<sup>[4]</sup>.

Current literature implicates N-acetyl-para-benzoquinone-imine, or N-acetyl-p-benzoquinoneimine (NAPQI), as the primary metabolite responsible for hepatotoxicity. An estimated 90% of APAP is metabolized in the liver *via* either glucuronidation or sulfation. Another approximate 5% is urinated unaltered. However, approximately 5% of APAP is metabolized by the cytochrome P450 2E1 pathway into NAPQI. In the scenario of APAP overdose, the normal route of metabolism becomes overburdened, so an overabundance of NAPQI is produced, causing hepatotoxicity. Glutathione can rescue this process, and is the reason NAC is used as a treatment modality. However, it is presumed only a portion of hepatotoxicity occurs *via* this mechanism. Lipid peroxidation *via* free radical formation, and mitochondrial dysfunction *via* increased permeability of the mitochondrial permeability transition, are also postulated as causes of APAP-associated hepatotoxicity<sup>[5,6]</sup>.

Clearly, a better understanding of how APAP specifically causes hepatic toxicity from a pathophysiologic perspective still needs to be determined. In addition, standard clinical laboratory testing may not reveal evidence of hepatic injury for up to 24 h following APAP ingestion. A considerable proportion of APAP-exposed individuals therefore receive unnecessary empiric treatment with an antidote before hepatic injury can be ruled out. To overcome this clinical problem, early diagnostic indicators of hepatic injury have been sought.

MicroRNA fragments (miRNAs), are short, chemically stable biomolecules, noncoding posttranslational regulators that bind to untranslated mRNA sequences to produce gene silencing<sup>[7-10]</sup>. Moreover, each miRNA targets several different mRNAs; the same target gene may be regulated by several different miRNAs in different biological situations, a process that allows enormous complexity and flexibility in their regulatory potential. miRNAs have been characterized as regulators of protein expression in diverse disease processes, including acute hepatic injury<sup>[9,10]</sup>. Importantly, miRNAs have already been successfully utilized as early biomarkers for esophageal squamous cell carcinoma detection in serum<sup>[7]</sup>, identifying Parkinson's disease onset and disease progression<sup>[8]</sup>, and diagnosis of hepatocarcinoma<sup>[9,10]</sup>, demonstrating miRNAs as an ideal area of research to determine other early biomarkers for disease states, notably APAP-associated hepatotoxicity<sup>[11]</sup>. In addition, miRNA fragments do not require the post-translational modifications necessary in protein production; with fewer human

miRNAs to evaluate (an estimated 1000 human miRNAs compared to approximately 20 000 proteins), there is an improved likelihood of identifying unique APAP-associated miRNA profiles<sup>[11]</sup>.

Recent work has also shown the medical utility of miRNA<sup>[12,13]</sup>. Interestingly, literature also supports its association specifically in the setting of acetaminophen toxicity. For instance, Wang *et al.*<sup>[11]</sup> (2009) showed increased levels of miR-122 and miR-192 in the plasma of acetaminophen overdosed mice, yet decreased levels of these miRNAs in the liver tissue. In addition, these determined markers changed with time and dosing corresponding to histologic liver damage. Of note, these profiles were not evaluated at the lethal APAP dosing of 500 mg/kg, a parameter requiring further investigation to specifically compare lethal and sublethal miRNA profiles. Furthermore, additional literature has described miRNA involvement in acetaminophen toxicity, as well as the utility of miRNAs as biomarkers useful in the setting of other hepatotoxic disorders, such as hepatitis B and C, alcoholic liver disease, non-alcoholic fatty liver disease, and primary biliary cirrhosis<sup>[14]</sup>.

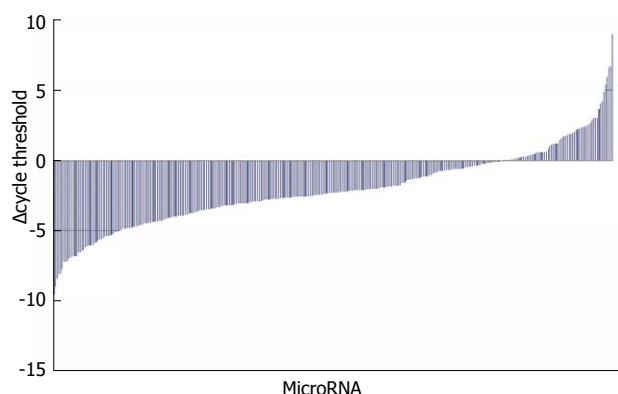
However, these miRNAs could be a potential marker for liver toxicity in the human patient as well, specifically in the setting of acetaminophen overdose. The identification of early plasma markers of acetaminophen toxicity is necessary and paramount. Early identification of acetaminophen toxicity would identify those requiring more expeditious treatment, potentially improving morbidity and mortality of these individuals. It would also possibly abrogate the need for patient admission, mitigating the resultant financial system burden and iatrogenic risk of hospital-acquired infections.

## MATERIALS AND METHODS

### Acetaminophen toxicity

C57Bl/6 wild-type mice were purchased from Jackson Laboratory (Bar Harbor, ME) and received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

For all experiments, 6-wk-old female C57/BL6 mice, with food deprivation 12 h prior to experimentation, were used for intraperitoneal (ip) injections. For lethal dosing APAP experiments, 20 C57/BL6 mice were each injected with acetaminophen 500 mg/kg (0.9% normal saline suspension) ip at time zero. For the sublethal dosing APAP experiments, 20 C57/BL6 mice were injected with acetaminophen 150 mg/kg acetaminophen (0.9% normal saline suspension) ip at time zero. At times 0.5 h, 2 h, 12 h, 24 h, and 48 h, 5 mice per group (both lethal and sublethal) were sacrificed *via* cervical dislocation. Just prior to sacrifice, 400 mL of cheek blood was obtained from each mouse. The whole blood was then centrifuged at 14 000 *g* for 10 min at room temperature. The plasma was removed, aliquoted, and stored at -80 °C. After sacri-



**Figure 1 Plasma microRNA both up- and downregulated in lethally (500 mg/kg) compared to sublethally (150 mg/kg) dosed acetaminophen mice at the 12 h time point.** A total of 528 microRNAs were screened using the reverse transcriptase<sup>2</sup> miRNA polymerase chain reaction (PCR) array of mouse whole genome, per the manufacturer's protocol (SABiosciences, Qiagen Sciences, MD). Quantitative PCR data were analyzed using manufacturer software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

fice, livers were snap frozen in liquid nitrogen for protein, stored in RNA stabilization reagent (RNAlater, Qiagen, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathologic analysis. Five mice were injected with saline only ip and sacrificed at time 48 h as controls.

### Hepatotoxicity verification

Alanine aminotransferase (ALT) was quantified by biochemical assay (D-Tek Analytical Laboratories Inc, San Diego, CA).

### Survival studies

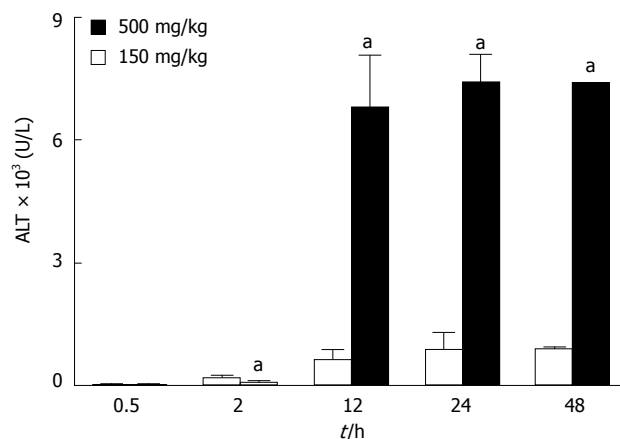
Ten mice were injected with APAP 500 mg/mL ip and an additional 10 mice were injected with APAP 150 mg/mL, all at time zero. Mice were monitored for 48 h and the mortality for each recorded and plotted using Kaplan-Meier survival statistics (GraphPad Prism Software, LaJolla, CA). All mice were housed, watered and fed under the same conditions throughout the experimental protocol.

### Histopathologic analysis

Sections of formalin-fixed, paraffin-embedded livers after sublethal and lethal APAP dosing were stained with hematoxylin and eosin and assessed for inflammatory infiltrate calculated with Microsuite (Olympus Soft Imaging Solution GmbH, Munster, Germany) image analysis software in 20 X objective.

### MicroRNA library screen

MicroRNA was purified from plasma using the MiR-Neasy Mini kit (Qiagen Sciences, MD). The cDNA was prepared using (reverse transcriptase<sup>2</sup>) RT<sup>2</sup> First Strand cDNA kit (SABiosciences, Qiagen Sciences, MD). The libraries were screened using pooled plasma samples from the 12 h time point for each APAP dosing parameter using saline injected mice as controls (5 mice/group). The



**Figure 2 Increased hepatotoxicity (measured in elevated alanine aminotransferase levels) in lethally dosed acetaminophen mice over time.** <sup>a</sup> $P < 0.05$  vs 0.5 h time point for the 150 mg/kg and 500 mg/kg treatment groups, respectively. ALT: Alanine aminotransferase.

screening libraries utilized were the RT<sup>2</sup> miRNA PCR arrays for mouse whole genome, per the manufacturer's protocol (SABiosciences, Qiagen Sciences, MD). Real-time quantitative polymerase chain reaction (QPCR) was performed using RT<sup>2</sup> QPCR SYBR green MasterMix (SABiosciences, Qiagen Sciences, MD) and the iCycler iQ Cyclor (Bio-Rad Laboratories, Inc, Hercules, CA).

QPCR data were analyzed using manufacturer software (<http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php>).

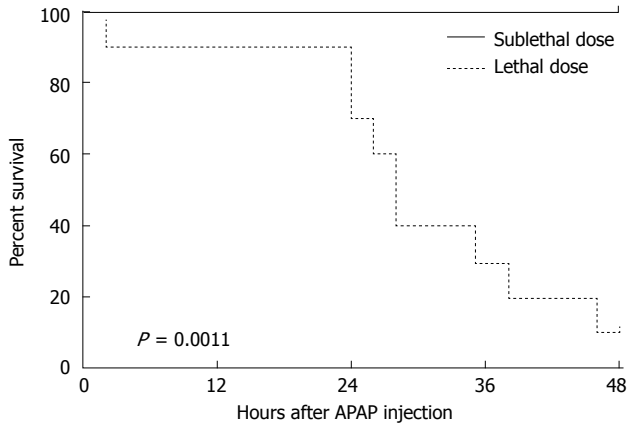
## RESULTS

### APAP toxicity confirmation: ALT levels and survival

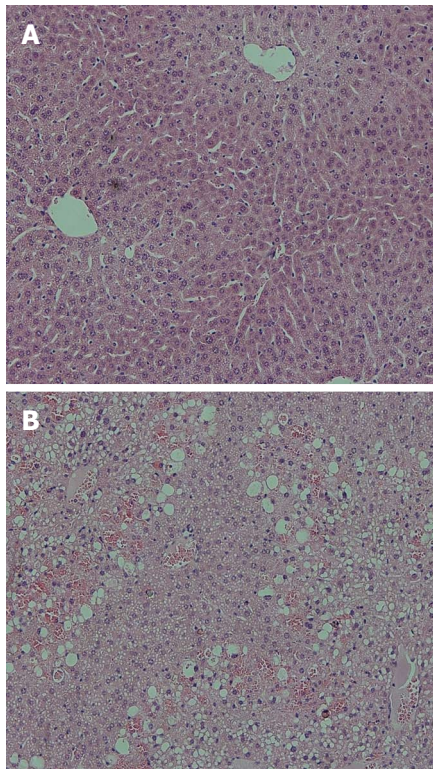
Numerous plasma miRNAs were both upregulated and downregulated, respectively, for the lethally compared to sublethally dosed APAP mice (Figure 1). Increase in serum levels of ALT is a well characterized marker of liver injury in the clinical setting as well as in animal models. Hepatotoxicity induced by APAP administration in our experiments was confirmed utilizing an ALT assay. An ALT average for the sublethal APAP dosing (150 mg/kg) peaked at 883 IU/L and for the lethal APAP dosing (500 mg/kg) peaked at 7396 IU/L, both at 24 h (Figure 2). There was a statistically significant increase in ALT levels in the lethal dosing compared to the sublethal dosing groups (642 IU/L compared to 6796 IU/L, respectively) starting at the 12 h time point ( $P < 0.001$ ). There remained statistically significant increased ALT levels for both the sublethal and lethal dosed groups at the 48 h time point compared to the 0.5 h and 2 h time points (Figure 2).

With administration of a high dose APAP (500 mg/kg), we found that there was 90% lethality compared to no lethality with the sublethal dosing (150 mg/kg) at the 48 h time point as demonstrated by the nonparametric maximum likelihood estimate of a Kaplan-Meier survival curve (Figure 3). This difference was statistically significant at  $P = 0.011$ , with ten mice utilized per dosing pa-





**Figure 3** Survival of lethally compared to sublethally dosed acetaminophen mice. Kaplan-Meier survival curve over time (h) after sublethal (150 mg/kg) and lethal (500 mg/kg) acetaminophen poisoning ( $P = 0.011$ ).



**Figure 4** Histopathologic analysis of liver after lethal compared to sublethal acetaminophen dosing. Hematoxylin and eosin liver tissue staining at 12 h of (A) sublethally (150 mg/kg) acetaminophen poisoned mice, with no signs of centrilobular inflammation or necrosis; and (B) lethally (500 mg/kg) acetaminophen poisoned mice with extensive centrilobular necrosis, enlarged hepatocytes, and highly vacuolated cytoplasm.

rameter analyzed (Graphpad Prism Software; LaJolla, CA).

#### APAP toxicity confirmation: Histology

For the sublethally dosed mice, the portal and periportal regions appear normal, with no signs of centrilobular necrosis or inflammation (Figure 4A). However, for the lethally dosed mice, obvious extensive centrilobular necrosis and inflammation were present, with distinctively en-

**Table 1** Greatest fold plasma microRNA changes in lethally dosed acetaminophen mice

MicroRNA	Fold increase	MicroRNA	Fold decrease
574-5p	203.7	342-3p	0.0005
135a*	173.6	195	0.0041
466g	110.7	375	0.0085
1196	82.7	29c	0.0134
466f-3p	71	148a	0.0152
877	64.4	652	0.0199
139-3p	59.7	202-5p	0.0317
686	48.8	200a	0.039
346	47.5	320	0.0422
149	34.9	374*	0.0508
485	34.5	9*	0.0556
409-3p	30.7	342-5p	0.0629
202-3p	28.1	192	0.0656
298	27.4	412	0.0713
15a	27.1	1	0.0775
341	26.2	199b	0.0775
296-3p	24.3	741	0.0902
466i	22.1	100	0.1145
1186	21.4	18b	0.1604
200a	20.5	122	0.2348

larged hepatocytes and highly vacuolated cytoplasm. The lethal dosed liver also demonstrated pyknotic nuclei with extensive ballooning vacuolar degeneration (Figure 4B).

#### MicroRNA profile of APAP toxicity

In an attempt to evaluate plasma miRNAs as potential indicators of APAP-induced liver damage, we screened plasma of mice after administration of a lethal or sublethal dose of APAP. Out of the 528 murine miRNAs analyzed, there were more than 40 potential miRNAs that were both greater than 2-fold up- and downregulated in the lethal (500 mg/kg) compared to sublethal (150 mg/kg) dosing (Table 1). The miRNAs listed were effectively detected suggesting the actual fold-change value is as large as the calculated and reported fold-change result (SABiosciences, Qiagen Sciences, MD). The small nucleolar RNA, C/D Box 68 (Snord68), was used as the internal control for each library evaluated.

Finally, we performed an extensive literature search to gather information on the known and putative targets of 12 miRNAs that were uniquely changed in the plasma after administration of the lethal dose of APAP (Table 2). Interestingly, we found 6 out of the top 12 miRNAs with the greatest fold change (both up- and downregulated) in the lethally compared to sublethally dosed APAP mice were associated with asthma. The other miRNAs found both highly up- and downregulated were found to be associated with hypoxia-inducible factor (HIF)-1<sup>[15]</sup>, follicle stimulating hormone<sup>[16]</sup>, type 1 diabetes<sup>[17]</sup>, procollagen type III<sup>[18]</sup>, colon cancer<sup>[19]</sup>, gastric carcinoma<sup>[20]</sup>, and elongation factor 2 tumor suppression<sup>[21]</sup> (Table 2).

## DISCUSSION

Our study reveals numerous miRNAs, notably 574-5p, 135a\*, 466g, 1196, 466f-3p, and 877, are upregulated



**Table 2** Lethal acetaminophen associated plasma microRNAs and potential correlative function

MicroRNA	Function
574-5p	Acute and chronic asthma (Garbacki <i>et al</i> <sup>[23]</sup> ); Procollagen type III A1 (Sterling <sup>[18]</sup> )
135a	Hypoxia-inducible factor-1 and nuclear factor- $\kappa$ B production (Gonsalves <i>et al</i> <sup>[15]</sup> )
466g	Acute and chronic asthma (Garbacki <i>et al</i> <sup>[23]</sup> )
1196	Follicle-stimulating hormone regulation (Yao <i>et al</i> <sup>[16]</sup> )
466f-3p	Acute and chronic asthma (Garbacki <i>et al</i> <sup>[23]</sup> ); Procollagen type III A1 (Sterling <sup>[18]</sup> )
877	Human type 1 diabetes (Zhou <i>et al</i> <sup>[17]</sup> )
139-3p	Colon and rectal cancer (Slattery <i>et al</i> <sup>[19]</sup> )
342-3p	HBV infection and HBV-positive hepatocarcinoma biomarker (Li <i>et al</i> <sup>[10]</sup> )
195	E2F tumor suppressor (Xu <i>et al</i> <sup>[21]</sup> )
375	Acute and chronic asthma (Garbacki <i>et al</i> <sup>[23]</sup> ); Pyruvate dehydrogenase kinase inhibition in gastric carcinomas (Tsukamoto <i>et al</i> <sup>[20]</sup> )
29c	Acute and chronic asthma (Garbacki <i>et al</i> <sup>[23]</sup> )
148a	HLA-G and risk asthma (Tan <i>et al</i> <sup>[22]</sup> )

HBV: Hepatitis B virus; E2F: Elongation factor 2; HLA-G: Human leukocyte antigen-G.

in the setting of lethal compared to sublethal APAP-associated hepatotoxicity, whereas miRNAs 342-3p, 195, 375, 29c, 148a and 652 are markedly downregulated. We demonstrate elevated ALT levels as well as histologic evidence supporting worsened hepatotoxicity in the setting of lethally dosed mice compared to non-lethally dosed mice (Figures 2 and 4). With 90% lethality in the lethally dosed mice in relation to no lethality in sublethally dosed mice ( $P = 0.0011$ ), this supports the premise that unique plasma miRNA profiles may correlate with non-*vs* life-threatening APAP dosing (Figure 3). The fold-change of a variety of miRNAs in the setting of lethally dosed mice compared to sublethal doses is of interest. Of note, more than 40 were both up- and downregulated, with the greatest fold miRNA changes reported (Table 1).

A literature search to investigate possible functions of the miRNAs both up- and downregulated was undertaken. Intriguingly, many of those most up- and downregulated in the lethally compared to sublethally dosed mice, namely 574-5p, 466g, 466f-3p, 375, 29c, and 148a, have also been implicated in the development of asthma<sup>[22,23]</sup>. For instance, 574-5p may be involved with asthma pathogenesis, with decreased miRNA 574-5p in chronic compared to acute asthma in a mouse model sensitized with ovalbumin<sup>[23]</sup>. In another study, a potential relationship between histocompatibility antigen-G, chronic asthma, and miRNA 29c was determined<sup>[22]</sup>. This consequently suggests a pathophysiologic relationship between APAP toxicity and asthma<sup>[22,23]</sup>.

Interestingly, prior research has shown an association between APAP use and asthma although the exact association is still unclear<sup>[24,25]</sup>. For instance, an adult case control study described a relationship between acetaminophen use and asthma<sup>[26]</sup>. Additional literature revealed an increased risk of wheeze in children whose mothers

used prenatal APAP<sup>[27]</sup>. The etiology still remains unclear. However, one theory is that decreased glutathione (due to depletion secondary to APAP toxicity) provides the opportunity for unchecked reactive oxygen species to promote asthma development<sup>[24]</sup>. Additional theories include increased prostaglandin E2 production secondary to elevated cyclooxygenase-2 activity in the presence of APAP promoting a T2 allergic response<sup>[25]</sup>. A third cause could be direct lung damage from NAPQI, a byproduct of APAP metabolism<sup>[28]</sup>. Clearly, more information is needed to further elucidate the relationship between APAP and risk of asthma.

The previous literature describing the function of the other miRNAs up- and downregulated in our study is more varied. For example, some literature reveals a miRNA 135a association with HIF 1- $\alpha$ <sup>[15]</sup>. Interestingly, previous literature has demonstrated HIF 1- $\alpha$  induction prior to APAP toxicity in the setting of lethal APAP dosing, with toxicity prevented by the presence of cyclosporine A, a HIF 1- $\alpha$  inhibitor which prevents mitochondrial permeability transition and oxidative stress<sup>[29]</sup>. Additional studies have also shown elevation of HIF 1- $\alpha$  in the setting of APAP toxicity, with increased HIF 1- $\alpha$  causing increased glucose transporter-1 expression<sup>[30]</sup>. Of note, miRNAs 195 and 342-3p have been shown as involved with hepato cytopathology in the setting of tumor suppression in hepatocellular carcinoma models<sup>[21]</sup> and hepatitis B virus hepatocarcinoma diagnosis<sup>[10]</sup>, respectively.

However, how these miRNAs in total affect hepatotoxicity in the setting of APAP poisoning still needs to be elucidated. Prior studies reveal upregulation of plasma miR-122 in the setting of APAP-associated liver toxicity, while our data suggest downregulation of plasma miR-122 at the 12 h time point. This discrepancy could be potentially explained by examination of upregulation of miR-122 at 1 h, 3 h and 24 h time points, not at a 12 h time point in previous literature. In addition, different APAP dosing levels were used in each study (300 mg/kg compared to 500 mg/kg), again demonstrating the dynamic nature of miRNA regulation across both time and clinical setting<sup>[11]</sup>. Of note, we found upregulation of plasma miR-298 and miR-370, whereas other researchers found downregulation of these miRNAs in the setting of APAP-associated hepatotoxicity<sup>[31]</sup>. Again, this may be due to evaluation at different time points (6 h compared to 12 h) and differing APAP dosing parameters (1000 mg/kg compared to 500 mg/kg)<sup>[31]</sup>. Together, these results demonstrate the need for further identification of additional plasma microRNA profiles at various time points and dosing levels.

Our ultimate goal would be to eventually have a miRNA APAP nomogram to be used for human patient care, similar to the previous effective Rumack-Matthew nomogram<sup>[32,33]</sup>. The problem with this current nomogram, however, is that it relies on knowing when a patient initially ingested APAP. This is often difficult, if patients are poor historians or have ingested mind-altering substances

such as alcohol at the time of evaluation. In addition, patients may have been taking APAP chronically, not acutely, making use of the Rumack-Matthew nomogram pointless. The importance of our work is to establish a novel miRNA nomogram that would be used for patients who have taken APAP at an unknown time or with chronic ingestions. In turn, this would avoid investigation of patients who have taken it chronically, thus preventing unnecessary treatment and iatrogenic ingestions. Additional studies in this field, with additional time points and dosing levels, however, are clearly still necessary.

The specificity of this profile also requires further improvement. For instance, in a recent report miRNA-122 was shown to be increased in the setting of APAP toxicity, although this increase was under the detection cut-off of our study<sup>[11]</sup>. Of note, miRNA-122 is also upregulated in hepatitis C settings which, by itself, is evidently not specific enough to uniquely identify APAP toxicity<sup>[14]</sup>. However, with additional future studies and data analysis, this may be possible. This approach may also be used as a model to develop profiles for additional disease processes, namely non-alcoholic fatty liver disease and hepatocellular carcinoma, since microRNA profiles are already being used as early biomarkers for numerous pathologic states<sup>[9,10]</sup>. Together, this may improve the diagnostic accuracy of hepatopathology, namely early APAP-induced hepatotoxicity. In turn, this may allow clinicians to better and more rapidly distinguish which patients who have ingested APAP will actually mandate therapy. Subsequently, this may result in decreasing the number of patients who receive unnecessary, expensive empiric treatment.

In conclusion, lethal dosing of APAP in a murine model is consistent with hepatotoxicity and up- and downregulation of a unique pattern of circulating plasma miRNAs, which is different from the plasma miRNA profile associated with sublethal APAP dosing. These differences may be useful in the future to distinguish lethal and sublethal APAP toxicity in humans.

## COMMENTS

### Background

Acetaminophen (APAP) continues to be an important cause of acute liver failure in the developed world, being the most common cause of death due to analgesic ingestion in the United States. The authors report unique microRNA (miRNA) profiles associated with lethal acetaminophen poisoning. Determining which specific miRNAs are associated with lethal acetaminophen toxicity may prove helpful in the future for prognosticating which patients will require N-acetylcysteine treatment.

### Innovations and breakthroughs

The importance of our work is to establish a novel miRNA nomogram that would be used for patients who have taken APAP at an unknown time or with chronic ingestions. This would be the first miRNA profile that could prognosticate patients who will require treatment for hepatotoxicity due to acetaminophen poisoning.

### Applications

The ultimate goal is to eventually have a miRNA APAP nomogram to be used for human patient care. The limitation of the current Rumack-Matthew nomogram for APAP toxicity is that it relies on knowing the time of APAP ingestion. This is often difficult if patients are poor historians or have ingested mind-altering substances at the time of evaluation, or have been chronically ingesting APAP.

### Terminology

Recent work has also shown the medical utility of miRNA. miRNAs, are short, chemically stable biomolecules that produce gene silencing. Furthermore, additional literature has described miRNA involvement in acetaminophen toxicity, as well as the utility of miRNAs as biomarkers.

### Peer review

The manuscript provides some interesting information on the potential use of miRNAs for the early diagnosis of APAP associated liver toxicity. The scientific goal of the study has significant clinical application but the interpretation of the data needs to be revised.

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## Pre-diagnostic levels of adiponectin and soluble vascular cell adhesion molecule-1 are associated with colorectal cancer risk

Mathilde Touvier, Léopold Fezeu, Namanjeet Ahluwalia, Chantal Julia, Nathalie Charnaux, Angela Sutton, Caroline Méjean, Paule Latino-Martel, Serge Hercberg, Pilar Galan, Sébastien Czernichow

Mathilde Touvier, Léopold Fezeu, Namanjeet Ahluwalia, Chantal Julia, Caroline Méjean, Paule Latino-Martel, Serge Hercberg, Pilar Galan, INSERM U557 (National Institute of Health and Medical Research), Inra, Cnam, University of Paris 13 (UREN), SMBH, 74 rue Marcel Cachin, F-93017 Bobigny, France

Nathalie Charnaux, Angela Sutton, Department of Biochemistry, Jean Verdier Hospital, F-93140 Bondy, France

Nathalie Charnaux, Angela Sutton, INSERM U698, University of Paris 13, F-93017 Bobigny, France

Serge Hercberg, Department of Public Health, Avicenne Hospital (AP-HP), University of Paris 13, F-93017 Bobigny, France

Sébastien Czernichow, Department of Nutrition, Ambroise Paré Hospital (AP-HP), University of Versailles Saint Quentin, F-92100 Boulogne-Billancourt, France

**Author contributions:** Touvier M and Czernichow S designed the research; Charnaux N, Sutton A, Hercberg S and Galan P collected the data; Touvier M analysed the data and drafted the manuscript; Touvier M, Fezeu L, Ahluwalia N, Julia C, Charnaux N, Sutton A, Méjean C, Latino-Martel P, Hercberg S, Galan P and Czernichow S interpreted the data, revised each draft and approved the final version of the manuscript.

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**Correspondence to:** Dr. Mathilde Touvier, PhD, INSERM U557 (National Institute of Health and Medical Research), Inra, Cnam, University of Paris 13 (UREN), SMBH, 74 rue Marcel Cachin, F-93017 Bobigny,

France. [m.touvier@uren.smbh.univ-paris13.fr](mailto:m.touvier@uren.smbh.univ-paris13.fr)

Telephone: +33-1-48388954 Fax: +33-1-48388931

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**METHODS:** A nested case-control study was designed to include all first primary incident colorectal cancer cases diagnosed between inclusion in the SUPplémentation en VItamines et Minéraux AntioXydants cohort in 1994 and the end of follow-up in 2007. Cases ( $n = 50$ ) were matched with two randomly selected controls ( $n = 100$ ). Conditional logistic regression models were used to investigate the associations between pre-diagnostic levels of hs-CRP, adiponectin, leptin, soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1, E-selectin, monocyte chemoattractant protein-1 and colorectal cancer risk. Area under the receiver operating curves (AUC) and relative integrated discrimination improvement (RIDI) statistics were used to assess the discriminatory potential of the models.

**RESULTS:** Plasma adiponectin level was associated with decreased colorectal cancer risk ( $P$  for linear trend = 0.03). Quartiles of sVCAM-1 were associated with increased colorectal cancer risk ( $P$  for linear trend = 0.02). No association was observed with any of the other biomarkers. Compared to standard models with known risk factors, those including both adiponectin and sVCAM-1 had substantially improved performance for colorectal cancer risk prediction ( $P$  for AUC improvement = 0.01, RIDI = 26.5%).

**CONCLUSION:** These results suggest that pre-diagnostic plasma adiponectin and sVCAM-1 levels are associated with decreased and increased colorectal cancer risk, respectively. These relationships must be confirmed in large validation studies.

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**Key words:** Colorectal cancer; Adiponectin; Soluble vascular cell adhesion molecule-1; Nested case-control study;

### Abstract

**AIM:** To examine the relationships between pre-diagnostic biomarkers and colorectal cancer risk and assess their relevance in predictive models.



## Prospective study

**Peer reviewers:** Dr. Inti Zlobec, PhD, Institute for Pathology, University Hospital Basel, Schoenbeinstrasse 40, CH-4031 Basel, Switzerland; Dr. Thomas Wex, PD, Clinic of Gastroenterology, Hepatology and Infectious Diseases, Otto-von-Guericke University Magdeburg, Leipziger Str. 44, 39120 Magdeburg, Germany

Touvier M, Fezeu L, Ahluwalia N, Julia C, Charnaux N, Sutton A, Méjean C, Latino-Martel P, Hercberg S, Galan P, Czernichow S. Pre-diagnostic levels of adiponectin and soluble vascular cell adhesion molecule-1 are associated with colorectal cancer risk. *World J Gastroenterol* 2012; 18(22): 2805-2812 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2805.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2805>

## INTRODUCTION

Colorectal cancer is the third most frequently diagnosed cancer worldwide, accounting for more than one million cases and 600 000 deaths every year<sup>[1]</sup>. The identification of pre-diagnostic biomarkers associated with subsequent colorectal cancer risk is a key challenge. Markers of adiposity, endothelial adhesion, and inflammation may be suitable candidates<sup>[2-5]</sup>. Adipose tissue is an endocrine organ that produces adipokines and plays a critical role in the regulation of inflammatory processes<sup>[6]</sup>. Leptin reflects body fat storage and acts as a pro-inflammatory adipokine. Conversely, adiponectin production is decreased in obesity and generally has anti-inflammatory properties. Adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and the chemokine monocyte chemoattractant protein-1 (MCP-1) are important in cell-cell and cell-basement membrane interactions. They are also intimately involved in inflammatory reactions<sup>[7]</sup>. C-reactive protein (CRP) is a widely used systemic biomarker for diagnosing acute and chronic inflammation<sup>[8]</sup>.

Previous cross-sectional studies suggest the potential involvement of these biomarkers in colorectal carcinogenesis, with higher blood levels of CRP<sup>[9]</sup>, leptin<sup>[10]</sup>, soluble adhesion molecules<sup>[11,12]</sup>, and lower levels of adiponectin<sup>[10,13]</sup> observed in patients with colorectal cancer compared to controls. The prognostic value of these markers has also been suggested by research with colorectal cancer patients<sup>[10,12]</sup>. However, few prospective studies have investigated the association between these biomarkers and colorectal cancer risk, and the current evidence is conflicting<sup>[14-19]</sup>. In addition, such studies did not evaluate the discriminatory capabilities of these biomarkers regarding colorectal cancer risk by contemporary statistical methods<sup>[20,21]</sup>.

Thus, our objectives were twofold: (1) to prospectively examine the relationships between biomarkers of adiposity, endothelial adhesion, and inflammation and development of colorectal cancer; and (2) to statistically compare the pertinence of models including these bio-

markers to standard models with known risk factors of colorectal cancer.

## MATERIALS AND METHODS

### Study population

The SUPplémentation en Vitamines et Minéraux AntioXydants (SU.VI.MAX) study is a population-based, double-blind, placebo-controlled, randomized trial initially designed to assess the effect of a daily antioxidant supplementation on the incidence of cardiovascular disease and cancer<sup>[22,23]</sup>. A total of 13 017 subjects were enrolled in 1994-1995. The intervention study lasted 8 years, and follow-up of health events was maintained until July 2007. Subjects provided written informed consent and the study was approved by the Ethics Committee for Studies with Human Subjects at the Paris-Cochin Hospital, "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale", No. 706 and the "Commission Nationale de l'Informatique et des Libertés", No. 334641.

### Baseline data collection

At enrolment, all participants underwent a clinical examination and anthropometric measurements carried out by study nurses and physicians. The participants also completed questionnaires on socio-demographic data, smoking, alcohol intake and physical activity. A fasting venous blood sample was obtained. Plasma aliquots were immediately prepared and stored frozen in liquid nitrogen.

### Case ascertainment

Confirmed or suspected cancer events were self-reported by subjects during the follow-up process. Investigations were conducted for all such events to obtain medical data from participants, physicians and/or hospitals. All information was reviewed by an independent expert committee and cancer cases were validated by pathological report and classified using the International Chronic Diseases Classification, 10th Revision, Clinical Modification.

### Nested case-control study

All first primary incident colorectal cancer cases diagnosed between inclusion in the SU.VI.MAX cohort in 1994 and July 2007 were included in the present study. For each cancer case, two controls were randomly selected among the remaining participants with complete follow-up data and without cancer diagnosis by the end of follow-up. Cases and controls were matched for sex, age (by 2-year strata), body mass index (BMI,  $<_{\text{N}} \geq 25 \text{ kg/m}^2$ ) and intervention group.

Baseline plasma samples of the selected subjects were used to determine the levels of highly-sensitive CRP (hs-CRP), leptin, adiponectin, soluble ICAM-1 (sICAM-1), soluble VCAM-1 (sVCAM-1), soluble E-selectin (sE-selectin) and MCP-1. Biomarker levels were determined with ELISA sandwich technique (R and D Laboratory Systems). Intra-assay (IACV) and inter-assay (IRCV) co-

**Table 1** Baseline characteristics of colorectal cancer cases and controls

	Cases ( <i>n</i> = 50)		Controls ( <i>n</i> = 100)		<i>P</i> value <sup>1</sup>
Age, yr	51.8	± 5.6	52.1	± 5.6	0.8
Gender					1.0
Men	28	56.0%	56	56.0%	
Women	22	44.0%	44	44.0%	
Intervention group					1.0
Yes	27	54.0%	54	54.0%	
No (placebo)	23	46.0%	46	46.0%	
BMI, kg/m <sup>2</sup>					1.0
< 25	24	48.0%	48	48.0%	
≥ 25	26	52.0%	52	52.0%	
Waist circumference, cm	88.2	± 12.9	82.2	± 12.1	0.01
Height, cm	169.3	± 7.1	167.8	± 8.5	0.3
Smoking status					0.9
Never smoker	23	46.0%	46	46.0%	
Former smoker	21	42.0%	40	40.0%	
Current smoker	6	12.0%	14	14.0%	
Alcohol intake, g/d	24	± 24.4	15.4	± 16.3	0.01
Physical activity					0.4
Low	10	20.0%	26	26.0%	
Moderate	18	36.0%	25	25.0%	
High	22	44.0%	49	49.0%	
Educational level, yr					0.9
< 12	30	60.0%	61	61.0%	
≥ 12	20	40.0%	39	39.0%	
Family history of colorectal cancer <sup>2</sup>					0.3
No	45	90.0%	84	84.0%	
Yes	5	10.0%	16	16.0%	
Plasma levels of biomarkers					
Adiponectin, µg/mL	9.0	± 4.7	10.9	± 7.5	0.2
Leptin, ng/mL	8.5	± 5.3	8.6	± 8.7	0.5
sVCAM-1, ng/mL	750.3	± 316.2	677.6	± 215.3	0.2
sICAM-1, ng/mL	249.7	± 80.3	247.8	± 67.3	0.9
sE-selectin, ng/mL	41.1	± 16.9	39.3	± 16.0	0.7
MCP-1, pg/mL	268.2	± 117.4	249	± 78.2	0.3
hs-CRP, mg/L	2.4	± 4.5	2.2	± 4.4	0.3

<sup>1</sup>*P* value for the comparison of cases and controls by Student *t* test or  $\chi^2$  test, as appropriate. Biomarker variables were log-transformed to improve normality. Values are mean ± SD or *n* % as appropriate. <sup>2</sup>In first degree relatives. BMI: Body mass index; hs-CRP: Highly sensitive C-reactive protein; sICAM-1: Soluble intercellular adhesion molecule-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; sE-selectin: Soluble E-selectin; MCP-1: Monocyte chemoattractant protein-1.

efficients of variation were all < 10%. hs-CRP had the lowest (1.6%) and MCP-1 had the highest (6.2%) IACV, and hs-CRP had the lowest (3.6) and sE-selectin had the highest (9.1%) IRCV.

### Statistical analyses

The participants' baseline characteristics were compared between colorectal cancer cases and controls using Student's *t*-tests or  $\chi^2$  tests. Associations between biomarkers and incident colorectal cancer were examined with conditional logistic regression models and expressed as odds ratios (OR) with 95% confidence intervals (CI). The ORs for sex-specific quartiles and for a 1 standard deviation (SD) increase in the corresponding biomarker were com-

puted in unadjusted and multivariate models. Multivariate models were adjusted for age, sex, BMI, height, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference and educational level.

The improvement in colorectal cancer prediction performance attributed to the biomarkers was assessed with both the area under the receiver operating curves (AUC) and the more recently proposed statistical tool, the Relative Integrated Discrimination Improvement (RIDI)<sup>[21]</sup>. The latter measures the percentage of increased discrimination upon addition of another variable to the prediction model. The Bootstrap method was used to derive the 95% CI for the RIDI estimates, which were based on 1000 replications. The added prediction performance was determined separately for each biomarker identified as statistically significantly associated with cancer risk (in the logistic regression analyses step), and then for a combination of these biomarkers simultaneously. Tests of significance for AUC improvement were one-sided, as improvement in model fit was expected. All other statistical tests were two-sided, and *P* < 0.05 was considered significant. Analyses were performed with SAS software (v9.1, Cary, NC, United States).

## RESULTS

A total of 50 incident colorectal cancer cases were diagnosed during follow-up (30 colon and 20 rectal cancers). Each case was matched with two randomly selected controls; thus, 150 subjects were included in the analyses. Median follow-up was 6.5 years in cases and 13 years in controls. Baseline characteristics of cases and non-cases are presented in Table 1. Compared to controls, cancer cases had a higher waist circumference and a higher alcohol intake.

In multivariate models, a one SD change in plasma adiponectin level was associated with a decreased colorectal cancer risk [OR (95% CI) = 0.45 (0.22-0.91), *P* = 0.03]. This association was also observed when adiponectin was considered as quartiles (OR for Q4 *vs* Q1 = 0.11 (0.01-0.93), *P* for linear trend = 0.03) (Table 2).

Quartiles of plasma sVCAM-1 level were positively associated with increased colorectal cancer risk (*P* for linear trend = 0.02) (Table 2). This association was borderline non-significant when sVCAM-1 was coded as a continuous variable (*P* = 0.07).

Unadjusted models (matching factors only) showed similar results (data not shown). A sensitivity analysis excluding cases that were diagnosed during the first two years of follow-up (7 cases) did not modify the findings, nor did sensitivity analyses excluding subjects with high hs-CRP values (> 15.5 ng/mL, i.e., mean + 3SD, *n* = 3 subjects; data not shown).

Indicators of the predictive potential of colorectal cancer risk models (Table 3) showed improvement when adiponectin alone was included in the multivariate model

**Table 2** Odds ratios and 95% confidence intervals for quartiles of each biomarker level and colorectal cancer risk from multivariate conditional logistic regression models<sup>1</sup>

	For a change in 1SD	Quartile1	Quartile2	Quartile3	Quartile4
Adiponectin					
OR	0.45	1 (ref)	0.83	0.42	0.11
95% CI	0.22-0.91		0.12-5.65	0.06-2.93	0.01-0.93
P for linear trend	0.03				0.03
Leptin					
OR	0.55	1 (ref)	0.19	2.22	0.29
95% CI	0.21-1.40		0.02-1.9	0.25-20.09	0.02-3.65
P for linear trend	0.2				0.6
sVCAM-1					
OR	1.69	1 (ref)	6.89	11.59	19.11
95% CI	0.96-2.98		0.72-66.4	0.64-209.81	1.4-261.27
P for linear trend	0.07				0.02
sICAM-1					
OR	0.74	1 (ref)	0.38	0.07	0.13
95% CI	0.40-1.40		0.04-3.23	0.01-0.76	0.01-1.93
P for linear trend	0.4				0.08
sE-selectin					
OR	0.95	1 (ref)	1.23	0.9	1.59
95% CI	0.49-1.81		0.13-11.49	0.1-8.14	0.16-15.62
P for linear trend	0.9				0.9
MCP-1					
OR	1.35	1 (ref)	1.67	1.02	2.02
95% CI	0.73-2.49		0.27-10.24	0.17-6.24	0.26-15.97
P for linear trend	0.3				0.4
hs-CRP					
OR	0.8	1 (ref)	0.73	2.22	1.53
95% CI	0.52-1.24		0.06-9.38	0.25-19.9	0.13-17.84
P for linear trend	0.3				0.6

<sup>1</sup>Adjusted for age, sex, body mass index, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference, height and educational level. *n* = 50 colorectal cancer cases and 100 controls. Cut-offs for sex-specific quartiles were: hs-CRP: 0.6, 1.2, 2.3 in men and 0.5, 0.9, 2.1 in women; sICAM-1: 198.7, 242.0, 287.4 in men and 193.0, 232.5, 286.0 in women; sVCAM-1: 539.0, 653.5, 798.6 in men and 523.0, 651.5, 875.7 in women; sE-selectin: 29.8, 42.5, 51.7 in men and 26.0, 36.3, 44.6 in women; MCP-1: 216.7, 263.5, 316.5 in men and 171.0, 212.0, 238.0 in women; Leptin: 3.1, 5.0, 8.2 in men and 5.4, 9.6, 15.4 in women; Adiponectin: 4.2, 6.6, 10.0 in men and 9.5, 13.9, 16.0 in women. OR: Odds ratio; CI: Confidence interval; hs-CRP: Highly sensitive C-reactive protein; sICAM-1: Soluble intercellular adhesion molecule-1; sVCAM-1: Soluble vascular cell adhesion molecule-1; sE-selectin: Soluble E-selectin; MCP-1: Monocyte chemoattractant protein-1; ref: Reference category.

(*P* for AUC improvement = 0.009). The RIDI statistic indicated a 12.2% (10.9-13.6) improvement. Improvement in the prediction of colorectal cancer risk was limited when sVCAM-1 only was introduced into the multivariate model (*P* for AUC improvement = 0.09), with 9.9% (8.7-11.0) improvement, as indicated by the RIDI statistic. Prediction was substantially improved when adiponectin and sVCAM-1 were simultaneously included in the multivariate model: *P* for AUC improvement was equal to 0.01, and the RIDI reached 26.5% (24.4-28.7).

## DISCUSSION

In this prospective study, pre-diagnostic plasma adiponectin level was associated with decreased colorectal cancer risk, independently of other known risk factors. On the contrary, plasma sVCAM-1 level was associated with increased colorectal cancer risk. Models including these two biomarkers showed significantly improved discriminatory capabilities compared to models including only established risk factors.

Lower levels of circulating adiponectin have been ob-

served in prevalent colorectal cancer cases compared to controls<sup>[10,13,24-26]</sup>. Single nucleotide polymorphism analyses have found that some variants of the adiponectin genes are related to either increased (rs822395, rs1342387) or decreased (rs266729) colorectal cancer risk<sup>[27]</sup>, although no association was detected in a recent study in the United Kingdom<sup>[28]</sup>. Another study suggested that variants of the adipokine genes may affect colorectal cancer risk in combination with variants in diabetes-related genes<sup>[29]</sup>. Studies with colorectal cancer patients showed that higher adiponectin levels were associated with a better prognosis<sup>[10,13,30]</sup>. It has been suggested that adiponectin may be used for estimation of advanced stage of cancer and for estimating risk of cancer recurrence<sup>[31]</sup>. However, to date, only three nested case-control studies have investigated the prospective association between adiponectin and colorectal cancer risk, showing inconsistent results<sup>[16-18]</sup>. Two studies did not find any associations; one of them included 381 male colorectal cancer cases<sup>[17]</sup> and the other included 306 colorectal cancer cases of both genders<sup>[16]</sup>. Consistent with our findings, the study of Wei *et al.*<sup>[18]</sup>, based on 179 male colorectal cancer cases, found an

**Table 3** Predictive potential of adiponectin and soluble vascular cell adhesion molecule-1 regarding colorectal cancer risk: Relative integrated discrimination improvement and improvement of area under the curve

	AUC	P value for AUC improvement	RIDI (%)	95% CI
Multivariate model <sup>1</sup>	0.89			
+ Adiponectin	0.98	0.009	12.2	10.9-13.6
+ sVCAM-1	0.92	0.09	9.9	8.7-11.0
+ Adiponectin + sVCAM-1	0.98	0.01	26.5	24.4-28.7

<sup>1</sup>Multivariate model was adjusted for age, sex, BMI, intervention group, alcohol intake, physical activity, smoking status, family history of colorectal cancer, waist circumference, height and educational level. Models including adiponectin and/or sVCAM-1 were compared to the multivariate model. *n* = 50 colorectal cancer cases and 100 controls. BMI: Body mass index; RIDI: Relative integrated discrimination improvement; AUC: Area under the receiver operating curve; sVCAM-1: Soluble vascular cell adhesion molecule-1; CI: Confidence interval.

inverse association between pre-diagnostic adiponectin levels and colorectal cancer risk. Circulating levels of adiponectin in those studies were comparable to the levels found in the present study. However, none of those three studies matched cases and controls on BMI. Adiponectin is strongly related to adiposity, which is, in turn, associated with an adverse effect on colorectal cancer development, especially in statin-positive patients, as recently shown by Ogino *et al.*<sup>[32]</sup>. Thus, matching on BMI is crucial and is a strength of our study compared to previous reports in the literature. Several mechanisms support the inverse relationship between adiponectin and colorectal cancer risk<sup>[33]</sup>. Adiponectin suppresses tumorigenesis in Apc(Min)(+/+) mice<sup>[34]</sup> and also suppresses colonic epithelial proliferation *via* inhibition of the mammalian target of the rapamycin (mTOR) pathway under a high-fat diet<sup>[35]</sup>. It inhibits colorectal cancer cell growth through the AMP-activated protein kinase/mTOR pathway<sup>[36]</sup> and possibly the PI3K/Akt signal pathway<sup>[37]</sup>. Adiponectin also attenuates interleukin-6-induced colon carcinoma cell proliferation *via* STAT-3<sup>[38]</sup>.

Several case-control studies have observed higher circulating levels of sVCAM-1 in colorectal cancer cases compared to controls<sup>[11,12,39-41]</sup>. In addition, it has been suggested that the serum level of sVCAM-1 may be a valuable prognostic marker in colorectal carcinoma<sup>[12,42]</sup>, reflecting both tumour progression and metastasis<sup>[39]</sup>. For instance, Mantur *et al.*<sup>[11]</sup> observed a significant correlation of serum levels of sVCAM-1 with tumor, node, metastases (TNM) stage and lymph node involvement in colorectal cancer patients. Yamada *et al.*<sup>[43]</sup> observed a positive association between concentrations of sVCAM-1 and risk of post-operative colorectal cancer recurrence. Consequently, investigations have been conducted to test for the chemopreventive potential of some molecules (e.g., celecoxib) *via* down-regulation of VCAM-1 in the colon cancer cell line HT29<sup>[44]</sup>.

However, to the best of our knowledge, our study is the first to investigate the prospective association be-

tween pre-diagnostic levels of sVCAM-1 and colorectal cancer risk. The observed positive association is supported by a mechanistic plausibility. Indeed, it has been demonstrated experimentally that sVCAM-1 stimulates angiogenesis and neovascularization<sup>[45,46]</sup> and is negatively correlated with the degree of tumour differentiation<sup>[41]</sup>. Cell adhesion molecule expression has been demonstrated in endothelial cells of small vessels at the invasive margin of tumour cells involved in metastatic spread<sup>[47]</sup>. The association among immunohistochemical cell adhesion molecule expression, tumour vascularity and leukocyte infiltration suggests an important role for these molecules in host immune response and in tumour progression<sup>[48]</sup>.

Epidemiologic studies usually estimate the strength of the association between a biomarker and disease risk. Assessment of the discriminatory capabilities of a biomarker in predicting risk of the studied pathology is another approach that may lead to slightly different but complementary information<sup>[21]</sup>. To the best of our knowledge, no study has previously evaluated the discriminatory capabilities of hs-CRP, leptin, adiponectin, sICAM-1, sVCAM-1, sE-selectin and MCP-1 in predicting colorectal cancer risk, using *ad-hoc* statistical methods such as the novel RIDI statistic<sup>[21]</sup>. Indeed, the use of the traditional AUC method as a comparative measure of prediction between models has certain limitations<sup>[49]</sup>, and the complementary use of the novel RIDI statistic appears to be more sensitive and accurate<sup>[21]</sup>. Several factors are already known to influence colorectal cancer risk (e.g., age, smoking status, physical activity, *etc.*) and are usually included in predictive models. As shown in Table 3, the RIDI statistic suggests that when quartiles of adiponectin and quartiles of sVCAM-1 plasma levels are added to the model, the ability of the model to predict colorectal cancer risk is improved by 26.5%, compared to a model including only well-established risk factors (age, smoking status, *etc.*). Thus, our results suggest that adiponectin, and possibly sVCAM-1, should not be ignored as predictors of colorectal cancer risk. In addition, the improvement in the predictive potential was substantially increased when both biomarkers were simultaneously added to the model. This might result from the mechanistic interrelations between adiposity and endothelial adhesion, notably through an inflammation pathway<sup>[6,50,51]</sup>. Large prospective and validation studies are needed to confirm and better quantify the predictive performance of these biomarkers in colorectal carcinogenesis.

Strengths of our study include its prospective design, the simultaneous measurement of seven biomarkers in the same individuals and, to our knowledge, the first assessment of the discriminatory capabilities of these biomarkers for estimating colorectal cancer risk by the novel RIDI statistic.

Some limitations should also be acknowledged. Firstly, the number of cases was limited in this exploratory study. This may explain some of the null results observed; however, it is unlikely to explain the observed relationships between adiponectin, sVCAM-1 and colorectal cancer



risk, which were statistically significant despite the limited statistical power. These associations are consistent with our initial hypothesis and are supported by available mechanistic data. Secondly, a single measurement of biomarker levels (at baseline) was performed and no indication was available regarding transient acute infection (cold, throat infection, *etc.*) concomitant with the blood draws. For some biomarkers such as hs-CRP, although the probability of differential misclassification bias between cases and controls is low, this limitation might have led to an attenuation of the strengths of the observed associations due to intra-individual variation. This may have limited our ability to detect an association between hs-CRP and colorectal cancer. Finally, the observed relationships might have been partly affected by unmeasured or residual confounders, even though such a possibility is limited since a broad range of usual risk factors were accounted for in the statistical analyses.

Our study adds to current knowledge of adiposity- and endothelial adhesion-related pathways in the development of colorectal cancer. For the first time, we have shown a prospective positive association between plasma sVCAM-1 levels and colorectal cancer risk. In addition, we observed an inverse relationship between pre-diagnostic adiponectin levels and colorectal cancer risk, which provides new insights given the conflicting literature. Our results suggest that the inclusion of adiponectin and sVCAM-1 plasma levels in prediction models of colorectal cancer risk may improve their discriminatory capabilities. Large prospective studies are needed to confirm the pertinence of these biomarkers in colorectal cancer risk prediction. If confirmed in validation studies, these results could lead to improved identification of individuals at risk of developing colorectal cancer, which could result in well-targeted cancer screening campaigns.

## COMMENTS

### Background

Previous studies suggest an association between biomarkers of adiposity, endothelial adhesion and inflammation and colorectal cancer risk, but prospective data are limited and evaluation of predictive performance is lacking.

### Research frontiers

Previous cross-sectional and case-control studies have suggested the potential involvement of such biomarkers in colorectal carcinogenesis, with higher blood levels of soluble adhesion molecules and lower levels of adiponectin in patients with colorectal cancer compared to controls. The prognostic value of these markers has also been suggested. Studies on single nucleotide polymorphisms further indicate that these markers may affect cancer risk. However, few prospective studies have investigated the association between these biomarkers and colorectal cancer risk, often providing conflicting evidence.

### Innovations and breakthroughs

This work shows a prospective positive association between plasma soluble vascular cell adhesion molecule-1 (sVCAM-1) levels and colorectal cancer risk, which has not been investigated previously. In addition, the authors observed an inverse relationship between pre-diagnostic adiponectin levels and colorectal cancer risk, which provides new insights given the conflicting literature. The inclusion of adiponectin and sVCAM-1 plasma levels in prediction models of colorectal cancer risk improved their discriminatory capabilities.

### Applications

This study adds to current knowledge of adiposity- and endothelial adhesion-

related pathways in the development of colorectal cancer. If confirmed in large validation studies, these results could lead to improved identification of individuals at risk of developing colorectal cancer, which could result in well-targeted cancer screening campaigns.

### Terminology

Leptin reflects body fat storage and acts as a pro-inflammatory adipokine. Conversely, adiponectin production is decreased in obesity and generally has anti-inflammatory properties. Adhesion molecules such as E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and the chemokine monocyte chemoattractant protein-1 are important in cell-cell and cell-basement membrane interactions. C-reactive protein is a widely used systemic biomarker for diagnosing acute and chronic inflammation.

### Peer review

The authors describe the potential role of two biomarkers in the diagnosis of colorectal cancer using a prospective study cohort initiated almost 18 years ago. The availability of this cohort and the derived material is a major strength of the study; even though a limited number of cases developed and were available for analysis. The study design and analytical work is not questionable, and the statistical analysis is "state of the art".

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## ***Patatin-like phospholipase domain containing-3* gene I148M polymorphism, steatosis, and liver damage in hereditary hemochromatosis**

Luca Valenti, Paolo Maggioni, Alberto Piperno, Raffaella Rametta, Sara Pelucchi, Raffaella Mariani, Paola Dongiovanni, Anna Ludovica Fracanzani, Silvia Fargion

Luca Valenti, Paolo Maggioni, Raffaella Rametta, Paola Dongiovanni, Anna Ludovica Fracanzani, Silvia Fargion, Università degli Studi di Milano, Fondazione Ca' Granda IRCCS Ospedale Maggiore Policlinico, 20122 Milano, Italy

Alberto Piperno, Sara Pelucchi, Raffaella Mariani, Università di Milano Bicocca, Ospedale San Gerardo Monza, 20090 Monza MI, Italy

**Author contributions:** Valenti L designed the study, performed the statistical analysis, drafted the manuscript and funded the study; Maggioni P, Rametta R, Pelucchi S, Mariani R, Dongiovanni P and Fracanzani AL acquired and interpreted the data and revised the manuscript for critical content; Piperno A and Fargion S interpreted the data, revised the manuscript for critical content and funded the study; all authors read, revised and approved the final version of the manuscript.

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**Correspondence to:** Luca Valenti, MD, Università degli Studi di Milano, Fondazione Ca' Granda IRCCS, Ospedale Maggiore Policlinico, via F Sforza, 20122 Milano, Italy. [luca.valenti@unimi.it](mailto:luca.valenti@unimi.it)

Telephone: +39-2-50320278 Fax: +39-2-50320296

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### **Abstract**

**AIM:** To investigate whether the *patatin-like phospholipase domain containing-3* gene (*PNPLA3*) I148M polymorphism is associated with steatosis, fibrosis stage, and cirrhosis in hereditary hemochromatosis (HH).

**METHODS:** We studied 174 consecutive unrelated homozygous for the C282Y HFE mutation of HH (C282Y+/+ HH) patients from Northern Italy, for whom the presence of cirrhosis could be determined based on

histological or clinical criteria, without excessive alcohol intake (< 30/20 g/d in males or females) or hepatitis B virus and hepatitis C virus viral hepatitis. Steatosis was evaluated in 123 patients by histology ( $n = 100$ ) or ultrasound ( $n = 23$ ). The *PNPLA3* rs738409 single nucleotide polymorphism, encoding for the p.148M protein variant, was genotyped by a Taqman assay (assay on demand, Applied Biosystems). The association of the *PNPLA3* I148M protein variant (p.I148M) with steatosis, fibrosis stage, and cirrhosis was evaluated by logistic regression analysis.

**RESULTS:** *PNPLA3* genotype was not associated with metabolic parameters, including body mass index (BMI), the presence of diabetes, and lipid levels, but the presence of the p.148M variant at risk was independently associated with steatosis [odds ratio (OR) 1.84 per p.148M allele, 95% confidence interval (CI): 1.05-3.31;  $P = 0.037$ ], independently of BMI and alanine aminotransferase (ALT) levels. The p.148M variant was also associated with higher aspartate aminotransferase ( $P = 0.0014$ ) and ALT levels ( $P = 0.017$ ) at diagnosis, independently of BMI and the severity of iron overload. In patients with liver biopsy, the 148M variant was independently associated with the severity (stage) of fibrosis (estimated coefficient  $0.56 \pm 0.27$ ,  $P = 0.041$ ). In the overall series of patients, the p.148M variant was associated with cirrhosis in lean ( $P = 0.049$ ), but not in overweight patients ( $P =$  not significant). At logistic regression analysis, cirrhosis was associated with BMI  $\geq 25$  (OR 1.82, 95% CI: 1.02-3.55), ferritin > 1000 ng/mL at diagnosis (OR 19.3, 95% CI: 5.3-125), and with the G allele in patients with BMI < 25 (OR 3.26, 95% CI: 1.3-10.3).

**CONCLUSION:** The *PNPLA3* I148M polymorphism may represent a permissive factor for fibrosis progression in patients with C282Y+/+ HH.



**Key words:** Fatty liver; Fibrosis; Hemochromatosis; HFE protein; Iron overload; *Patatin-like phospholipase domain containing-3* gene; Steatosis

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## INTRODUCTION

Hereditary hemochromatosis (HH) is a genetic disorder of iron metabolism characterized by defective release or activity of hepcidin, the hepatic hormone that inhibits iron absorption by binding and inactivating ferroportin<sup>[1]</sup>. HH is most frequently related to hampered hepcidin up-regulation by iron stores as a consequence of homozygosity for the C282Y mutation in the *HFE* gene<sup>[2]</sup>. The resultant increase in serum iron leads to progressive accumulation in the liver and other parenchymal organs, however, although hepatic iron overload leads to progressive liver fibrosis and cirrhosis in some affected individuals, the phenotypic expression is unpredictable and highly variable<sup>[3]</sup>.

Indeed, liver disease is the most frequent clinical manifestation of homozygous for the C282Y *HFE* mutation of HH (C282Y+/+ HH), but it is now clear that only a proportion of subjects carrying this genotype will ever develop hepatic fibrosis<sup>[4]</sup>. Most of C282Y +/+ male subjects develop expanded iron stores during life, whereas due to the physiological iron losses during fertile age, the female gender represents a major protective factor. In population based screening studies it has been shown that between 75% and 94% of C282Y+/+ males develop elevated transferrin saturation, and that 64% to 68% will have an increased serum ferritin<sup>[4-8]</sup>. However, even in males, the prediction of risk of clinical disease remains uncertain<sup>[9]</sup>.

The recognition of the incomplete penetrance of HH has led to a search for genetic and other modifiers of clinical expression. HH expression may be influenced at different levels<sup>[9]</sup>: (1) by factors affecting iron loading, including sex and genetic factors (genes regulating hepcidin expression, beta-thalassemia trait<sup>[10]</sup>); (2) by factors influencing the progression to liver disease, such as hepatic steatosis<sup>[11]</sup>, viral hepatitis, genes regulating pro-inflammatory cytokines and oxidative injury<sup>[12,13]</sup>; and (3) by those regulating both, such as alcohol intake and

hepatitis C virus (HCV) infection<sup>[14,15]</sup>.

There is established evidence that increased body mass (BMI) and the metabolic syndrome<sup>[16]</sup> are strong risk factors for hepatic steatosis<sup>[17]</sup> and that steatosis accelerates the progression of liver diseases by favoring oxidative stress and hepatocellular damage. In 214 C282Y +/+ patients<sup>[11]</sup>, a significant association between steatosis and the presence of fibrosis was detected. This relationship remained significant after adjustment for confounding factors such as alcohol intake and iron loading.

Recently, the rs738409 C > G single nucleotide polymorphism (SNP) of *patatin-like phospholipase domain containing-3* gene (*PNPLA3*), encoding for the I148M protein variant (p.I148M), has been identified as a determinant of liver fat content and of the susceptibility to develop steatohepatitis and progressive fibrosis<sup>[18-23]</sup>. Importantly, *PNPLA3* genotype influences liver fat independently of body mass, dyslipidemia, and insulin resistance<sup>[24,25]</sup>.

Since hepatic steatosis has been reported to influence HH expression, the aim of this study was to determine whether the *PNPLA3* I148M variant predisposes to the development of steatosis, and to progressive liver damage, as evaluated fibrosis stage and the presence of cirrhosis, in patients with pure C282Y+/+ HH stratified according to the presence of overweight.

## MATERIALS AND METHODS

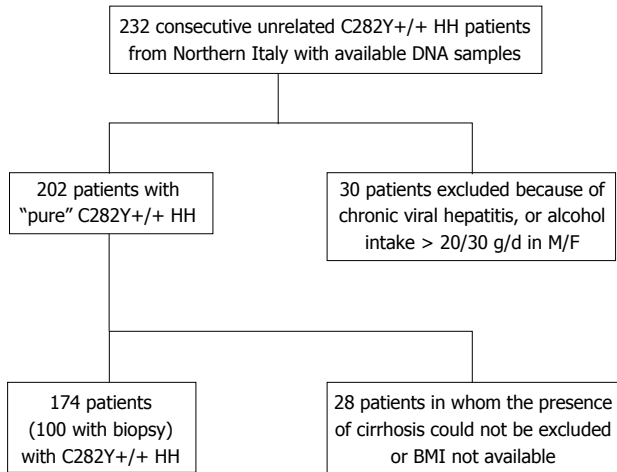
### Patients

From 232 consecutive unrelated C282Y+/+ HH patients referred to two centers in the Milan area of Northern Italy, we excluded subjects with alcohol intake > 30/20 g per day in male/female, hepatitis B virus (HBV) and/or HCV infections, and other cofactors of liver disease ( $n = 30$ ), and those with an uncertain diagnosis of cirrhosis or incomplete clinical data ( $n = 28$ ), and finally included 174 patients in the analysis (Figure 1). DNA samples were available for all patients.

Diagnosis of cirrhosis was based upon liver histology ( $n = 100$ ) or clinical evidence ( $n = 74$ ): in particular, cirrhosis was diagnosed by liver histology in 26 patients, and by clinical criteria in 6 cases (in the presence of hepatic decompensation or of portal hypertension; liver biopsy was not indicated for ethical reasons), whereas it was excluded by liver histology in 74 cases, and by clinical criteria in the remaining 68 cases (when liver biopsy was not indicated and not performed for ethical reasons).

Tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen and Perls for iron. Steatosis was considered present when involving at least 5% of hepatocytes and graded according to Kleiner<sup>[26]</sup>. Tissue iron was graded according to Scheuer<sup>[27]</sup>. Fibrosis was scored according to Ishak<sup>[28]</sup>. The minimum biopsy size was 1.7 cm and the number of portal areas was 10. For data analysis, a fibrosis stage of 6 was attributed to patients with a clinical diagnosis of cirrhosis.

Ultrasonographic diagnosis of steatosis at diagnosis



**Figure 1 Study flow chart.** C282Y+/+ HH: Homozygous for the C282Y HFE mutation of hereditary hemochromatosis; M/F: Male/female; BMI: Body mass index.

by an experienced operator (available in 123) was based on evident ultrasonographic contrast between the hepatic and right renal parenchyma of the right intercostal sonogram in the midaxillary line, or abnormally intense, high-level echoes arising from the hepatic parenchyma, and was graded on a three-grade scale as none, mild, or severe in accordance with intensity<sup>[29]</sup>.

Cirrhosis was considered clinically absent only if all these conditions were satisfied: (1) age < 40 years; (2) alanine aminotransferase (ALT) within normal levels; and (3) ferritin < 1000 ng/mL. These criteria have been shown to rule out not only cirrhosis, but also advanced fibrosis with high specificity in patients with C282Y+/+ HH without viral hepatitis and excessive alcohol intake<sup>[30]</sup>.

Overweight was considered present when BMI > 25 kg/m<sup>2</sup>. For each patient we collected data on sex, age, geographical origins, BMI, alcohol consumption, aspartate aminotransferase (AST), ALT and  $\gamma$ -glutamyl transferase (GGT) levels, ferritin, transferrin saturation percentage, total cholesterol, high density lipoprotein cholesterol and triglycerides levels, glucose, and type 2 diabetes<sup>[31]</sup>. Clinical features of the patients included are shown in Table 1. Informed written consent was obtained from each patient included. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Institutional Review Board of the Institutions involved.

### Genetic analysis

DNA was extracted from peripheral blood by the phenol-chloroform method. Success rate in extracting DNA was 100% for each study group. The PNPLA3 rs738409 SNP was genotyped by a Taqman assay (assay on demand for rs738409, Applied Biosystems, Foster City, CA, United States) by personnel unaware of patients and controls clinical status. Post-polymerase chain reaction allelic discrimination was carried out measuring allele-specific fluorescence on the Opticon2 detection system (MJ Research, Waltham, MA, United States). Random samples

**Table 1** Demographic, anthropometric, clinical, and histological features, as evaluated at diagnosis, of 174 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis subdivided according to the PNPLA3 I148M genotype

	All patients	PNPLA3 I148M genotype			P value
		I/I	I/M	M/M	
n (%)	174	82 (47)	70 (40)	22 (13)	
Age (yr)	47 $\pm$ 13	46 $\pm$ 13	47 $\pm$ 14	50 $\pm$ 11	0.22
Gender F (%)	49 (28)	24 (30)	19 (27)	6 (27)	0.79
DAI (g)	10 (0-20)	10 (0-20)	5 (0-20)	10 (0-20)	0.98
BMI (kg/m <sup>2</sup> )	24 $\pm$ 3	24 $\pm$ 3	24 $\pm$ 3	25 $\pm$ 2	0.11
Diabetes (%)	16 (9)	7 (9)	6 (9)	3 (14)	0.57
Total cholesterol (mg/dL)	191 $\pm$ 44	197 $\pm$ 37	186 $\pm$ 50	182 $\pm$ 44	0.25
HDL cholesterol (mg/dL)	53 $\pm$ 16	53 $\pm$ 14	53 $\pm$ 17	57 $\pm$ 15	0.47
Triglycerides (mg/dL)	120 $\pm$ 66	123 $\pm$ 64	122 $\pm$ 74	99 $\pm$ 35	0.30
TS %	80 $\pm$ 16	80 $\pm$ 16	79 $\pm$ 15	81 $\pm$ 23	0.94
Ferritin (ng/mL)	1000 (509-1800)	1018 (490-1813)	947 (516-1732)	1053 (504-2101)	0.36
AST (IU/mL)	34 $\pm$ 21	31 $\pm$ 16	35 $\pm$ 24	44 $\pm$ 29	0.019
ALT (IU/mL)	46 $\pm$ 32	41 $\pm$ 26	47 $\pm$ 34	55 $\pm$ 45	0.06
GGT (IU/mL)	24 (16-37)	24 (17-37)	24 (15-38)	23 (14-30)	0.95
Steatosis <sup>1</sup> (%)	55 (48)	18 (31)	29 (58)	8 (53)	0.014
Advanced fibrosis % (Ishak 4) %	18 (10)	9 (11)	7 (10)	2 (9)	0.95
Cirrhosis (Ishak 5-6) %	32 (18)	13 (16)	14 (20)	5 (23)	0.39

<sup>1</sup>Available in 123 patients. I: Isoleucine; M: Methionine; n: Number; F: Female; DAI: Daily alcohol intake; BMI: Body mass index; HDL: High density lipoprotein cholesterol; TS: Transferrin saturation; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT:  $\gamma$ -glutamyl transferase.

were confirmed by direct genotyping which provided concordant results in all cases<sup>[19]</sup>. Quality controls were performed to verify the reproducibility of the results. Valid genotypic data were obtained for 100% of subjects analyzed.

### Statistical analysis

Values are expressed as mean  $\pm$  SD or median (interquartile range) according to distribution. Mean values were compared by analysis of variance or Wilcoxon, and frequencies by *F* test and  $\chi^2$  test for trend, when appropriate. The study had a > 85% power to detect a two-fold higher risk of cirrhosis in carriers of the 148M allele, but only 33% to detect a 33% increased risk. Independent predictors of AST and ALT levels were analyzed by generalized linear model. The association of the PNPLA3 p.148M variant with fibrosis was evaluated by ordinal logistic regression analysis, and with the presence of steatosis and cirrhosis was evaluated by multivariate logistic regression analysis. *P* values were considered significant when < 0.05 (two-tailed). Analyses were carried out with JMP 6.0 statistical analysis software (SAS Institute Inc., Cary, NC, United States).

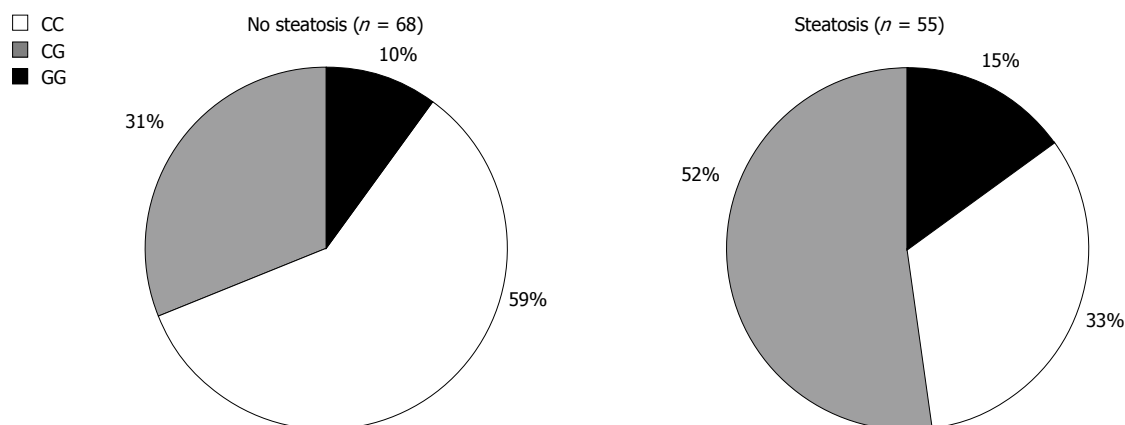


Figure 2 Frequency distribution of the rs738409 C > G single nucleotide polymorphism, encoding for the I148M protein variant, in 123 patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis subdivided according to the presence of steatosis ( $P = 0.015$ ).

Table 2 Independent predictors of steatosis and cirrhosis in Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, as evaluated by logistic regression analysis

	OR	95% CI	P value
Independent predictors of steatosis $n = 123$			
BMI (per kg/m <sup>2</sup> )	1.22	1.06-1.42	0.008
ALT (per IU/mL)	1.01	0.99-1.02	0.353
PNPLA3 genotype (per p allele)	1.84	1.05-3.31	0.037
Independent predictors of cirrhosis $n = 174$			
Ferritin (ng/mL)	1.001	1.000-1.002	< 0.0001
Diabetes	0.54	0.26-1.16	0.101
BMI (>/m <sup>2</sup> )	1.83	1.02-3.57	0.055
PNPLA3 p allele present (BMI > 25)	0.76	0.37-1.53	0.453
PNPLA3 p allele present (BMI ≤ 25)	3.26	1.28-10.30	0.024

OR: Odds ratio; CI: Confidence interval; BMI: Body mass index; ALT: Alanine aminotransaminase; p.148M: PNPLA3 rs738409 148Met protein variant.

## RESULTS

### Association of PNPLA3 gene genotype with steatosis

We first sought to confirm the association of the G allele encoding for the p.148M variant with liver fat in C282Y+/+ HH. The frequency distribution of the rs738409 PNPLA3 SNP ( $P =$  not significant for Hardy-Weinberg equilibrium testing) in patients subdivided according to the presence of steatosis is shown in Figure 2 ( $P = 0.014$ ). The frequency of the G allele was 0.41 in patients with and 0.26 in those without steatosis ( $P = 0.011$ ), and did not change after the exclusion of patients with ultrasonographic evaluation of the presence of steatosis. Independent predictors of steatosis at logistic regression analysis, considered as independent variables selected by a stepwise mixed regression model, are shown in Table 2. Steatosis was independently associated with BMI ( $P = 0.008$ ) and PNPLA3 genotype [odds ratio (OR) 1.84 per G allele, 95% confidence interval (CI): 1.05-3.31;  $P = 0.037$ ].

### Association of PNPLA3 gene genotype with liver enzymes

As expected, PNPLA3 genotype was not significantly asso-

ciated with demographic or anthropometric features, daily alcohol intake, metabolic parameters, including the presence of diabetes, and the severity of iron overload (Table 1). However, we observed an association between PNPLA3 and transaminases, which was significant for AST levels [ $P$  for trend (i.e., for increasing levels with increasing number of 148M alleles) = 0.019 for AST and  $P$  for trend = 0.06 for ALT], whereas GGT levels were not affected. Independent predictors of AST and ALT levels in the generalized linear model are shown in Table 3; variables included were selected by a stepwise mixed regression model. Both AST and ALT levels were significantly and independently correlated with younger age, higher iron parameters (TS% and ferritin levels), GGT levels, BMI, and the number (0-2) of 148M PNPLA3 alleles ( $P = 0.0014$  and  $P = 0.017$  for AST and ALT levels, respectively).

### Association of PNPLA3 gene genotype with severity of fibrosis and cirrhosis

We next evaluated whether PNPLA3 genotype influences fibrosis stage. At ordinal regression analysis conducted in patients with liver biopsy or clinical diagnosis of cirrhosis ( $n = 106$ ; shown in Table 4), fibrosis stage (0-6) was independently associated with gender, ALT and GGT values, and PNPLA3 p.148M alleles (estimated coefficient of correlation  $0.56 \pm 0.27$ ,  $P = 0.04$ ). Possibly due to the relatively low number of patients studied, PNPLA3 genotype was not significantly associated with cirrhosis in the whole cohort (Table 1), although the presence of the 148M allele was nominally significantly associated with cirrhosis in patients with BMI < 25 ( $P = 0.05$ ,  $P = 0.1$  after Bonferroni correction; Figure 3). Importantly, positivity for the PNPLA3 148M variant was associated with an increase in the prevalence of steatosis in subjects with BMI < 25, which reached levels similar to those of overweight patients (BMI < 25: 17/37, 46% vs 7/32, 22%,  $P = 0.036$ ; BMI ≥ 25: 22/31, 71% vs 10/26, 38%;  $P = 0.017$  for patients positive and negative for the 148M variant, respectively). Independent predictors of cirrhosis are shown in Table 2. At logistic regression analysis, cirrhosis was associated with BMI ≥ 25 (OR 1.82, 95% CI:

**Table 3** Independent predictors of aspartate aminotransferase and alanine aminotransaminase levels in 174 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, in the multivariate generalized linear model

	AST			ALT		
	Estimate	95% CI	P value	Estimate	95% CI	P value
Age (yr)	-0.27 ± 0.09	-0.45 - -0.09	0.0034	-0.55 ± 0.14	-0.81 - -0.27	0.0001
TS (%)	0.14 ± 0.07	-0.01 - 0.29	0.063	0.28 ± 0.11	0.05 - 0.50	0.016
Ferritin (ng/mL)	0.01 ± 0.001	0.008 - 0.012	< 0.0001	0.01 ± 0.001	0.009 - 0.014	< 0.0001
GGT (IU/mL)	0.10 ± 0.03	0.03 - 0.16	0.003	0.20 ± 0.05	0.10 - 0.29	< 0.0001
BMI (kg/m <sup>2</sup> )	0.86 ± 0.42	0.03 - 1.69	0.042	3.36 ± 0.63	2.12 - 4.60	< 0.0001
PNPLA3 genotype (per p.148M allele)	5.46 ± 1.68	2.15 - 8.67	0.0014	6.05 ± 2.51	1.09 - 11.00	0.017

AST: Aspartate aminotransferase; ALT: Alanine aminotransaminase; CI: Confidence interval; TS: Transferrin saturation; GGT:  $\gamma$ -glutamyl transferase; BMI: Body mass index; p.148M: PNPLA3 rs738409 148Met protein variant.

**Table 4** Independent predictors of fibrosis stage in 106 Italian patients with homozygous for the C282Y HFE mutation of hereditary hemochromatosis, at ordinal logistic regression analysis

	Estimate	P value
Age (yr)	-0.03 ± 0.02	0.077
Gender (female)	-0.57 ± 0.26	0.029
BMI (kg/m <sup>2</sup> )	-0.08 ± 0.08	0.311
Diabetes	0.57 ± 0.34	0.09
ALT	-0.02 ± 0.008	0.013
GGT (IU/mL)	0.02 ± 0.01	0.035
Ferritin (ng/mL)	0.0001 ± 0.00	0.421
PNPLA3 genotype (per p.148M allele)	0.56 ± 0.27	0.041

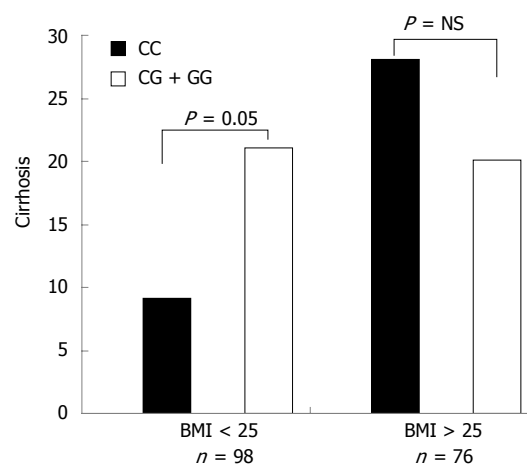
BMI: Body mass index; ALT: Alanine aminotransaminase; GGT:  $\gamma$ -glutamyl transferase; p.148M: PNPLA3 rs738409 148Met protein variant.

1.02-3.55), ferritin > 1000 ng/mL at diagnosis (OR 19.3, 95% CI: 5.3-125), and with the G allele in patients with BMI < 25 (OR 3.26, 95% CI: 1.3-10.3).

## DISCUSSION

In this study, we evaluated the effect of the PNPLA3 rs738409, encoding for the p.I148M variant, on steatosis and liver damage in patients affected by C282Y+/+ HH without other causes of liver damage. Our results confirm the association of the PNPLA3 148M allele with the presence of steatosis and liver enzymes, independently of iron overload, which represents the major cause of progressive liver damage in HH patients. Furthermore, PNPLA3 rs738409 was also associated with fibrosis stage, and with the presence of cirrhosis, albeit only in the presence of normal BMI.

Although data on steatosis were not available in the whole series of patients evaluated, the association of PNPLA3 with steatosis in HH was expected based on data obtained in the general population, in patients with non-alcoholic fatty liver disease (NAFLD), and in other liver diseases. In addition, the magnitude of the observed association was in line with previous reports<sup>[18,19,32]</sup>. Due to the retrospective design of the study, insulin resistance evaluation was not available for all patients, however, previous studies have excluded a major effect of PNPLA3

**Figure 3** Effect of the rs738409 G allele, encoding for the 148M PNPLA3 variant, on liver cirrhosis in 174 patients with HH subdivided according to the presence of overweight. NS: Not significant; BMI: Body mass index.

genotype on insulin resistance, and the 148M variant was not associated with diabetes in this study.

As steatosis has been reported to influence fibrosis progression in C282Y+/+ patients, independently of alcohol intake and iron loading<sup>[11]</sup>, the main aim of the present study was to evaluate whether PNPLA3 genotype influences liver damage progression in HH. We found that the PNPLA3 148M allele was a strong predictor of transaminase levels, and in particular of AST levels, which are generally more strongly linked with chronic liver damage (fibrosis stage) than ALT<sup>[11,33]</sup>. PNPLA3 polymorphism has been reported to represent a major determinant of transaminase levels in the general population, in patients with NAFLD, and in obese subjects at risk of steatosis<sup>[18,19,34,35]</sup>. Our findings indicate, that this is also true for patients with C282Y+/+ HH. It is likely that this association reflects the predisposing effect of the 148M PNPLA3 allele on steatosis. Interestingly, BMI, which was the other determinant of steatosis in our series of patients, was also associated with transaminases. Furthermore, we demonstrated that PNPLA3 genotype was associated with fibrosis stage in patients with HH, consistent with the hypothesis that the 148M allele of PNPLA3 influences the progression of liver damage in



HH, even if this has to be confirmed in a larger series of patients.

The association of the 148M variant with the risk of cirrhosis, a turning point in the natural history of patients with HH due to the frequent progression to hepatocellular carcinoma<sup>[36,37]</sup>, is also in line with this hypothesis. Interestingly, data obtained by our and other groups indicate that the 148M variant is significantly associated with cirrhosis and hepatocellular carcinoma in patients with HCV-related chronic hepatitis, thus suggesting that this can be true also for liver diseases of different etiology<sup>[32,38]</sup>.

Based on the previously reported interaction between the effect of the PNPLA3 I148M mutation and body mass<sup>[35]</sup>, the other major determinant of steatosis in our series of patients, we analyzed the effect of PNPLA3 rs738409 on cirrhosis risk in patients stratified according to the presence of overweight. We found an association between PNPLA3 genotype and cirrhosis, but this was restricted to subjects with normal BMI. There are several possible explanations for this finding. The first is that the result is due to chance. Because of the relatively limited number of subjects included, the study power to detect an association with cirrhosis was relatively low, i.e., > 85% to detect a two-fold higher risk of cirrhosis in carriers of the 148M allele, but only 33% to detect a 33% increased risk. Secondly, this was a retrospective study with data evaluation at the time of diagnosis, therefore we could not exclude the fact that some patients “normalized” their body mass after the development of cirrhosis due to the malnutrition typical of this condition. However, increased BMI was independently associated with steatosis and tended to be associated also with cirrhosis risk, and the shift in body weight should have equally affected patients with and without the 148M allele. Lastly, we cannot exclude that the PNPLA3 148M variant, by favoring steatosis development in subjects with normal BMI, may play a permissive role in the progression of liver damage in lean patients with C282Y+/+ HH, whereas in overweight subjects steatosis is mainly related to metabolic factors, reducing the role played by PNPLA3. Additional studies are required to clarify this issue, and to evaluate whether the 148M variant may predispose to hepatocellular carcinoma development also in HH and in patients carrying HFE mutations<sup>[39-41]</sup>.

In conclusion, we showed that in Italian patients with C282Y+/+ HH the PNPLA3 I148M polymorphism is associated with the risk of steatosis, increased liver enzymes, higher stage of fibrosis, and possibly with an increased risk of cirrhosis at diagnosis, particularly in subjects with normal body mass. Future studies should evaluate whether PNPLA3 I148M genotype might be clinically useful for selecting HH patients for biopsy, or to determine screening intervals for hepatocellular carcinoma in cirrhotics.

## COMMENTS

### Background

Hereditary hemochromatosis, characterized by progressive accumulation of

iron in tissues, is a very frequent genetic disease in individuals of European descent. The most frequent clinical manifestation is liver disease, which may lead to liver cancer. However, disease expression is highly variable. Previous work has led to hypothesize that genetic factors and liver fat accumulation (i.e., “steatosis”) are implicated in this process. Recently, the common I148M patatin-like phospholipase domain containing-3 genetic polymorphism has been recognized together with obesity as a key factor regulating fat accumulation in the liver, contributing significantly to the liver disease burden in the general population.

### Research frontiers

The identification of genetic factors involved in the penetrance and expression of hereditary hemochromatosis is a very active area of research, as such markers would be helpful to identify subjects at risk during screening and to personalize treatment and follow-up in patients presenting with liver disease.

### Innovations and breakthroughs

The key findings of the study are that the PNPLA3 genetic variant, present in 40% of patients, was a key determinant, together with overweight, of hepatic fat accumulation and of alterations of biochemical indices of liver damage. Furthermore, this marker was also associated with chronic fibrotic damage detected by liver biopsy. Importantly, the PNPLA3 variant put at risk of steatosis also normal weight patients, who would be normally protected, allowing the development of progressive liver damage and cirrhosis, with potential clinical complications.

### Applications

These results raise new hope to offer better, personalized treatment to patients with hemochromatosis, which will be tested in future studies. In particular, intensified follow-up and preventive treatments could be proposed to subjects at risk of developing liver cancer, the leading cause of death in patients with clinically overt hemochromatosis, which is also favored by steatosis.

### Terminology

Hereditary hemochromatosis is a genetic disorder of iron metabolism characterized by defective release or activity of hepcidin, the hepatic hormone that inhibits iron absorption, leading to progressive accumulation in the liver and other parenchymal organs; PNPLA3 is an enzyme with phospholipase activity, which is expressed in the liver, and likely involved in the breakdown triglycerides; genetic polymorphisms: inherited variant of the DNA, which is detected in > 1% of the population and is not associated *per se* with a pathologic phenotype.

### Peer review

This is an interesting clinical study, which provides evidence for association between the PNPLA3 148M polymorphism and progression of liver fibrosis. The study is well designed and the data are novel.

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## An epidemiological study of collagenous colitis in southern Sweden from 2001-2010

Lina Vigren, Martin Olesen, Cecilia Benoni, Klas Sjöberg

Lina Vigren, Cecilia Benoni, Klas Sjöberg, Division of Gastroenterology, Department of Clinical Sciences, Skåne University Hospital, Lund University, SE-205 02 Malmö, Sweden  
 Martin Olesen, University and Regional Laboratories, Region Skåne, Department of Pathology, Skåne University Hospital, SE-205 02 Malmö, Sweden

**Author contributions:** Vigren L and Sjöberg K made substantial contributions to conception and design, analysed the data and contributed to the acquisition of data, analysis and interpretation; Vigren L, Olesen M, Benoni C and Sjöberg K drafted the article and revised it critically for important intellectual content; Olesen M performed the histopathological analysis; all authors approved the version to be published.

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**Correspondence to:** Lina Vigren, MD, Division of Gastroenterology, Department of Clinical Sciences, Skåne University Hospital, Lund University, SE-205 02 Malmö, Sweden. [lina.u.vigren@skane.se](mailto:lina.u.vigren@skane.se)

Telephone: +46-410-55000 Fax: +46-410-55127

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### Abstract

**AIM:** To estimate the incidence of collagenous colitis (CC) in southern Sweden during 2001-2010.

**METHODS:** Cases were identified by searching for CC in the diagnostic registers at the Pathology Departments in the county of Skåne. The catchment area comprised the south-west part of the county (394 307 inhabitants in 2010) and is a mixed urban and rural type with limited migration. CC patients that had undergone colonoscopy during the defined period and were living in this area were included in the study regardless of where in Skåne they had been diagnosed. Medical records were scrutinized and uncertain cases were reassessed to ensure that only newly diagnosed CC cases were included. The diagnosis of CC was based on both clinical and histopathological criteria. The clinical crite-

ria were non-bloody watery diarrhoea. The histopathological criteria were a chronic inflammatory infiltrate in the lamina propria, a thickened subepithelial collagen layer  $\geq 10$  micrometers ( $\mu\text{m}$ ) and epithelial damage such as flattening and detachment.

**RESULTS:** During the ten year period from 2001-2010, 198 CC patients in the south-west part of the county of Skåne in southern Sweden were newly diagnosed. Of these, 146 were women and 52 were men, i.e., a female: male ratio of 2.8:1. The median age at diagnosis was 71 years (range 28-95/inter-quartile range 59-81); for women median age was 71 (range 28-95) years and was 73 (range 48-92) years for men. The mean annual incidence was  $5.4/10^5$  inhabitants. During the time periods 2001-2005 and 2006-2010, the mean annual incidence rates were  $5.4/10^5$  for both periods [95% confidence interval (CI): 4.3-6.5 in 2001-2005 and 4.4-6.4 in 2006-2010, respectively, and 4.7-6.2 for the whole period]. Although the incidence varied over the years (minimum 3.7 to maximum  $6.7/10^5$ ) no increase or decrease in the incidence could be identified. The odds ratio (OR) for CC in women compared to men was estimated to be 2.8 (95% CI: 2.0-3.7). The OR for women 65 years of age or above compared to below 65 years of age was 6.9 (95% CI: 5.0-9.7), and for women 65 years of age or above compared to the whole group the OR was 4.7 (95% CI: 3.6-6.0). The OR for age in general, i.e., above or 65 years of age compared to those younger than 65 was 8.3 (95% CI: 6.2-11.1). During the last decade incidence figures for CC have also been reported from Calgary, Canada during 2002-2004 ( $4.6/10^5$ ) and from Terrassa, Spain during 2004-2008 ( $2.6/10^5$ ). Our incidence figures from southern Sweden during 2001-2010 ( $5.4/10^5$ ) as well as the incidence figures presented in the studies during the 1990s (Terrassa, Spain during 1993-1997 ( $2.3/10^5$ ), Olmsted, United States during 1985-2001 ( $3.1/10^5$ ), Örebro, Sweden during 1993-1998 ( $4.9/10^5$ ), and Iceland during 1995-1999 ( $5.2/10^5$ ) are all in line with a north-south gradient, something that has been suggested before both for CC and inflammatory bowel disease.



**CONCLUSION:** The observed incidence of CC is comparable with previous reports from northern Europe and America. The incidence is stable but the female: male ratio seems to be decreasing.

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**Key words:** Collagenous colitis; Epidemiology; Incidence; Microscopic colitis

**Peer reviewers:** Hugh J Freeman, Professor, MD, CM, FRCPC, FACP, Department of Medicine, University of British Columbia, UBC Hospital 2211 Westbrook Mall, Vancouver, BC V6T 1W5, Canada; Shotaro Nakamura, MD, Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

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## INTRODUCTION

In 1976, a pathology colleague at our hospital, Clas G Lindström, received rectal biopsies for a second opinion. The patient was a 48-year-old woman with chronic non-bloody watery diarrhoea and based on his observations he described the first ever case of collagenous colitis (CC)<sup>[1]</sup>. In his case report, Lindström actually described a typical case of CC, i.e., a middle-aged woman with chronic non-bloody watery diarrhoea. Independently, a group from Canada also reported this entity<sup>[2]</sup>. Other common CC symptoms include abdominal pain, weight loss and faecal incontinence<sup>[3-5]</sup>. The diagnosis of CC can only be established by microscopic examination of colonic mucosal biopsies. Endoscopy usually reveals a macroscopically normal mucosa, although some changes may be seen with special staining methods, e.g. altered vessel formation and disturbed pattern of the mucous membrane<sup>[6,7]</sup>. Histopathologically, CC is characterised by a thickened subepithelial collagen layer, combined with a chronic inflammatory infiltrate in the lamina propria and surface epithelial damage. In 1989, Lazenby<sup>[8]</sup> described lymphocytic colitis (LC), a similar condition clinically and histopathologically, but without a thickened subepithelial collagen layer. Collagenous colitis and LC are included in the umbrella term microscopic colitis (MC).

About 10% of patients investigated for non-bloody diarrhoea and with a macroscopically normal colonic mucosa are diagnosed with MC<sup>[8-11]</sup> even though an incidence as high as 29% has been observed<sup>[12]</sup>. These figures indicate that the condition could be prevalent. Incidence data on CC is available from the mid 1990s<sup>[13]</sup>. Initially, CC was considered to be a rare disease, but with time it has become evident that the incidence of CC is higher than was first anticipated.

The aim of this study was to estimate the incidence of CC in a well-defined population in southern Sweden during the ten year period from 2001-2010.

## MATERIALS AND METHODS

### Catchment area

The catchment area in this study comprised the south-west part of the county of Skåne (the county had 1 243 329 inhabitants on December 31st, 2010). The catchment area (including the cities of Malmö and Trelleborg, and the villages Vellinge and Svedala) is a mixed urban and rural type with limited migration (from 2001 to 2010 the population increased by 13%, from 349 693 to 394 307 inhabitants). The town of Malmö has the third largest population in Sweden, while Trelleborg is a small town. Vellinge and Svedala are smaller communities. In the catchment area there are two hospitals, one in Malmö and one in Trelleborg. Colonoscopy is carried out at both hospitals as well as by some private practitioners. There is only one Pathology Department in the catchment area (in Malmö) and all mucosal colonic biopsy specimens from the hospitals in Malmö and Trelleborg as well as from the private practitioners are sent to this Pathology Department.

### Patients

Patients living in the catchment area who underwent colonoscopy due to watery diarrhoea and were diagnosed with CC from 2001-2010, were included in the study. Cases were identified by searching for the diagnosis of CC in the diagnostic register at the Pathology Department in Malmö. The medical records for the identified CC patients were subsequently scrutinized for clinical data to ensure that only newly diagnosed cases were included.

Histopathologically uncertain cases as well as cases not diagnosed by an experienced gastrointestinal pathologist were reassessed by a pathologist specialised in gastrointestinal pathology (Martin Olesen). Patients not fulfilling the histopathological criteria for CC were excluded. Patients with a histopathological diagnosis of “unspecific chronic inflammation” and with watery diarrhoea were also re-evaluated to identify putative missed CC cases.

All CC patients living in the catchment area were included regardless of where in Skåne they were diagnosed. To eliminate the risk of missing patients living in the catchment area during the study period in 2001-2010, but having had a diagnostic colonoscopy with biopsies in adjacent areas outside the catchment area (i.e., in the remaining area of Skåne), the diagnostic registers at the other three Pathology Departments in Skåne were scrutinized for cases with CC. In accordance with this, patients not living in the catchment area from 2001-2010, but diagnosed at our Pathology Department were excluded.

All information regarding the size of the population as well as age and sex distribution was obtained from Statistics Sweden, the central bureau for national socioeconomic information.

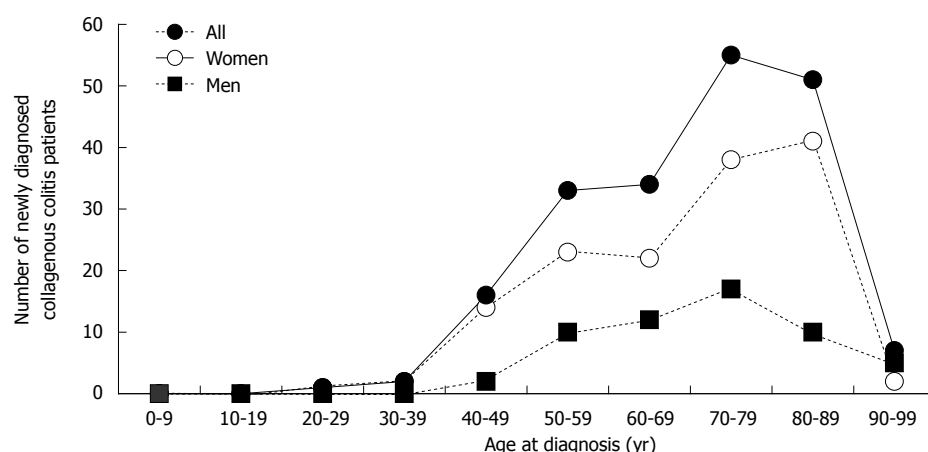


Figure 1 Age- and sex-specific annual incidence of collagenous colitis in southern Sweden.

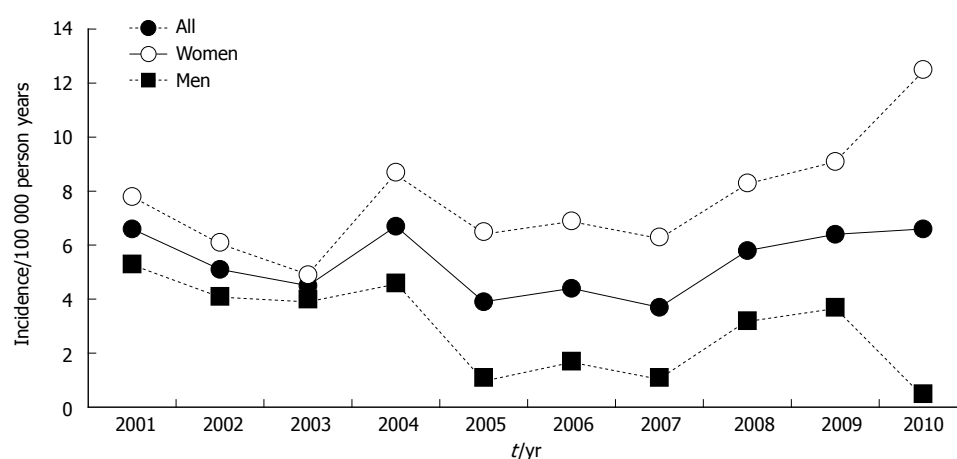


Figure 2 Annual incidence of collagenous colitis in southern Sweden.

### Diagnostic criteria

The diagnosis of CC was based on both clinical and histopathological criteria. The clinical criterion was non-bloody watery diarrhoea. The histopathological criteria were as follows: (1) A chronic inflammatory infiltrate in the lamina propria; (2) A thickened subepithelial collagen layer  $\geq 10$  micrometers ( $\mu\text{m}$ ); and (3) Epithelial damage such as flattening and detachment.

The subepithelial collagen layer was measured with an ocular micrometer in a well orientated section of the mucosa. Measurement of the subepithelial collagen layer was obtained using special stains for collagen fibres (Masson's trichrome or van Gieson) and/or reticulin fibres (Sirius red).

### Statistical analysis

For the purpose of calculating the incidence rate, it was assumed that the entire population in the catchment area was at risk. The incidence calculations were based on the date of diagnosis and 95% confidence interval (CI) were included. The calculation of mean annual incidence/100 000 as well as age-related incidence was based on the number of inhabitants on December 31st of each year. Data on the studied population are presented as

median and range/inter-quartile range (IQ) (25th-75th percentiles). Comparisons between groups were carried out using odds ratios (OR) and corresponding 95% CI.

### Ethics

The study was approved by the Committee of Research Ethics at Lund University.

## RESULTS

### Patients

In the ten year period from 2001-2010, 198 CC patients were newly diagnosed. Of these, 146 were women and 52 were men (female:male ratio 2.8:1). The median age at diagnosis was 71 years (range 28-95/IQ 59-81); for women median age was 71 (range 28-95) years and was 73 (range 48-92) years for men (Figure 1).

### Incidence

During the time periods 2001-2005 and 2006-2010, the mean annual incidence rates were  $5.4/10^5$  for both periods (95% CI: 4.3-6.5 in 2001-2005 and 4.4-6.4 in 2006-2010, respectively, and 4.7-6.2 for the whole period). Conse-

**Table 1** Mean annual incidence of collagenous colitis year by year during 2001-2010

Year	Annual incidence/10 <sup>5</sup> (total)	Annual incidence/10 <sup>5</sup> (women)
2001	6.6	7.8
2002	5.1	6.1
2003	4.5	4.9
2004	6.7	8.7
2005	3.9	6.5
2006	4.4	6.9
2007	3.7	6.3
2008	5.8	8.3
2009	6.4	9.1
2010	6.6	12.5

quently, although the incidence rates varied over the years (minimum 3.7 to maximum 6.7/10<sup>5</sup>) no increase or decrease could be identified (Figure 2, Tables 1 and 2).

The OR for CC in women compared to men was estimated to be 2.8 (95% CI: 2.0-3.7). The OR for women 65 years of age or above compared to below 65 years of age was 6.9 (95% CI: 5.0-9.7), and for women 65 years of age or above compared to the whole group the OR was 4.7 (95% CI: 3.6-6.0). The OR for age in general, i.e., above or 65 years of age compared to those younger than 65 was 8.3 (95% CI: 6.2-11.1).

## DISCUSSION

Information on the incidence of CC is available from one centre in the United States, one in Canada and a few in Europe (including Örebro in the central part of Sweden). Our study has added information on the epidemiology of CC in the southern part of Sweden. We report a mean annual incidence of CC of 5.4/10<sup>5</sup> for the period 2001-2010, which is in line with previous data from Örebro in central Sweden during 1993-1998 (4.9/10<sup>5</sup>)<sup>[11]</sup>, and is in accordance with the reported figures from Olmsted County, Minnesota, United States from 1997-2001 (6.2/10<sup>5</sup>)<sup>[14]</sup>, Iceland from 1995-1999 (5.2/10<sup>5</sup>)<sup>[15]</sup> and Calgary, Alberta, Canada from 2002-2004 (4.6/10<sup>5</sup>)<sup>[15,16]</sup>. However, these findings are in contrast to the incidence reported from Terrassa, Spain during 2004-2008 (2.6/10<sup>5</sup>)<sup>[17]</sup> (Table 2). Although the findings are not contradictory or novel in comparison with, for example, the report from Örebro<sup>[11]</sup>, the number of cases is large and the epidemiological information is updated in a new region that has not been studied before.

Most relevant from the Swedish perspective, are the extensive investigations from Örebro, in the central part of Sweden, where the incidence rates were calculated over a 15-year period from 1984-1998. An increased incidence of CC from 1.8/10<sup>5</sup> in 1984-1993 to 3.7/10<sup>5</sup> in 1993-1995 and 6.1/10<sup>5</sup> in 1996-1998 was reported<sup>[11,12]</sup>. In Spain, the incidence increased from 1.1/10<sup>5</sup> in 1993-1997 to 2.6/10<sup>5</sup> in 2004-2008<sup>[10,17]</sup>. In accordance with these reported increases, the Minnesota data from 1985 to 2001 show the same phenomenon. In 1985-1997, the incidence was 1.6/10<sup>5</sup> compared to 7.1/10<sup>5</sup> in 1998-2001<sup>[14]</sup>. An explana-

**Table 2** Mean annual incidence and female:male ratio of collagenous colitis in different countries/100 000 inhabitants

Ref.	Time period	N	Mean annual incidence <sup>1</sup>	Female:male ratio
Raclot <i>et al</i> <sup>[32]</sup>	1987-1992		0.6	
Bohr <i>et al</i> <sup>[13]</sup>	1984-1993	30	1.8	9
Pardi <i>et al</i> <sup>[14]</sup>	1985-2001	46	3.1	4.4
Fernández-Bañares <i>et al</i> <sup>[10]</sup>	1993-1997	23	2.3	4.8
Olesen <i>et al</i> <sup>[11]</sup>	1993-1998	51	4.9	7.5
Agnarsdottir <i>et al</i> <sup>[15]</sup>	1995-1999	71	5.2	7.9
Williams <i>et al</i> <sup>[16]</sup>	2002-2004	75	4.6	
Fernández-Bañares <i>et al</i> <sup>[17]</sup>	2004-2008	40	2.6	3.4
Present study	2001-2010	198	5.4	2.8

<sup>1</sup>Southern (left) and northern (right) latitudes. N: Number of new collagenous colitis patients.

tion for these reported increases in incidence rates could be an increased awareness of CC and more frequent use of colonoscopies with biopsies in the diagnostic procedure, although there was probably a true increase in incidence as well. The incidence of other gastrointestinal disorders such as ulcerative colitis, Crohn's disease and coeliac disease has increased in the Western world during the last few decades<sup>[18-21]</sup> and the influence of a common environmental factor (or several) cannot be ruled out. Interestingly, coeliac disease- similar to CC- has also increased over the last few decades and these diseases are related to each other<sup>[22]</sup>. Lansoprazole and other proton pump inhibitors are, in addition to nonsteroidal anti-inflammatory drugs, associated with a higher risk of CC<sup>[22-30]</sup>. The possibility that other factors such as infectious agents and components in our food could contribute to the increased incidence has also been contemplated.

The time period of ten years in our study is fairly long and provides information on possible fluctuations in the CC incidence rate in our catchment area during the period 2001-2010. Despite some variation in the annual incidence we did not observe any significant increase or decreases in the incidence during the study period. This is in contrast with the reported increases mentioned above. The stable incidence in our study might be due to the fact that CC was described at our hospital in Malmö by Lindström as early as 1976, i.e., 35 years ago. Accordingly, CC has been known among medical doctors in this area for more than 30 years, and as a consequence regular use of colonoscopies, with multiple biopsies throughout the entire length of the colon, has been standard in the diagnostic procedure of chronic watery diarrhoea for a long time. Furthermore, there has been a close clinical and scientific collaboration between gastroenterologists and pathologists within the field of CC.

During the last decade incidence figures for CC have been reported from Canada during 2002-2004 (4.6/10<sup>5</sup>) and from Spain during 2004-2008 (2.6/10<sup>5</sup>)<sup>[16,17]</sup>. There was a considerable difference in the incidence of CC in these studies despite the fact that the study periods were similar and relatively recent. Fernandez-Banares highlighted the possibility of a north-south gradient with a

higher incidence further north compared to those closer to the equator<sup>[17]</sup>. This possibility is further strengthened by our study. A larger number of studies have been listed in Table 2 to illustrate this phenomenon. This is also in line with the north-south gradient suggested for inflammatory bowel disease<sup>[31]</sup>.

The female: male ratio in the present study (2.8:1) was significantly lower than that in previous reports (Table 2). The previous Swedish studies from Örebro reported a female: male ratio as high as 9 and 7.5 in 1984-1993 and 1993-1998, respectively. Interestingly, it might very well be that the ratio may have decreased over the years as indicated in Table 2, which to the best of our knowledge, has not been reported before.

The OR calculated for age in general, women compared to men, older women compared to younger women, and older women compared to the whole group indicated that the risk of acquiring CC is higher in older persons, especially women, which is in line with observations from several other studies. Based on the levels for the different OR it could be speculated that age (OR 8.3 for age in general and 6.9 for older women) contributes more than sex (OR 2.8) to the risk of CC.

The geographical area studied, the south-west part of Skåne, a county in southern Sweden, has several advantages for carrying out epidemiological studies. The organisation for medical care is well defined, with two hospitals and one Pathology Department. The catchment area *per se* is also well defined. In addition, the migration rate in the area is limited. Furthermore, the use of personal identity numbers in Sweden that follow the individual throughout her or his entire life span makes it possible to identify every individual with CC in the region and to determine whether she or he lives within the catchment area. Accordingly, the conditions for conducting an epidemiologic CC study in Sweden are favourable. Another strength of this CC study was the precautions taken to identify patients who belonged to the catchment area but who had been diagnosed in another part of the county, by scrutinizing residents with CC at all Pathology Departments in Skåne. This study is also fairly large with 198 identified CC cases during the period (Table 2).

In conclusion, we observed a mean annual incidence of CC in southern Sweden of  $5.4/10^5$ , in line with previous data. In contrast to previously reported increases in the incidence rate, we report a stable incidence during the ten year study period from 2001-2010. The female: male ratio (2.8:1) was lower than previously reported. Based on data from available studies it seems that the female: male ratio is decreasing. In accordance with previously presented data it also seems that CC is more common in northern countries.

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## COMMENTS

### Background

Collagenous colitis (CC) predominantly affects middle-aged women and results in chronic watery diarrhoea. It has become evident that the incidence of CC is much higher than was first anticipated.

### Research frontiers

Information on the incidence of CC is available from one centre in the United States, one in Canada and a few in Europe. The incidence of CC in Örebro, in central Sweden, was  $4.9/10^5$  from 1993-1998. This level is in accordance with the reported figures from Olmsted County, Minnesota, United States during 1997-2001 ( $6.2/10^5$ ), Iceland during 1995-1999 ( $5.2/10^5$ ) and Calgary, Alberta, Canada during 2002-2004 ( $4.6/10^5$ ). However, these findings are in contrast to the incidence reported from Terrassa, Spain in 2004-2008 ( $2.6/10^5$ ), which is much lower.

### Innovations and breakthroughs

The study added information on the epidemiology of CC in the southern part of Sweden. The authors observed a mean annual incidence of CC in southern Sweden of  $5.4/10^5$ , which is in line with previous data. In contrast to previously reported increases in the incidence rate, the authors report a stable incidence in the ten year study period from 2001-2010. The female: male ratio (2.8:1) was lower than previously reported. Based on data from available studies it seems that the female: male ratio is decreasing. In accordance with previously presented data, it also seems that CC is more common in northern countries.

### Applications

During the 25 years since the first epidemiological study, the incidence of CC increased from 1.8 to  $5.4/100\,000$  inhabitants. It now seems to have levelled out. At the same time the female: male ratio has decreased. Consequently, more men (in relation to women) have been affected in recent years. Despite this, age is associated with a higher OR for disease than sex. The risk of disease is higher in northern than in southern Europe. The following remain to be clarified: Why the incidence is no longer increasing, why the female: male ratio has decreased and why people in northern Europe have a higher risk of disease. Environmental factors could have a substantial impact on CC; smoking, nonsteroidal anti-inflammatory drugs and proton pump inhibitors are known triggers but it can not be ruled out that several other factors are responsible for the high incidence of CC.

### Terminology

Incidence represents how many persons are affected by a certain disease/year in each group of 100 000 inhabitants within a defined area. Collagenous colitis mean inflammation in the large intestine that is predominantly visible via examination with a microscope, where the collagen layer can be observed in the epithelium. The disease results in chronic watery diarrhoea.

### Peer review

The manuscript is relatively well written, and the results are moderately interesting.

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## Evaluation of SNPs in miR-196-a2, miR-27a and miR-146a as risk factors of colorectal cancer

Renata Hezova, Alena Kovarikova, Julie Bienertova-Vasku, Milana Sachlova, Martina Redova, Anna Vasku, Marek Svoboda, Lenka Radova, Igor Kiss, Rostislav Vyzula, Ondrej Slaby

Renata Hezova, Alena Kovarikova, Milana Sachlova, Martina Redova, Marek Svoboda, Igor Kiss, Rostislav Vyzula, Ondrej Slaby, Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czech  
Julie Bienertova-Vasku, Anna Vasku, Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Kamenice 5, 625 00 Brno, Czech

Martina Redova, Ondrej Slaby, Central European Institute of Technology, Masaryk University, 625 00 Brno, Czech

Lenka Radova, Laboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry, Palacky University and Palacky University Affiliated Hospital Olomouc, 775 15 Olomouc, Czech

**Author contributions:** Hezova R and Kovarikova A performed DNA purifications and single nucleotide polymorphism analysis; Sachlova M, Vasku A, Svoboda M, Kiss I and Vyzula R collected DNA samples and clinical data from patients and controls involved in the study; Radova L performed statistical evaluation of the collected data; Bienertova-Vasku J, Redova M and Slaby O participated in manuscript preparation; and Slaby O designed the study, performed the data analysis and interpretation, and critically revised the manuscript.

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**Correspondence to:** Ondrej Slaby, PhD, Department of Comprehensive Cancer Care, Masaryk Memorial Cancer Institute, Zluty kopec 7, 656 53 Brno, Czech. [slaby@mou.cz](mailto:slaby@mou.cz)

Telephone: +420-5-43136902 Fax: +420-5-43136902

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**METHODS:** In order to investigate the effect of these SNPs in CRC, we performed a case-control study of 197 cases of sporadic CRC and 212 cancer-free controls originating from the Central-European Caucasian population using TaqMan Real-Time polymerase chain reaction and allelic discrimination analysis.

**RESULTS:** The genotype and allele frequencies of SNPs were compared between the cases and the controls. None of the performed analysis showed any statistically significant results.

**CONCLUSION:** Our data suggest a lack of association between rs11614913, rs895819 and rs2910164 and colorectal cancer risk in the Central-European Caucasian population, a population with an extremely high incidence of sporadic colorectal cancer.

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**Key words:** Association study; Colorectal cancer; MicroRNA; Single nucleotide polymorphism

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### Abstract

**AIM:** To investigate whether selected single nucleotide polymorphisms (SNPs) in miR-196a2, miR-27a and miR-146a genes are associated with sporadic colorectal cancer (CRC).

### INTRODUCTION

Sporadic colorectal cancer represents a typical multifactorial

rial disease with an intense crosstalk of the genetic background with the environment, including lifestyle habits and diet. Certain populations present higher rates of sporadic colorectal cancer, independently of diet and lifestyle habits than others<sup>[1]</sup>, which supports the hypothesis that individual genetic background is involved in the etio-pathogenesis of the disease. An extremely high incidence of colorectal cancer<sup>[1]</sup> has been repeatedly reported for the Central-European Caucasian population, significantly exceeding the peak incidence observed in the United States and other developed countries<sup>[2]</sup>. This population is, therefore, highly likely to carry a strong genetic predisposition to sporadic colorectal cancer and could be a good model population for sporadic colorectal cancer.

MicroRNAs (miRNAs) are short non-coding RNAs, 18 to 25 nucleotides in length, which regulate gene expression<sup>[3]</sup>. Single nucleotide polymorphisms (SNPs) may occur at the level of the miRNA biogenesis pathway genes, pri-miRNA, pre-miRNA or mature miRNA sequences. Such polymorphisms may be functional with regard to the biogenesis and actions of the mature miRNA. Specific SNPs are located at predicted miRNA target sites within 3' of untranslated regions of mRNAs. These SNPs have the potential to affect the efficiency of miRNA binding at their target sites as well as to create or disrupt binding sites. Resulting gene dysregulation may involve changes in phenotype and may eventually prove critical for the susceptibility to and the onset of cancer, as well as for prognosis and therapy response prediction<sup>[1]</sup>.

The most frequently studied miRNA-associated SNP in cancer is rs11614913 in the pre-miRNA region of miR-196-a2. Hu *et al*<sup>[4]</sup> observed the association of the rs11614913: T > C variant genotype with a significantly increased risk of breast cancer [odds ratio (OR) 1.23; 95% confidence interval (CI): 1.02-1.48]. A number of case-control studies were consequently performed in breast<sup>[5,6]</sup>, lung<sup>[7,8]</sup>, gastric<sup>[9]</sup>, esophageal<sup>[10]</sup>, hepatocellular<sup>[11]</sup> and head and neck cancer<sup>[12]</sup>. More recently, two contradictory studies were published evaluating rs11614913 as a potential risk factor for colorectal cancer in the Chinese population (T *vs* C allele-OR 1.320; CI: 1.056-1.649, *P* = 0.014<sup>[13]</sup> *vs* OR 1.065; CI: 0.803-1.414, *P* = 0.665<sup>[14]</sup>). SNP rs895819, located in the terminal loop of a pre-miR-27a oncogene, was initially evaluated in familial breast cancer, whereas the G allele was associated with reduced familial breast cancer risk (*P* = 0.0215). The opposite of this association was observed by Sun *et al*<sup>[15]</sup> in a gastric cancer case-control study where subjects with variant genotypes (AG + GG) showed a significantly increased risk of gastric cancer relative to AA carriers (OR 1.48; 95% CI: 1.06-2.05; *P* = 0.019). AG to C SNP (rs2910164) located within the sequence of the miR-146a precursor was first studied by Shen *et al*<sup>[16]</sup> due to the fact that predicted miR-146a target genes include BRCA1 and BRCA2, i.e., key breast and ovarian cancer susceptibility genes. Breast and ovarian cancer patients who had at least one miR-146a variant allele were diagnosed at an earlier age. Subsequently, the distribution of the miR-146a polymorphism

rs2910164 was evaluated in breast<sup>[6]</sup>, esophageal<sup>[17]</sup>, hepatocellular<sup>[18]</sup> and thyroid cancer<sup>[19]</sup>.

Thus, a significant association with the risk of various types of solid cancers, with the exception of colorectal cancer, has been repeatedly reported for SNPs: rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a; consequently, we decided to perform a case-control study evaluating these three SNPs and the risk of sporadic colorectal cancer in a Central-European Caucasian population.

## MATERIALS AND METHODS

### Patients and controls

The study included patients with newly diagnosed sporadic colorectal cancer treated at the Masaryk Memorial Cancer Institute, Czech Republic between January 2008 and December 2010. The patient cohort consisted of 197 subjects [105 men, 92 women; age (mean  $\pm$  SD): 63  $\pm$  9 years] with histologically confirmed colorectal adenocarcinomas, whereas the control cohort included a total of 202 cancer-free blood donor volunteers recruited from the same institute with a similar age distribution (93 men, 109 women; mean age: 65  $\pm$  14 years) and no previous history of any type of cancer. Due to its invasiveness, colonoscopy was not performed to exclude colorectal cancer (CRC) in the control cohort; however, all subjects were symptom free and no anemia was present. All study subjects were Caucasian. The hospital ethical committee approved the study and all study subjects supplied a written informed consent which was subsequently archived.

### DNA isolation and genotyping

Genomic DNA was isolated from the full peripheral blood using the MagNA Pure DNA Isolator (Roche). DNA concentration was measured on the Nanodrop ND-1000 (NanoDrop Technologies, Inc.). For analysis of rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a, Real-Time polymerase chain reaction (PCR) allelic discrimination was performed on Step-One Real-Time PCR (Applied Biosystems, United States) using standard TaqMan genotyping assays according to the manufacturer's instructions. In brief, probes, primers and TaqMan universal PCR Master Mix were obtained from Applied Biosystems. A reaction solution of 10  $\mu$ L contained 0.5  $\mu$ L TaqMan Genotyping Assay mix (consisting of 20X Mix of unlabeled PCR primers and TaqMan minor groove binder probe, 6-carboxy-fluorescein and VIC dye-labeled), 8  $\mu$ L of PCR mixture reagent and 10 ng of genomic DNA. Reactions were run according to the manufacturer's instructions. The PCR consisted of pre-PCR read at 60  $^{\circ}$ C for 30 s, holding stage at 95  $^{\circ}$ C for 10 min, 50 cycles of denaturing at 92  $^{\circ}$ C for 15 s, annealing 60  $^{\circ}$ C for 1 min 30 s and post-PCR read at 60  $^{\circ}$ C for 30 s.

### Statistical analysis

The Hardy-Weinberg equilibrium was tested for each

**Table 1** Logistic regression analysis of genotype frequencies of single nucleotide polymorphisms rs11614913, rs895819 and rs2910164 in colorectal cancer cases and controls in the Czech population

		Control		CRC		OR <sup>1</sup>	95% CI	P value
		n	%	n	%			
miR-27a	A/A	93	43.87	88	44.67	1		0.996 <sup>2</sup>
	A/G	94	44.34	86	43.65	0.98	(0.64-1.49)	0.950
	G/G	25	11.79	23	11.68	1.04	(0.54-1.98)	0.970
	AG + GG vs AA					1.01	(0.68-1.51)	0.954
	[G] vs [A]					0.999	(0.71-1.39)	0.995
miR-146a	Trend	212		197		0.99	(0.8-1.22)	0.9118 <sup>a</sup>
	G/G	124	58.49	115	58.38	1		0.761 <sup>a</sup>
	C/G	79	37.26	70	35.53	0.93	(0.61-1.41)	0.740
	C/C	9	4.25	12	6.09	1.31	(0.52-3.27)	0.556
	CG + CC vs GG					1.03	(0.69-1.54)	0.879
miR-196-a2	[C] vs [G]					1.37	(0.56-3.33)	0.494
	Trend	212		197		0.97	(0.79-1.19)	0.7558 <sup>a</sup>
	C/C	87	41.04	82	41.62	1		0.6098 <sup>a</sup>
	C/T	103	48.58	89	45.18	0.95	(0.62-1.45)	0.794
	T/T	22	10.38	26	13.2	1.32	(0.69-2.54)	0.415
	CT + TT vs CC					1.01	(0.68-1.51)	0.951
	[T] vs [C]					1.04	(0.75-1.45)	0.811
	Trend	212		197		1.08	(0.8-1.46)	0.5987 <sup>a</sup>

P-values are calculated according to Wald's test. <sup>a</sup>P-values according to likelihood ratio-test; <sup>1</sup>Age and sex adjusted; CRC: Colorectal cancer; OR: Odds ratio; CI: Confidence interval.

polymorphism using the  $\chi^2$  test in patients and controls separately. Allelic frequencies were estimated by the "counting method" and differences in allele frequencies between case and control subjects were tested using the likelihood ratio  $\chi^2$  tests for 2  $\times$  2 tables (two alleles, case vs control subjects). The homozygote of the most frequent allele was used as a reference for calculating the OR. For an OR and 95% confidence interval, logistical regression was used based on a model for sex and age of the patients. Data analysis was performed using the Statistica v. 9.0 (Statsoft Inc., Tulsa, OK, United States) program package. Values of  $P < 0.05$  were considered statistically significant.

## RESULTS

All polymorphisms met the criteria of the Hardy-Weinberg equilibrium in the individual patient and control groups. Logistic regression modeling was used to estimate the odds ratios of the investigated genotypes and alleles of SNPs rs2910164, rs11614913 and rs3746444 in CRC cases as well as in the controls (Table 1). All of the examined polymorphisms displayed a clear lack of statistically significant associations with colorectal cancer risk.

## DISCUSSION

Sporadic colorectal cancer is a multifactorial disease with multiple genetic determinants of varied significance. Numerous SNP analyses of sporadic colorectal cancer were conducted in order to clarify the genetic background. It has been hypothesized that polymorphic genetic variants involved in metabolism, DNA repair and apoptosis are linked to susceptibility to colorectal cancer<sup>[20]</sup>. Although

alterations in miRNA function have been detected in a broad spectrum of hematological malignancies and solid tumors<sup>[21-23]</sup>, including CRC<sup>[24]</sup>, only two studies performed to date have focused on miRNA-associated SNPs in CRC; although these studies were carried out in the Chinese population<sup>[13,14]</sup>, their results were contradictory. Although it has been hypothesized that SNPs in miRNA genetic regions may affect the transcription of pri-miRNA transcripts, processing of miRNA precursors to mature miRNAs or miRNA target interactions, genetic variants in pre-miRNA regions are rare and unlikely to be functionally important, mainly due to the serious pressure imposed by natural selection on the evolutionary conserved pre-miRNA sequences<sup>[5]</sup>.

In our study, we performed a case-control study of the three most frequently studied SNPs in miRNA genes (rs11614913 in miR-196-a2, rs895819 in miR-27a and rs2910164 in miR-146a), to investigate the degree of risk of CRC in the Central-European Caucasian population.

As it has been experimentally validated that the rs11614913 polymorphism located in the miR-196-a2 mature sequence affects the maturation and effect of target mRNA possibility, it is biologically plausible that genetic variation of hsa-miR-196a2 could modulate cancer susceptibility. In accordance with this finding, rs11614913 is one of the most frequently studied SNPs associated with miRNAs in case-control studies of a wide range of solid cancers<sup>[5-14]</sup>. For example, Hu *et al.*<sup>[8]</sup> reported that the CC homozygous genotype of rs11614913 located in miR-196a2 was associated with a statistically significant increase in the mature miR-196a and a worse prognosis in non-small-cell lung cancer (NSCLC), proposing that this SNP could serve as a prognostic marker of NSCLC. Another Chinese study reported a clear association between



CC and CC/CT genotypes of rs11614913 and increased risk of breast cancer (OR 1.23; 95% CI: 1.02-1.48)<sup>[4]</sup>. When reviewed together, the majority of these studies described significant associations of the rs11614913-C allele with susceptibility and/or poor prognosis of lung cancer<sup>[7,8]</sup>, gastric cancer<sup>[9]</sup>, esophageal cancer<sup>[10]</sup>, hepatocellular carcinoma<sup>[11]</sup> and head and neck cancer<sup>[12]</sup>. More recently, two Chinese studies focusing on an association between this SNP and susceptibility to CRC and its progression were performed<sup>[13,14]</sup>.

Although the frequency of CC homozygotes of rs11614913 was higher in CRC patients than in healthy controls (41.62% *vs* 41.04%) in our study, the genotypes carrying the C allele (CT and CC) expressed the opposite trend in frequencies (64.21% in CRC *vs* 65.33% in controls). Moreover, the frequency of the C allele in CRC patients (64.21%) was not significantly lower than in healthy controls (65.33%). Furthermore, no significant association between the miR-196a2 polymorphism and the risk of CRC was observed in our study. These results are in agreement with the findings by Chen *et al.*<sup>[14]</sup>. On the other hand, Zhan's group described the C allele as a risk factor for CRC in the Chinese population. Neither of the Chinese studies reported any associations between the rs11614913 polymorphism and CRC progression, including tumor grade, stage, lymph node and distant metastasis<sup>[13,14]</sup>. The discrepancy in the potential significance of rs11614913 in CRC reported by the above-mentioned independent studies may be due to different molecular pathogenetic mechanisms as different contributors to cancer or population-specific factors such as the different genetic backgrounds of the studied cohorts.

MiR-27a, in general, is a very important miRNA involved in the development of chemoresistance in solid cancer<sup>[15]</sup>. This study presents the first case-control investigation of the role of the A/G polymorphism (rs895819) in miR-27a in CRC; however, no significant associations were observed. Although the frequency of AA homozygotes was higher in CRC patients than in healthy controls (44.67% *vs* 43.87%), the frequencies of the genotypes carrying the A allele (AA and AG) did not show significant differences between the study cohorts (66.50% in CRC patients *vs* 66.04% in healthy controls). In gastric cancer, it has been reported that the variant genotypes of rs895819 located at miR-27a conferred a 48% increased risk of developing gastric cancer in the Chinese population; moreover, this trend tended to be age-specific. The authors concluded that elevated levels of miR-27a, through regulating the Zinc finger and BTB domain containing 10 (ZBTB10), result in the over-expression of Sp proteins and Sp-dependent genes, which play important roles in gastric cancer cell survival and angiogenesis<sup>[15,25]</sup>. Furthermore, Yang *et al.*<sup>[26]</sup> found that the G-allele of rs895819, located in the terminal loop of the pre-miR-27a oncogene, is associated with reduced familial breast cancer risk (OR = 0.88; 95% CI: 0.78-0.99; *P* = 0.0287).

MiRNA-146a and its G to C common polymorphism, rs2910164, located within the sequence or the miR-146a

precursor represent another miRNA hotspot evaluated in CRC for the first time in the present study. This SNP leads to change from a G:U pair to a C:U mismatch, and consequently, to reduced levels of pre- and mature miR-146a<sup>[17,19]</sup>. As BRCA1 and BRCA2, key breast and ovarian cancer susceptibility genes, are predicted targets of miR-146a, the majority of studies have been focused on breast and ovarian cancer. The results of Chen *et al.*<sup>[16]</sup>, who primarily studied rs2910164 in breast cancer and postulated that breast and ovarian cancer patients who had at least one variant allele were diagnosed at an earlier age (*P* = 0.029, *P* = 0.014, respectively), were not confirmed by further and larger independent case-control studies performed by Hu *et al.*<sup>[4]</sup> and Catucci *et al.*<sup>[6]</sup>. Garcia *et al.*<sup>[27]</sup> concluded that the rs2910164: G > C SNP in the miR-146a gene is not associated with breast cancer risk in BRCA1 and BRCA2 mutation carriers. This case-control study, i.e., the first study to investigate the role of the miR-146a polymorphism, rs2910164, in CRC risk, found no significant association. Although our results did not indicate any significant relationship between the above-mentioned, miRNA-associated, SNPs and risk of CRC, we believe that a more detailed and comprehensive characterization of miRNA SNPs will improve our understanding of the miRNAs involved in CRC onset and progression which is necessary for the development of novel diagnostic and therapeutic strategies and approaches to this deadly disease.

## COMMENTS

### Background

The Central-European Caucasian population displays an extraordinarily high incidence of sporadic colorectal cancer and although there is a long-term tendency towards a decrease in mortality rates attributed to colorectal cancer, it still represents a major cause of death in the population. Susceptibility to colorectal cancer is typically multifactorial and can be characterized by intensive crosstalk between the genetic background of an individual and environmental factors. Susceptibility genes are involved in metabolic pathways controlled by epigenetic mechanisms where microRNA-associated regulations may play an important role.

### Research frontiers

MicroRNAs (miRNAs) are small non-coding RNAs regulating gene expression. It has been recently suggested that single nucleotide polymorphisms (SNPs) in genes encoding mir196-a2, miR-27a and mir146-a may be associated with increased risk of various types of solid cancer. However, no such study of colorectal cancer has been conducted so far. This study investigated three SNPs (rs11614913 in miR-196-a2, rs895819 in hsa-miR-27a and rs2910164 in miR-146a) which were previously reported to be significantly associated with various types of solid cancer.

### Innovations and breakthroughs

To the best of our knowledge, this is the first study focusing on the significance of rs11614913 in miR-196-a2, rs895819 in hsa-miR-27 and rs2910164 in miR-146a in sporadic colorectal cancer. The study was conducted using a highly homogenous Central-European Caucasian population with extremely high rates of sporadic colorectal cancer.

### Applications

The significance of SNPs in genes encoding miRNAs remains controversial. Positive associations of the investigated polymorphisms were reported in various types of solid cancer. Based on the results, however, the investigated SNPs in miRNA genes do not seem to be major genetic determinants of genetic susceptibility to sporadic colorectal cancer in the Central-European population.

# Terminology

Genetic susceptibility refers to inherited predisposition to increased risk of developing a certain disease, typically a multifactorial disease. mRNAs mean short non-coding RNAs, 17-22 nucleotides in length, which regulate gene expression and thereby play significant roles in cancer.

# Peer review

The manuscript is well presented and supported by data.

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## Progression of remnant gastric cancer is associated with duration of follow-up following distal gastrectomy

Shuheï Komatsu, Daisuke Ichikawa, Kazuma Okamoto, Daito Ikoma, Masahiro Tsujiura, Yukihisa Nishimura, Yasutoshi Murayama, Atsushi Shiozaki, Hisashi Ikoma, Yoshiaki Kuriu, Masayoshi Nakanishi, Hitoshi Fujiwara, Toshiya Ochiai, Yukihito Kokuba, Eigo Otsuji

Shuheï Komatsu, Daisuke Ichikawa, Kazuma Okamoto, Daito Ikoma, Masahiro Tsujiura, Yukihisa Nishimura, Yasutoshi Murayama, Atsushi Shiozaki, Hisashi Ikoma, Yoshiaki Kuriu, Masayoshi Nakanishi, Hitoshi Fujiwara, Toshiya Ochiai, Yukihito Kokuba, Eigo Otsuji, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachihirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

Author contributions: Komatsu S, Ichikawa D, Okamoto K, Murayama Y, Shiozaki A, Ikoma H, Kuriu Y, Nakanishi M, Fujiwara H, Ochiai T, Kokuba Y and Otsuji E performed research; Komatsu S, Ikoma D, Tsujiura M and Nishimura Y analyzed the data; and Komatsu S wrote the paper.

Correspondence to: Daisuke Ichikawa, MD, Division of Digestive Surgery, Department of Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachihirokoji, Kamigyo-ku, Kyoto 602-8566, Japan. [ichikawa@koto.kpu-m.ac.jp](mailto:ichikawa@koto.kpu-m.ac.jp)  
Telephone: +81-75-2515527 Fax: +81-75-2515522

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### Abstract

**AIM:** To re-evaluate the recent clinicopathological features of remnant gastric cancer (RGC) and to develop desirable surveillance programs.

**METHODS:** Between 1997 and 2008, 1149 patients underwent gastrectomy for gastric cancer at the Department of Digestive Surgery, Kyoto Prefectural University of Medicine, Japan. Of these, 33 patients underwent gastrectomy with lymphadenectomy for RGC. Regarding the initial gastric disease, there were 19 patients with benign disease and 14 patients with gastric cancer. The hospital records of these patients were reviewed retrospectively.

**RESULTS:** Concerning the initial gastric disease, the

RGC group following gastric cancer had a shorter interval [ $P < 0.05$ ; gastric cancer *vs* benign disease: 12 (2-22) *vs* 30 (4-51) years] and were more frequently reconstructed by Billroth- I procedure than those following benign lesions ( $P < 0.001$ ). Regarding reconstruction, RGC following Billroth- II reconstruction showed a longer interval between surgical procedures [ $P < 0.001$ ; Billroth- II *vs* Billroth- I : 32 (5-51) *vs* 12 (2-36) years] and tumors were more frequently associated with benign disease ( $P < 0.001$ ) than those following Billroth- I reconstruction. In tumor location of RGC, after Billroth- I reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth- II reconstruction, RGC occurred more frequently at the anastomotic site. The duration of follow-up was significantly associated with the stage of RGC ( $P < 0.05$ ). Patients diagnosed with early stage RGC such as stage I - II tended to have been followed up almost every second year.

**CONCLUSION:** Meticulous follow-up examination and early detection of RGC might lead to a better prognosis. Based on the initial gastric disease and the procedure of reconstruction, an appropriate follow-up interval and programs might enable early detection of RGC.

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**Key words:** Remnant gastric cancer; Surveillance; Follow-up; Reconstruction; Distal gastrectomy

**Peer reviewers:** Dr. Ashok Kumar, MD, Department of Surgical Gastroenterology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow 226014, India; Liang-Shun Wang, MD, Professor, Vice-superintendent, Shuang-Ho Hospital, Taipei Medical University, No. 291, Jhongheng Rd., Jhonghe City, New Taipei City 237, Taiwan, China; Takaaki Arigami, MD, PhD, Department of Surgical Oncology and Digestive Surgery, Field of Oncology, Kagoshima University Graduate School of Medical



and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 891-0175, Japan

Komatsu S, Ichikawa D, Okamoto K, Ikoma D, Tsujiura M, Nishimura Y, Murayama Y, Shiozaki A, Ikoma H, Kuriu Y, Nakanishi M, Fujiwara H, Ochiai T, Kokuba Y, Otsuji E. Progression of remnant gastric cancer is associated with duration of follow-up following distal gastrectomy. *World J Gastroenterol* 2012; 18(22): 2832-2836 Available from: URL: <http://www.wjg-net.com/1007-9327/full/v18/i22/2832.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2832>

## INTRODUCTION

The incidence of remnant gastric cancer (RGC) following distal gastrectomy has been reported to account for 1%-2% of all gastric cancers in Japan<sup>[1,2]</sup>. Previously, RGC was reported to be caused by multiple factors, and the incidence, pathological features, and potential mechanisms have been extensively investigated<sup>[3-5]</sup>. Specifically, RGC is commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequently poor prognosis<sup>[6,7]</sup>. However, recently, the incidence and etiology of RGC have been changing<sup>[8]</sup> because of the long latency periods, decreasing prevalence of gastrectomy for benign disease<sup>[6,9]</sup>, early detection and improved outcomes in patients with gastric cancers<sup>[10,11]</sup>. Moreover, recent advances in diagnostic and treatment techniques have led to a higher detection rate of early RGC following distal gastrectomy<sup>[12]</sup>. Consequently, endoscopic therapy such as endoscopic mucosal resection or endoscopic submucosal dissection is applicable for treatment of early-stage RGC<sup>[13,14]</sup>. Indeed, more than half of the RGC patients were treated for T1 or T2, node-negative and early stage cancer at our institution and almost 80% of patients with RGC were curatively resected. Therefore, it is necessary to re-evaluate the risk factors of RGC to develop an optimal new surveillance program and treatment guide. However, there is limited information available to help guide the treatment of patients with RGC. This study was designed to re-evaluate the clinicopathological characteristics and surgical outcomes of RGC and to develop desirable surveillance programs.

## MATERIALS AND METHODS

### Patients

Between 1997 and 2008, 1149 patients underwent gastrectomy for gastric cancer. Of these, 33 consecutive patients with primary RGC were treated in the Department of Digestive Surgery, Kyoto Prefectural University of Medicine. All patients underwent gastrectomy with lymphadenectomy for RGC. The clinicopathologic findings of these patients were determined retrospectively based on their hospital records. Macroscopic, microscopic and histopathological classifications of gastric cancers were based on the Japanese Classification of Gastric Carcinomas<sup>[15]</sup> and tumor-node-metastasis staging system<sup>[16]</sup>.

Histologic types were classified as differentiated (papillary, moderately or well-differentiated adenocarcinoma) and undifferentiated (poorly or undifferentiated adenocarcinoma, signet-ring cell carcinoma, and mucinous adenocarcinoma).

### Follow-up program after initial gastrectomy

The follow-up program after initial gastrectomy at our institution is comprised of a regular physical examination and laboratory blood tests, chest X rays, an upper gastrointestinal series or endoscopy and ultrasonography or computer tomography for the first 5 years, and yearly endoscopy thereafter, if possible.

### Evaluation of clinical associations between remnant gastric cancer and various clinical factors

The correlations between clinical factors and an initial factor such as previous disease or method of reconstruction in initial surgery were examined. Moreover, the follow-up interval is very important for screening recurrence and second primary gastric cancers. Therefore, correlation between follow-up periods and progression was evaluated in RGC.

### Statistical analysis

The patient was included as a cause-specific death when the cause of death was specified as recurrent RGC.  $\chi^2$  test and Fisher's exact probability test were performed for categorical variables, while Student's *t*-test and Mann-Whitney *U*-test for unpaired data with continuous variables were performed to compare the clinicopathological characteristics between two groups. Kruskal-Wallis *H* test was used as a nonparametric procedure that can be used to compare more than two groups for analyses of follow-up interval. A *P* value less than 0.05 was considered significant.

## RESULTS

### Clinicopathologic characteristics of patients with primary remnant gastric cancer

The mean patient age was 68 years, and the male:female ratio was 2.7:1. Regarding the initial gastric disease, there were 19 patients with benign disease and 14 patients with gastric cancer. The median interval between the 1st and 2nd surgery was 20 years. Reconstruction during the 1st surgery was mainly Billroth- I or Billroth- II. En bloc resection of the tumor by total remnant gastrectomy was performed with jejunal mesentery and D2 lymphadenectomy and concomitant organ resection. Eighteen patients additionally received splenectomy, four patients received distal pancreatectomy, two patients received partial colon resection and two patients received liver resection. Reconstructions were performed in 16 patients by Billroth- I, 16 patients by Billroth- II and one by Roux-en-Y procedure for all resected RGC tumors. Tumors were located at the anastomotic site in 16 (61%) patients, corpus and/or cardia in nine (34%), and throughout the



Table 1 Association between clinicopathologic characteristics and initial disease *n* (%)

Variables	<i>n</i>	Initial disease		<i>P</i> value
		Benign ( <i>n</i> = 19)	Cancer ( <i>n</i> = 14)	
Age (yr) (mean)		70 (51)	66 (49)	0.26
Gender				
Male	24	15 (63)	9 (38)	0.35
Female	9	4 (44)	5 (56)	
Interval from initial surgery				
Year (median)		30 (4-51)	12 (2-22)	< 0.05
Reconstruction of 1st surgery				
Billroth- I	16	4 (25)	12 (75)	< 0.001
Billroth- II	16	15 (94)	1 (6)	
R-Y	1	0 (0)	1 (100)	
Location of RGC				
Anastomotic site	11	9 (82)	2 (18)	0.08
Suture line	7	2 (29)	5 (71)	
Others	15	8 (53)	7 (47)	
Histological type				
Differentiated	13	8 (62)	5 (38)	0.71
Undifferentiated	20	11 (55)	9 (45)	
Lymphatic invasion				
Negative	16	8 (50)	8 (50)	0.39
Positive	17	11 (65)	6 (35)	
Venous invasion				
Negative	16	8 (50)	8 (50)	0.39
Positive	17	11 (65)	6 (35)	
Tumor size				
cm (mean)		51 (46)	61 (54)	0.40
Depth of tumor				
T1	10	4 (40)	6 (60)	0.18
T2, 3, 4	23	15 (65)	8 (35)	
Lymph node metastasis				
Negative	20	10 (50)	10 (50)	0.27
Positive	13	9 (69)	4 (31)	
Stage				
I	17	8 (47)	9 (53)	0.21
II, III, IV	16	11 (69)	5 (31)	

Significant values are shown in boldface type. *P* values were derived from  $\chi^2$  or Fisher's exact test and were considered significant at < 0.05. R-Y: Roux-en Y; RGC: Remnant gastric cancer.

whole remnant in one (4%) patient. Consequently, more than half of the RGC patients demonstrated T1 or T2, undifferentiated, node-negative and early stage cancer. In 78.8% (26/33) of patients, resections were performed with curative intent.

Association between clinicopathologic characteristics and initial disease

Clinicopathologic findings of 33 patients with primary RGC are listed in Table 1 according to the nature of the primary disease. Patients with RGC following gastric cancer showed a significantly shorter interval between the 1st and 2nd surgery [*P* < 0.05, gastric cancer *vs* benign disease: 12 (2-22) *vs* 30 (4-51) years] and were more frequently reconstructed by the Billroth- I method than those following benign disease (*P* < 0.005). Other factors did not significantly differ between the two groups.

Association between clinicopathologic characteristics and reconstruction of 1st surgery

Table 2 shows details of 33 RGC patients according to

Table 2 Association between clinicopathologic characteristics and reconstruction of 1st surgery *n* (%)

Variables	<i>n</i>	Reconstruction at first surgery		<i>P</i> value
		Billroth- I ( <i>n</i> = 16)	Billroth- II ( <i>n</i> = 16)	
Age (yr) (mean)		68 (50)	69 (50)	0.64
Gender				
Male	24	13 (54)	11 (46)	0.69
Female	8	3 (38)	5 (63)	
Interval from initial surgery				
Year (median)		12 (2-36)	32 (5-51)	< 0.001
Initial gastric disease				
Benign	19	4 (21)	15 (79)	< 0.001
Cancer	13	12 (92)	1 (8)	
Location of RGC				
Anastomotic site	11	2 (18)	9 (82)	0.11
Suture line	7	5 (71)	2 (29)	
Others	14	9 (64)	5 (36)	
Histological type				
Differentiated	13	8 (62)	5 (38)	0.47
Undifferentiated	19	8 (42)	11 (58)	
Lymphatic invasion				
Negative	15	6 (40)	9 (60)	0.48
Positive	17	10 (59)	7 (41)	
Venous invasion				
Negative	16	8 (50)	8 (50)	0.72
Positive	16	8 (50)	8 (50)	
Tumor size				
mm (mean)		51	56	0.67
Depth of tumor				
T1	10	6 (60)	4 (40)	0.76
T2, 3, 4	22	10 (45)	12 (55)	
Lymph node metastasis				
Negative	14	9 (47)	10 (53)	1
Positive	13	7 (54)	6 (46)	
Stage				
I	17	9 (53)	8 (47)	1
II, III, IV	15	7 (47)	8 (53)	

Significant values are shown in boldface type. *P* values were derived from  $\chi^2$  or Fisher's exact test and were considered significant at < 0.05. RGC: Remnant gastric cancer.

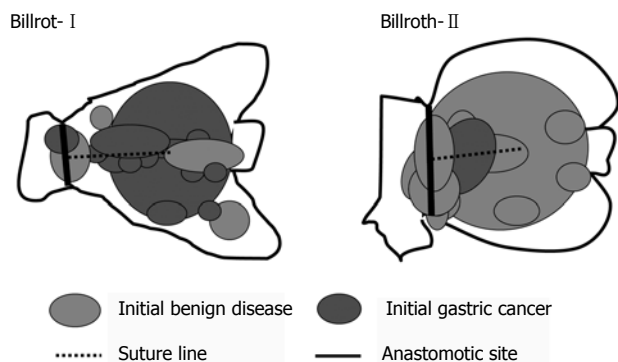
the method of reconstruction. RGC following Billroth- II reconstruction showed a longer interval between surgical procedures (*P* < 0.001) and tumors were more frequently associated with benign disease (*P* < 0.001) than those following Billroth- I reconstruction. Figure 1 shows the tumor location of 32 RGC following distal gastrectomy according to the method of reconstruction. After Billroth- I reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth- II reconstruction, RGC occurred more frequently at the anastomotic site.

The duration of follow-up after distal gastrectomy

The duration of follow-up was significantly associated with the stage of progression in RGC (*P* < 0.05). Patients diagnosed with early stage RGC such as stage I - II tended to have been followed up almost every second year (Figure 2).

DISCUSSION

Gastric cancer is the second leading cause of cancer-

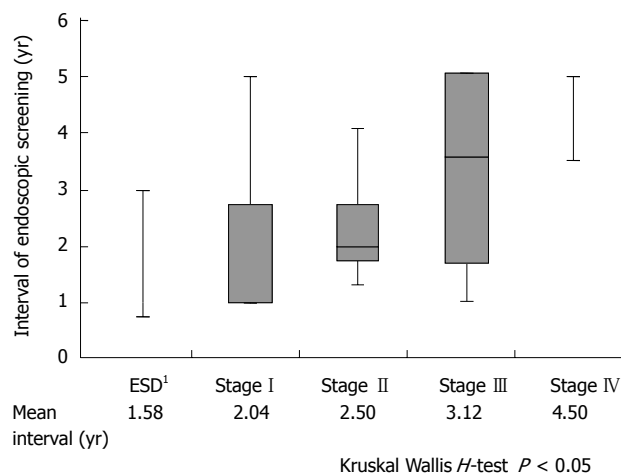


**Figure 1** Location of 32 remnant gastric cancer tumors following distal gastrectomy according to the method of reconstruction. After Billroth- I reconstruction, remnant gastric cancer (RGC) occurred more frequently near the suture line and remnant gastric wall; whereas, RGC after Billroth- II reconstruction occurred more frequently at the anastomotic site.

related death in the world<sup>[17]</sup>. However, recent advances in diagnostic methods, less invasive treatment techniques and better peri-operative management have increased the early detection of gastric cancer and decreased the mortality and morbidity rates<sup>[18-20]</sup>. Consequently, the number of cured patients has been increasing and some of these patients are at risk of acquiring second primary cancer in the remnant stomach. This implies that more cases of RGC will be encountered in the future.

In previous reports, RGC was commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequent poor prognosis<sup>[6,7]</sup>. However, recently, the incidence and etiology of RGC following distal gastrectomy may be changing due to diagnostic and technological advances. In our study, more than half of the RGC patients were treated for T1 or T2, node-negative and early stage cancer, contrary to that in previous series (Table 1). Almost 80% of patients were curatively resected with intensive lymphadenectomy. Thereby, the survival curves of primary proximal gastric cancer (PGC) and RGC were similar and without a significant difference, although patients with RGC tended to have a higher incidence of undifferentiated cancer, vascular invasion, and T4 component than patients with PGC (data not shown). Therefore, RGC is not always advanced at diagnosis and if so, intensive surgery for RGC does not necessarily mean a poor prognosis in comparison to that for primary gastric cancer. Therefore, it is necessary to re-evaluate the risk factors of RGC to develop an optimal new endoscopic surveillance program and treatment guide.

Regarding surveillance systems for early detection and curative treatment of RGC, periodic endoscopic examinations of the gastric remnant are shown to be extremely important in our study (Figure 1). However, a follow-up program that is too intensive may not be beneficial to the patient. The initial gastric disease and the interval between the 1st and 2nd surgery could affect the incidence of RGC. In our study, RGC following gastric cancer had a significantly shorter interval between 1st and 2nd surgery than that following benign disease [ $P <$



**Figure 2** Association of endoscopic follow-up intervals and the stage of progression. The follow-up interval was significantly associated with the stage of progression in remnant gastric cancer ( $P < 0.05$ ). <sup>1</sup>Patients treated with endoscopic submucosal dissection (ESD), who were not included in this study, are presented for the purpose of comparison.

0.05; gastric cancer *vs* benign disease: 12 (2-22) years *vs* 30 (4-51) years]. However, surveillance systems for gastric cancer should be especially considered because of decreasing gastrectomy for benign disease. Furthermore, 86% of all initial gastric cancer patients underwent Billroth- I reconstruction at our institution and their median interval between 1st and 2nd surgery was 12 (2-36) years. Moreover, the duration of follow-up was significantly associated with the stage of RGC progression and an early detection of RGC led to better prognosis (Figure 2). Taken together, annual surveillance endoscopic screening should be required for at least 12 years following distal gastrectomy. Furthermore, after 12 years of follow-up, surveillance endoscopy should be recommended every second year because we found that patients diagnosed with early stage RGC such as stage I - II tended to have been followed almost every second year. In particular, meticulous endoscopy examination should be performed near the suture line and remnant gastric wall after Billroth- I reconstruction and also should be performed at the anastomotic site after Billroth- II reconstruction.

In conclusion, due to recent advances in diagnostic and treatment technologies, the etiology of RGC has been changing. Meticulous follow-up examination and early detection of RGC might lead to a better prognosis. Considering both the initial gastric disease and the procedure of reconstruction, an appropriate follow-up interval and programs should facilitate the detection of early RGC.

## COMMENTS

### Background

Recently, the incidence and etiology of remnant gastric cancer (RGC) have been changing because of the long latency periods, decreasing prevalence of gastrectomy for benign disease, early detection and improved outcomes in patients with gastric cancers. Moreover, recent advances in diagnostic and treatment technique have led to a higher detection rate of early RGC following distal gastrectomy.

## Research frontiers

It is necessary to re-evaluate the risk factors of RGC and develop an optimal new surveillance program and treatment guide. However, there is limited information available to help guide the treatment of patients with RGC. In this study, the authors re-evaluated the clinicopathological characteristics and surgical outcomes of RGC and developed desirable surveillance programs.

## Innovations and breakthroughs

In this study, more than half of the RGC patients were demonstrated to have T1 or T2, undifferentiated, node-negative and early stage cancer. The duration of follow-up was significantly associated with the stage of progression in RGC. Patients diagnosed with early stage RGC such as stage I-II tended to have been followed almost every second year. After Billroth-I reconstruction, RGC occurred more frequently near the suture line and remnant gastric wall. After Billroth-II reconstruction, RGC occurred more frequently at the anastomotic site.

## Applications

RGC following gastric cancer had a significantly shorter interval between 1st and 2nd surgery than those following benign disease. Annual surveillance endoscopic screening should be required for at least 12 years following distal gastrectomy. Furthermore, after 12 years of follow-up, surveillance endoscopy should be recommended every second year.

## Terminology

The incidence of RGC following distal gastrectomy has been reported to account for 1%-2% of all gastric cancers in Japan. In previous reports, RGC was commonly found at an advanced stage, resulting in low rates of curative resection (38%-40%) and a consequent poor prognosis.

## Peer review

Authors have given new thoughts while designing this study. The paper is nicely written.

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## Role of ascites adenosine deaminase in differentiating between tuberculous peritonitis and peritoneal carcinomatosis

Seung Joo Kang, Ji Won Kim, Jee Hyun Baek, Se Hyung Kim, Byeong Gwan Kim, Kook Lae Lee, Ji Bong Jeong, Yong Jin Jung, Joo Sung Kim, Hyun Chae Jung, In Sung Song

Seung Joo Kang, Joo Sung Kim, Hyun Chae Jung, In Sung Song, Department of Internal Medicine and Liver Research Institute, Seoul National University College of Medicine, Seoul 110-744, South Korea

Ji Won Kim, Byeong Gwan Kim, Kook Lae Lee, Ji Bong Jeong, Yong Jin Jung, Department of Internal Medicine, Seoul National University Boramae Hospital, Seoul 157-707, South Korea

Jee Hyun Baek, Se Hyung Kim, Department of Radiology, Seoul National University College of Medicine, Seoul 110-744, South Korea

**Author contributions:** Kang SJ and Kim JW designed the study and wrote the manuscript; Baek JH and Kim SH reviewed the all abdomen computed tomographs in this study; Kim BG, Lee KL, Jeong JB and Jung YJ co-ordinated and provided the patient's data; and Kim JS, Jung HC and Song IS analyzed the data and involved in editing the manuscript.

**Correspondence to:** Ji Won Kim, MD, PhD, Associate Professor, Department of Internal Medicine, Seoul National University Borame Hospital, 395, Shindaebang 2-Dong, Dongjak-Gu, Seoul 157-707, South Korea. [giwkim@hanmail.net](mailto:giwkim@hanmail.net)

Telephone: +82-2-8402712 Fax: +82-2-8310174

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### Abstract

**AIM:** To investigate the usefulness of tumor markers and adenosine deaminase in differentiating between tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC).

**METHODS:** A retrospective analysis of data was performed on consecutive patients who underwent peritoneoscopic and abdominal computed tomography (CT) evaluations. Among 75 patients at the Seoul National University Hospital from January 2000 to June 2010 who underwent both tests, 27 patients (36.0%) and 25

patients (33.3%) were diagnosed with TBP and PC, respectively. Diagnosis was confirmed by peritoneoscopic biopsy.

**RESULTS:** Serum c-reactive protein ( $7.88 \pm 6.62$  mg/dL vs  $3.12 \pm 2.69$  mg/dL,  $P = 0.01$ ), ascites adenosine deaminase ( $66.76 \pm 32.09$  IU/L vs  $13.89 \pm 8.95$  IU/L,  $P < 0.01$ ), ascites lymphocyte proportion ( $67.77 \pm 23.41\%$  vs  $48.36 \pm 18.78\%$ ,  $P < 0.01$ ), and serum-ascites albumin gradient ( $0.72 \pm 0.49$  g/dL vs  $1.05 \pm 0.50$  g/dL,  $P = 0.03$ ) were significantly different between the two groups. Among tumor markers, serum and ascites carcinoembryonic antigen, serum carbohydrate antigen 19-9 showed significant difference between two groups. Abdominal CT examinations showed that smooth involvement of the parietal peritoneum was more common in the TBP group (77.8% vs 40.7%) whereas nodular involvement was more common in the PC group (14.8% vs 40.7%,  $P = 0.04$ ). From receiver operating characteristic (ROC) curves ascites adenosine deaminase (ADA) showed better discriminative capability than tumor markers. An ADA cut-off level of 21 IU/L was found to yield the best results of differential diagnosis; sensitivity, specificity, positive predictive value, and negative predictive value were 92.0%, 85.0%, 88.5% and 89.5%, respectively.

**CONCLUSION:** Besides clinical and radiologic findings, ascitic fluid ADA measurement is helpful in the differential diagnosis of TBP and PC.

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**Key words:** Tuberculous peritonitis; Peritoneal carcinomatosis; Adenosine deaminase; Peritoneoscopy

**Peer reviewer:** Bernabe Matias Quesada, MD, Department of Surgery, Hospital Cosme Argerich, Talcahuano 944 9°A, Buenos Aires 1013, Argentina



Kang SJ, Kim JW, Baek JH, Kim SH, Kim BG, Lee KL, Jeong JB, Jung YJ, Kim JS, Jung HC, Song IS. Role of ascites adenosine deaminase in differentiating between tuberculous peritonitis and peritoneal carcinomatosis. *World J Gastroenterol* 2012; 18(22): 2837-2843 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2837.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2837>

## INTRODUCTION

Tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC) are two of the most common causes of exudative ascites in South Korea and both diseases require rapid recognition for the appropriate therapeutic management<sup>[1-3]</sup>. In a clinical situation, etiological diagnosis of the two diseases is very difficult because of the lack of specific differential clinical, radiological, or laboratory findings. Peritoneoscopy is thought to be the method of choice in the diagnosis of the two diseases<sup>[4-6]</sup>. However, the diagnostic failure rate of peritoneoscopy can reach as high as approximately 14%; the main reason for failure is interference from adhesions due to tumor, tuberculosis or previous surgery<sup>[4]</sup>.

The purpose of this study was to clarify the differences in clinical, radiological, laboratory and peritoneoscopic findings between TBP and PC and to evaluate the diagnostic capacity of ascites adenosine deaminase (ADA) and tumor markers for the differentiating the two diseases.

## MATERIALS AND METHODS

### Patients

Between January 2000 and June 2010, patients over 18 years of age with exudative ascites of unknown etiology who underwent abdominal computed tomography (CT) scan and peritoneoscopy for diagnosis were enrolled in this study. All patients had laboratory tests such as complete blood count, serum biochemical tests, tumor markers from blood and ascites fluid, ascites cytology, ascites cell count and biochemical tests, and ascites ADA. Diagnosis of TBP was made if one of the following criteria was met: (1) ascites was positive for acid-fast bacilli stain and culture; (2) tuberculosis polymerase chain reaction test from ascites or peritoneoscopic biopsy specimen was positive; or (3) caseating granuloma was noted in the peritoneal biopsy specimen. PC was diagnosed if cancer cells from ascites cytology were detected or cancer cells were documented from the peritoneoscopic biopsy specimen. This study protocol was approved by the Ethics Committee at Seoul National University Hospital.

### Peritoneoscopy

Patients underwent peritoneoscopy under local anesthesia with systemic analgesics. A lidocaine injection was done from skin to fascia at about 1 cm left side from umbilicus. After local anesthesia, a Veress needle was inserted,

and then air insufflation was performed through the needle. After removal of the Veress needle, a trocar was inserted into peritoneum and peritoneoscope (Olympus; Tokyo, Japan) was inserted through the trocar into the peritoneum. After ascites fluid was drained for examination, a detailed observation of the peritoneum and intra-abdominal organs was performed. Experienced endoscopists performed all peritoneoscopic procedures and observation. Pictures of all important peritoneoscopic findings were taken and stored in a picture archiving and communication system. Two endoscopists (Kang SJ, Kim JW) reviewed the peritoneoscopic images to assess the nature of the ascites fluid and look for abnormalities such as nodules, patches, and adhesions. Nodules were classified according to size as  $\leq 1$  cm or  $> 1$  cm. Patches were classified according to their location (parietal or visceral peritoneum). Membranous patches were defined as plaques.

### Radiologic examination

All patients had abdominal CT scan examination. The following CT scanners were used in this study: Hi Speed/RP single channel CT scanner (GE Healthcare, Milwaukee, Wisconsin, United States) ( $n = 14$ ), MX 8000 four-channel CT scanner (Marconi Medical Systems, Cleveland, Ohio, United States) ( $n = 32$ ), LightSpeed eight-channel CT scanner (GE Healthcare) ( $n = 12$ ), Sensation 16 16-channel CT scanner (Siemens Medical Solutions, Erlangen, Germany) ( $n = 13$ ), and Brilliance 64 64-channel CT scanner (Philips Healthcare, Cleveland, Ohio, United States) ( $n = 4$ ). Section thickness and reconstruction interval were both 7mm for the single channel CT scanner and 5mm for the four-channel and eight-channel CT scanners, for the 16- and 64-channel CT scanners, section thickness and reconstruction interval were 3mm and 2.5 mm, respectively. Scanning was performed from the dome of the diaphragm through the pubic symphysis. Contrast-enhanced CT scan was performed after injection of nonionic contrast material (iopromide, Ultravist 370; Bayer Healthcare, Germany).

All scans were obtained on a GE 9800 (General Electric, Milwaukee, Wisconsin, United States) or a Somatom DR3 (Siemens, Erlangen, Germany) scanner with a slice thickness of 10 mm at 10- to 13-mm intervals from the dome of the diaphragm to the pubic symphysis. Two radiologists (Baek JH, Kim SH) reviewed abdominal CT scans of patients and looked at ascites (presence of loculation), parietal involvement patterns (smooth thickening, irregular or nodular thickening, seeding nodules), mesenteric changes, mesenteric thickening, mesenteric nodules (micronodule, macronodule), omental thickness, omental changes (smudged, nodular, omental cake), and intestinal involvement.

### Statistical analysis

Values for continuous variables were presented as mean  $\pm$  SD or median with ranges and as the number of individuals (and the percentage in each group) for the cat-

**Table 1** Demographics and clinical characteristics of the patients *n* (%)

	Tuberculous peritonitis ( <i>n</i> = 27)	Peritoneal carcinomatosis ( <i>n</i> = 25)	<i>P</i> value
Age (yr)	58.04 ± 12.61	61.12 ± 11.67	0.37
Gender (M:F)	11:16	16:9	0.09
Duration of symptoms			0.01
< 1 mo	11 (40.7)	2 (8.0)	
≥ 1 mo	16 (59.3)	23 (92.0)	
Symptoms			
Abdominal pain	12 (44.4)	15 (60.0)	0.26
Abdominal distension	25 (92.6)	21 (84.0)	0.33
Weight loss	8 (29.6)	11 (44.0)	0.28
Loss of appetite	12 (44.4)	8 (32.0)	0.36
Night sweating	3 (11.1)	0 (0.0)	0.09
Fever	16 (59.3)	0 (0.0)	< 0.01
Diarrhea	2 (7.4)	3 (12.0)	0.58

M/F: Male/female.

egorical variables. Nominal data were compared by using the Fisher exact test or Pearson  $\chi^2$  test, and continuous variables were compared by using the Student *t* test or Mann-Whitney *U*-test. A receiver-operating characteristic (ROC) curve was plotted and the area under the curve (AUC) was calculated to determine the predictive ability of different ascites ADA and tumor markers level cutoff values for differentiation. For all analyses, a *P* value of < 0.05 (two-tailed) was taken as statistically significant. All statistical analyses were performed with SPSS 15.0K for Windows (SPSS South Korea, Seoul, South Korea).

## RESULTS

### Clinical characteristics of patients

A total of 75 patients underwent abdominal CT scan and diagnostic peritoneoscopy. Of that population, 27 patients were diagnosed with TBP and 25 patients were diagnosed with PC according to the definition stated in the methods section. Other patients were diagnosed with various diseases such as pelvic inflammatory disease, continuous ambulatory peritoneal dialysis peritonitis, systemic lupus erythematosus (SLE), or peritoneal lymphomatosis. PC group included only adenocarcinomas from the various origins (6 pancreatic cancers, 4 ovary cancers, 5 malignancies of unknown origin, 4 colorectal cancers, 3 advanced gastric cancers and 3 other cancers). Clinical characteristics of patients are shown in Table 1. In the TBP group, there were 11 men (40.7%) and 16 women (59.3%), ranging from 28 to 83 years of age (mean ± SD = 58.04 ± 12.61 years). The PC group had more males (16 men and 9 women), but no significant difference in gender was found between the two groups (*P* = 0.09). There were 11 patients (40.7%) with duration of symptoms < 1 mo in the TBP group, while most patients in the PC group (*n* = 23, 92.0%) developed symptoms > 1 mo before they were diagnosed (*P* = 0.01). In the TBP group, 3 patients had night sweats, whereas no patients in the PC group complained of that symptom (*P* = 0.09). Fever

**Table 2** Laboratory features including tumor markers in serum and ascites of the patients

	Tuberculous peritonitis ( <i>n</i> = 27)	Peritoneal carcinomatosis ( <i>n</i> = 25)	<i>P</i> value
Serum lab			
Serum WBC (/mm <sup>3</sup> )	6083.7 ± 3115.9	6429.6 ± 2466.6	NS
Serum lymphocyte (%)	16.70 ± 8.39	22.01 ± 7.19	0.02
Serum CRP (mg/dL)	7.88 ± 6.62	3.12 ± 2.69	0.03
Serum CEA (ng/mL)	1.79 ± 1.09	10.26 ± 25.38	0.02
Serum CA 19-9 (U/mL)	10.63 ± 8.52	2283.56 ± 6211.99	0.05
Serum CA 125 (U/mL)	591.36 ± 440.95	676.73 ± 1088.28	NS
Ascites lab			
Ascites WBC (/mm <sup>3</sup> )	1325.9 ± 955.1	951.1 ± 773.1	NS
Ascites lymphocyte (%)	67.77 ± 23.41	48.36 ± 18.78	< 0.01
Ascites albumin (g/dL)	2.30 ± 0.75	2.32 ± 0.76	NS
SAAG	0.72 ± 0.49	1.05 ± 0.50	0.03
Ascites ADA (IU/L)	66.76 ± 32.09	13.89 ± 8.95	< 0.01
Ascites CEA (ng/mL)	1.36 ± 0.83	682.77 ± 1955.34	0.01
Ascites CA 19-9 (U/mL)	17.53 ± 24.15	12344.10 ± 33569.78	NS
Ascites CA 125 (U/mL)	1069.20 ± 578.74	1188.56 ± 1439.06	NS

WBC: White blood cells; CRP: C-reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 125: Carbohydrate antigen 125; SAAG: Serum-ascites albumin gradient; ADA: Adenosine deaminase; NS: Not significant. All statistical significance tests were performed by Mann-Whitney *U*-test.

was the predominant manifestation in the TBP group (16, 59.3%), whereas no patient in the PC group developed fever (*P* < 0.01).

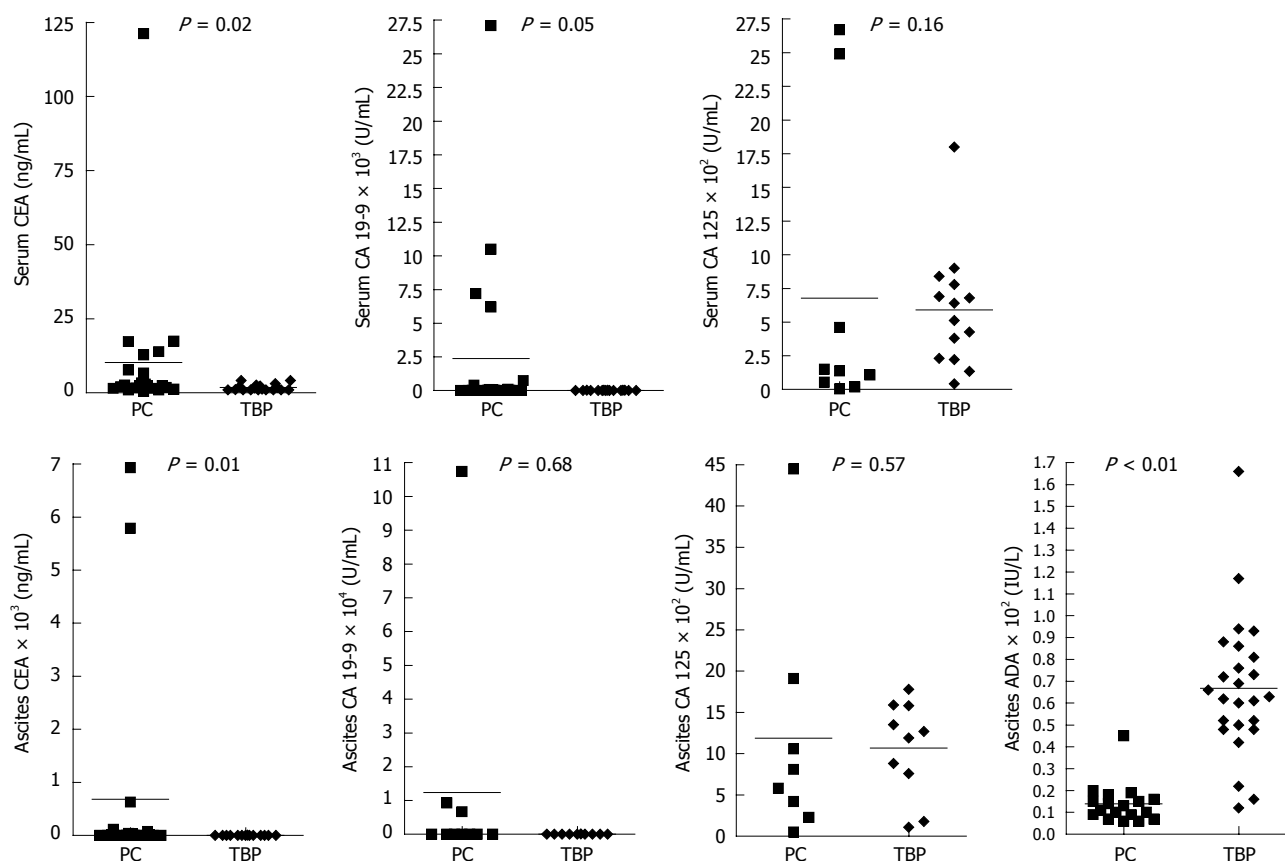
### Laboratory findings and tumor markers in serum and ascites of patients

Results of blood and ascites laboratory tests including tumor markers are summarized in Table 2. Serum c-reactive protein (CRP) was significantly higher in the TBP group. Among ascites laboratory findings, TBP group showed severer lymphocytosis, lower serum ascites albumin gradient, and higher ascites ADA.

Tumor markers [cercinoembryonic antigen (CEA), CA 19-9, CA 125] in serum and ascites in both groups are also presented in Table 2 and their scatter plots are presented in Figure 1. In PC group, serum and ascites CEA and serum CA 19-9 were higher than TBP group. Serum and ascites CA 125 were elevated in both groups and showed no significant differences.

### Radiological characteristics of patients

Ascites was found in the abdominal CT scan of all patients. The parietal involvement pattern was significantly different between the PC and TBP groups as shown in Table 3. In the TBP group, 21 patients (77.8%) showed smooth thickening of the parietal peritoneum, whereas smooth thickening was found in 10 patients (40.0%) in the PC group. Irregular and nodular parietal involvement was noted in 11 patients (44.0%) in the PC group whereas only 4 patients (14.8%) showed irregular or nodular involvement in the TBP group (*P* = 0.04). Thickening of mesentery was found in 15 (55.6%) and 8 (32.0%) patients in TBP and PC group, respectively (*P* = 0.09).



**Figure 1** Scatter plots shows the distributions of tumor markers and adenosine deaminase in serum and ascites between peritoneal carcinomatosis group and tuberculous peritonitis group. All tests were performed by Mann-Whitney *U*-test. PC: Peritoneal carcinomatosis; TBP: Tuberculous peritonitis; ADA: Adenosine deaminase; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; CA 125: Carbohydrate antigen 125.

**Table 3** Abdominal computed tomography characteristics of the patients *n* (%)

	Tuberculous peritonitis ( <i>n</i> = 27)	Peritoneal carcinomatosis ( <i>n</i> = 25)	<i>P</i> value
Ascites loculation	11 (40.7)	10 (40.0)	0.96
Parietal involvement			0.04
No	2 (7.4)	3 (12.0)	
Smooth thickening	21 (77.8)	10 (40.0)	
Irregular or nodular	4 (14.8)	11 (44.0)	
Seeding nodule	0 (0.0)	1 (4.0)	
Mesenteric change	23 (85.2)	17 (68.0)	0.14
Thickening of mesentery	15 (55.6)	8 (32.0)	0.09
Mesenteric nodule			0.31
No	15 (55.6)	12 (48.0)	
Micronodule	12 (44.4)	10 (40.0)	
Macronodule	0 (0.0)	2 (8.0)	
Omental thickness (mm)	20.48 ± 11.03	23.00 ± 15.77	0.51
Omental change			0.56
No	2 (7.4)	2 (8.0)	
Smudged	8 (29.6)	4 (16.0)	
Nodular	3 (11.1)	2 (8.0)	
Omental cake	13 (48.1)	17 (68.0)	
Intestinal involvement	3 (11.1)	5 (20.0)	0.53

In the TBP group, mesenteric nodularities were seen in 12 patients (44.4%) and all nodules were micronodules. Twelve patients (48.0%) in the PC group showed mesenteric nodules, which were composed of 10 micronodules

and 2 macronodules. There was no discriminative difference in omental thickness and patterns of change between the two groups.

### Peritoneoscopic findings of patients

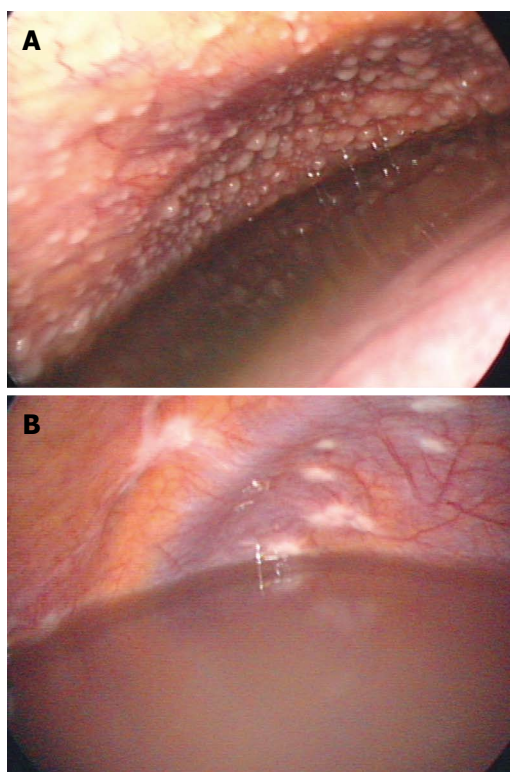
Peritoneoscopic images were reviewed by 2 experienced endoscopists (Kang SJ and Kim JW), and findings are summarized in Table 4. Twenty patients (74.1%) in the TBP group were found to have micronodules on the peritoneum (example of micronodule was shown in Figure 2A), and this number was greater than the number of TBP patients found to have micronodules on CT scan. Macronodules were not observed in TBP group. In the PC group, micronodules were detected in 8 patients, and macronodules were observed in 9 patients (*P* = 0.01). The distribution of whitish patches and presence of plaques were not significantly different (*P* = 0.41) between the two groups (example of patch lesion in Figure 2B). Adhesions between the peritoneum and abdominal organs were seen in 14 patients in the TBP group and 10 patients in the PC group (*P* = 0.41).

### Laboratory parameters for differentiation between TBP and PC groups

Among the evaluated laboratory parameters, parameters that showed significant difference between two groups were serum CRP, CEA, CA 19-9 and ascites ADA, CEA. AUC was calculated using ROC curves and these results

**Table 4** Peritoneoscopic findings of the patients *n* (%)

	Tuberculous peritonitis ( <i>n</i> = 27)	Peritoneal carcinomatosis ( <i>n</i> = 25)	<i>P</i> value
Nodules on peritoneum			< 0.01
No	7 (25.9)	7 (28.0)	
< 1 cm micronodule	20 (74.1)	8 (32.0)	
> 1 cm macronodule	0 (0.0)	9 (36.0)	
Peritoneum			0.41
Multiple whitish patches on parietal peritoneum	2 (7.4)	5 (20.0)	
Multiple whitish patches on parietal and visceral peritoneum	0 (0.0)	1 (4.0)	
No patches	19 (70.4)	15 (60.0)	
Whitish plaques	4 (14.8)	3 (12.0)	
Adhesion	14 (51.9)	10 (40.0)	0.41

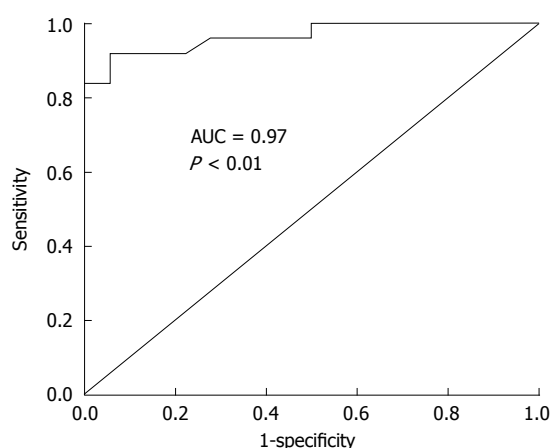
**Figure 2** Peritoneoscopic pictures of tuberculous peritonitis and peritoneal carcinomatosis. A: Peritoneoscopic picture of tuberculous peritonitis of female patient. Multitudinous miliary nodules are seen on the parietal peritoneum. Biopsy revealed caseous granulomatous inflammation and the biopsy specimen stained positive for acid-fast bacilli; B: Colon cancer with peritoneal seeding. Multiple irregular whitish patch lesions were found on the parietal peritoneum. Poorly differentiated adenocarcinoma was documented on biopsy.

were presented in Table 5. Among these markers, ascites ADA level was the strongest factor that differentiated TBP and PC patients. The ability to consider ascites ADA levels as a biomarker for differentiating TBP and PC patients was evaluated using ROC curve analysis. The AUC for TBP was 0.966 [95% confidence interval (CI): 0.916-1.00;  $P < 0.01$ ] (Figure 3). The sensitivity, specificity and positive and negative predictive values of ascites ADA were 92.0%, 94.4%, 95.8% and 89.5% at cut-off

**Table 5** Discriminative capability of tumor markers and adenosine deaminase between tuberculosis peritonitis and peritoneal carcinomatosis using receiver operating characteristics curve

	AUC	95% CI	<i>P</i> value
Serum CRP	0.705	0.537-0.872	0.033
Serum CEA	0.721	0.562-0.880	0.017
Serum CA 19-9	0.693	0.527-0.860	0.044
Ascites ADA	0.966	0.916-1.000	< 0.001
Ascites CEA	0.823	0.686-0.960	0.002

ADA: Adenosine deaminase; CI: Confidence interval; CRP: C-reactive protein; CEA: Carcinoembryonic antigen; CA 19-9: Carbohydrate antigen 19-9; AUC: Area under the curve.

**Figure 3** Receiver operating characteristic curve of ascites adenosine deaminase for differentiating between tuberculous peritonitis and peritoneal carcinomatosis. AUC of this receiver operating characteristic curve is 0.97 (95% CI: 0.92-1.00,  $P < 0.01$ ). CI: Confidence interval; AUC: Area under the curve.

level of 21.0 IU/L and 88.0%, 94.4%, 95.7% and 85.0% at 32.0 IU/L. When compared to cut-off level of 32.0, cut-off level of 21.0 shows higher sensitivity and negative predictive value and same specificity and positive predictive value.

## DISCUSSION

TBP and PC are two of the most common causes of exudative ascites<sup>[1]</sup>. Differentiating between the two disease entities is difficult, and laparoscopy is thought to be the gold standard for diagnosis. Laparoscopy with biopsy has shown impressive sensitivity and specificity rates of 93% and 98% respectively<sup>[7]</sup>. Yet even with laparoscopy, some patients cannot be diagnosed, because the biopsy findings can be insufficient for diagnosis. Furthermore, some patients cannot be evaluated by laparoscopy because of their poor general condition or technical failure. In those cases, clinical and laboratory findings can be helpful for differentiating between TBP and PC. This study demonstrates that ascites ADA is valuable laboratory finding in differentiating between TBP and PC. To the best of our knowledge, this is the first comprehensive series to date that compares tumor markers, abdominal CT scan, and



laparoscopic findings between tuberculous peritonitis and PC patients.

It is well known that ascites ADA has high sensitivity and specificity in the early diagnosis of TBP<sup>[8-10]</sup>. When used for diagnosing exudative ascites, at the cut-off level of 30 IU/L, ADA has demonstrated sensitivity of approximately 94%<sup>[4]</sup>. One study about pediatric patients report 100% sensitivity and 97% specificity for diagnosis of TBP<sup>[11]</sup>. Of interest, in our study ADA also had the high diagnostic value in differentiating tuberculosis peritonitis and PC and showed highest differentiating value (largest AUC) at the cut-off level of 21 IU/L. This suggests that when two diseases, TBP and PC, are strongly suspected from clinical, laboratory and radiological findings, and other diseases causing exudative ascites are reliably excluded, an ADA cut-off level of 21 IU/L that is lower than the usual cut-off level used for diagnosis could be used for discrimination. But this study only includes 27 TBP patients and 25 PC patients, about the precise cut-off level of ascites ADA for discrimination of two diseases, further verification with large numbers of patients are required.

Ascites ADA is accurate but insensitive for detecting TBP in liver cirrhosis patients living in the United States where the prevalence of tuberculosis is low. In the United States, which has a low tuberculous burden, 59% of TBP patients have liver cirrhosis<sup>[12]</sup>. This means that in developed country where tuberculosis burden is low, TBP is mainly developed in immune compromised patients such as liver cirrhosis. In South Korea, the tuberculous burden is intermediate, so in our study only 14.8% (4 out of 27) of TBP patients had liver cirrhosis<sup>[13]</sup>. Low portion of cirrhotic patients partly explains high ascites ADA performance. Furthermore, 75% of TBP patients with liver cirrhosis (3 out of 4 patients) had a higher ascites ADA level than normal. So even cirrhotic patients, ascites of TBP patients shows high ADA. This also explains the sensitivity of ascites ADA for diagnosing TBP in our study.

Increased serum and ascites CA-125 levels, detected in up to 80% of women with late-stage ovarian cancer, have demonstrated great value in treatment monitoring and recurrence detection of ovary cancer<sup>[14-16]</sup>. In TBP, serum CA-125 levels are as high as ovarian cancer associated with peritoneal infiltration, and, by the end of the fourth month of anti-tuberculous therapy in a patient with TBP, serum CA-125 levels have returned to normal<sup>[17-19]</sup>. Similar to previous reports, this study demonstrated that both TBP and PC patients have elevated serum and ascites CA-125 levels<sup>[20-22]</sup>. As a result, ascites and serum CA-125 are cautiously interpreted in differentiating between TBP and PC.

Radiological findings of TBP are similar to PC. With a model of multivariate logistic analysis using mesenteric macronodules, omental lining, irregularity of omentum, and splenic abnormalities, the sensitivity for predicting tuberculous peritonitis was 69%, whereas the sensitivity for PC was 91%<sup>[23]</sup>. In our study, only the parietal involvement pattern was significantly different between the

TBP and PC groups. A thickened mesentery and loss of normal mesenteric configuration are known to be characteristics of TBP and helpful for diagnosis, but our results did not show these findings to be helpful in differentiating between two diseases<sup>[24]</sup>. When parietal involvement patterns were used as marker for differentiation, the sensitivity and specificity for diagnosing TBP in this study were 60.5% and 75.0%, respectively. This characteristic is insufficient as a marker for differentiation of diseases but can be used in adjunct to other examinations.

Peritoneoscopy is the diagnostic tool of choice in patients with exudative ascites of unknown origin<sup>[2,4]</sup>. Peritoneal tubercles and ascites are the main features of TBP and appear in more than 90% of TBP cases<sup>[25-27]</sup>. In our study, micronodules on the peritoneum and ascites were seen in 20 of 27 patients (74.1%). The low micronodule detection rate compared to previous studies may be due to the discovery of patch and plaque lesions in 6 patients (2 patch lesions and 4 plaque lesion, 22.2%) of the TBP group during peritoneoscopy, because nearly all suspicious patch lesions were biopsied whenever possible. For diagnosis of PC, peritoneoscopy has higher sensitivity and specificity than helical CT scan with 5-mm slice thickness, and similar results were found in this study<sup>[28]</sup>. The diagnostic failure rate of peritoneoscopy is substantially high, however, reaching about 14%; the main reason for diagnostic failure is interference from adhesions from previous surgery or tumor adhesion<sup>[7]</sup>.

Interpretation of our findings requires careful consideration of several aspects. First, the positive and negative predictive values are usually affected by pretest probability, so those values of the ascites ADA in this study may be variable in countries of low tuberculosis prevalence. Second, PC group is composed of various cancers. So discriminative ability of each tumor marker such as CEA for specific cancer could not fully tested in this study, so larger numbers of patients are needed to assess the predicting ability of each ascites tumor marker for each type of cancer.

In conclusion, clinical findings such as duration of symptom less than 1 mo and fever are helpful for differentiating TBP and PC of various causes. Among laboratory findings, ascites ADA was the most valuable discriminative marker. For differentiation of two diseases, in addition to clinical findings and radiologic characteristics, ascites ADA should also be considered.

## COMMENTS

### Background

Tuberculous peritonitis (TBP) and peritoneal carcinomatosis (PC) are two of the most common causes of exudative ascites but in a clinical situation, etiological diagnosis of the two diseases is very difficult. Peritoneoscopy with biopsy is thought to be the method of choice in the differential diagnosis of the two diseases but the morbidity related with peritoneoscopic procedure and diagnostic failure rate reaches as high as approximately 8% and 14%, respectively. So we investigate the diagnostic capacities of adenosine deaminase (ADA) and tumor markers for differentiating the two diseases.

### Research frontiers

Diagnostic abilities of serum ADA and tumor markers in differentiating two

diseases are hotspots in recent studies. Serum c-reactive protein, serum carcinoembryonic antigen (CEA), serum CA 19-9, ascites ADA and ascites CEA were significantly different between two disease groups. Among them, ascites ADA showed largest area under the curves (AUC) in receiver operating characteristic curves (AUC = 0.966; 95% confidence interval: 0.916-1.00;  $P < 0.01$ ). In addition to ascites ADA, clinical findings such as symptom duration less than 1 mo and fever was more frequent finding in tuberculosis peritonitis. In abdomen computed tomography findings, smooth thickening was the most common in TBP whereas in PC group nodular pattern was the most common finding.

### Innovations and breakthroughs

This study showed that among laboratory findings, ascites ADA was the most valuable marker for discriminating TBP and PC.

### Applications

This analysis for the diagnostic capabilities of ADA implicates that ascites ADA may be helpful for differentiation of two diseases.

### Peer review

It is a very nice paper with an excellent statistic work and very interesting findings.

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## XAF1 is frequently methylated in human esophageal cancer

Xiang-Yu Chen, Qiao-Yu He, Ming-Zhou Guo

Xiang-Yu Chen, Qiao-Yu He, Department of Gastroenterology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450052, Henan Province, China

Ming-Zhou Guo, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing 100853, China

**Author contributions:** Chen XY and He QY performed all the experiments and wrote the manuscript; Guo MZ was involved in editing and correcting of the manuscript, designing the study and correcting the manuscript.

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**Correspondence to:** Ming-Zhou Guo, PhD, MD, Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, No. 28 Fuxing Road, Beijing 100853, China. [mzguo@hotmail.com](mailto:mzguo@hotmail.com)

Telephone: +86-10-66937651 Fax: +86-10-68180325

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### Abstract

**AIM:** To explore epigenetic changes in the gene encoding X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) during esophageal carcinogenesis.

**METHODS:** Methylation status of XAF1 was detected by methylation-specific polymerase chain reaction (MSP) in four esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and partially methylated in TE3 cell lines), nine cases of normal mucosa, 72 cases of primary esophageal cancer and matched adjacent tissue. XAF1 expression was examined by semi-quantitative reverse transcriptional polymerase chain reaction and Western blotting before and after treatment with 5-aza-deoxycytidine (5-aza-dc), a demethylating agent. To investigate the correlation of XAF1 expression and methylation status in primary esophageal cancer, immunohistochemistry for XAF1 expression was performed in 32 cases of esophageal cancer and matched adjacent tissue. The association of methylation status and clinical

copathological data was analyzed by logistic regression.

**RESULTS:** MSP results were as follows: loss of XAF1 expression was found in three of four esophageal cell lines with promoter region hypermethylation (completely methylated in KYSE30, KYSE70 and BIC1 cell lines and partially in TE3 cells); all nine cases of normal esophageal mucosa were unmethylated; and 54/72 (75.00%) samples from patients with esophageal cancer were methylated, and 25/72 (34.70%) matched adjacent tissues were methylated (75.00% vs 34.70%,  $\chi^2 = 23.5840$ ,  $P = 0.000$ ). mRNA level of XAF1 measured with semi-quantitative reverse transcription polymerase chain reaction was detectable only in TE3 cells, and no expression was detected in KYSE30, KYSE70 or BIC1 cells. Protein expression was not observed in KYSE30 cells by Western blotting before treatment with 5-aza-dc. After treatment, mRNA level of XAF1 was detectable in KYSE30, KYSE70 and BIC1 cells. Protein expression was detected in KYSE30 after treatment with 5-aza-dc. Immunohistochemistry was performed on 32 cases of esophageal cancer and adjacent tissue, and demonstrated XAF1 in the nucleus and cytoplasm. XAF1 staining was found in 20/32 samples of adjacent normal tissue but was present in only 8/32 samples of esophageal cancer tissue ( $\chi^2 = 9.143$ ,  $P = 0.002$ ). XAF1 expression was decreased in cancer samples compared with adjacent tissues. In 32 cases of esophageal cancer, 24/32 samples were methylated, and 8/32 esophageal cancer tissues were unmethylated. XAF1 staining was found in 6/8 samples of unmethylated esophageal cancer and 2/24 samples of methylated esophageal cancer tissue. XAF1 staining was inversely correlated with XAF1 promoter region methylation (Fisher's exact test,  $P = 0.004$ ). Regarding methylation status and clinicopathological data, no significant differences were found in sex, age, tumor size, tumor stage, or metastasis with respect to methylation of XAF1 for the 72 tissue samples from patients with esophageal cancer.

**CONCLUSION:** XAF1 is frequently methylated in esophageal cancer, and XAF1 expression is regulated by promoter region hypermethylation.



**Key words:** X chromosome-linked inhibitor of apoptosis-associated factor 1; Esophageal cancer; Methylation; Methylation-specific polymerase chain reaction; Semi-quantitative reverse transcriptional polymerase chain reaction

**Peer reviewer:** Dr. Subbaramiah Sridhar, Medical College of Georgia, BBR 2544, Medical College of Georgia, 15th Street, Augusta, GA 30912, United States

Chen XY, He QY, Guo MZ. XAF1 is frequently methylated in human esophageal cancer. *World J Gastroenterol* 2012; 18(22): 2844-2849 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2844.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2844>

## INTRODUCTION

Esophageal cancer is the eighth most common cancer worldwide. The incidence of esophageal cancer has increased rapidly in the past 20 years, and about 380 000 patients die each year from the disease<sup>[1-3]</sup>. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer. It is highly invasive, rapidly metastatic, and results in a poor postoperative quality of life<sup>[4,5]</sup>. The mechanisms contributing to ESCC carcinogenesis are poorly understood. Recent studies have shown that aberrant promoter DNA methylation contributes to gene silencing and may participate in the carcinogenesis of human cancer. In-depth investigation of the relationship between DNA methylation and gene expression in ESCC will facilitate research in tumor pathogenesis and guide clinical practice.

Inhibitors of apoptosis (IAPs) are antiapoptotic factors in cancer cells that render cells resistant to apoptosis by inhibition of core death executioners, the caspases, or by neutralizing antagonists<sup>[6]</sup>. In the IAP family, X chromosome-linked IAP (XIAP) has been recognized as the most versatile caspase inhibitor<sup>[7,8]</sup>. In many models of cancer, XIAP is overexpressed<sup>[9]</sup>. XIAP-associated factor 1 (XAF1) is one of the antagonists that has been identified as a mediator of XIAP by rescuing XIAP-suppressed caspase activity<sup>[10]</sup>. XAF1 is also a new candidate tumor suppressor. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation<sup>[11-17]</sup>. Zou *et al*<sup>[15]</sup> found that loss of XAF1 expression is associated with tumor progression in human gastric and colon cancers. Lee *et al*<sup>[14]</sup> also discovered that downregulation of XAF1 expression is correlated with human urogenital malignancies. However, the relationship between the expression level of XAF1 and the methylation status of XAF1 in esophageal cancer has not been demonstrated.

In this study, we investigated whether promoter region methylation was associated with the progression of esophageal cancer and analyzed the relationship between

XAF1 expression and promoter region methylation. We identified XAF1 as a potential esophageal cancer biomarker for prognosis and a target for future therapeutic agents. Detection of the methylation status of XAF1 appears to be promising as a predictive factor in primary ESCC.

## MATERIALS AND METHODS

### Human tissue samples and cell lines

Tissue samples taken from 72 cases of ESCC and 72 matched adjacent normal tissues were used in this study. Nine cases of normal esophageal epithelia were removed during endoscopy biopsy and then snap frozen. All samples were collected from the Chinese PLA General Hospital under the guidelines approved by the Institutional Review Board of the Chinese PLA General Hospital.

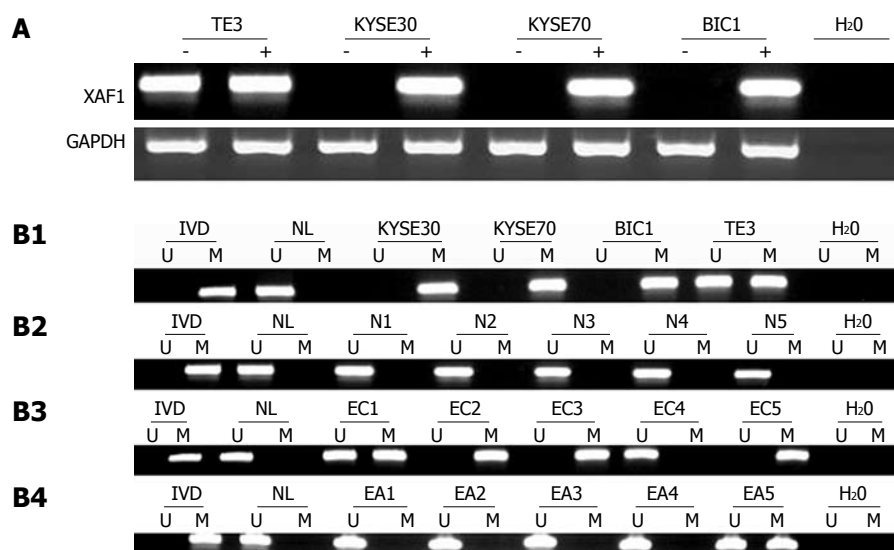
Four esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3) were examined in this study. All esophageal cancer cell lines were previously established from primary esophageal cancer and were maintained in 90% RPMI 1640 (Invitrogen, Carlsbad, CA, United States) supplemented with 10% fetal bovine serum. Cells were passaged once at a ratio of 1:3. Cells were then allowed to grow to total confluence (about 10<sup>6</sup> cells) on a 75-cm<sup>2</sup> culture flask (NEST Biotechnology, Jiangsu, China).

Esophageal cancer cell lines were split to low density (30% confluence) 12 h before treatment. Cells were treated with 2 mol/L 5-aza-2'-deoxycytidine (5-aza-dc) (Sigma, St. Louis, MO, United States), a demethylating agent, which was added fresh every 24 h for a total of 96 h. At the end of the treatment, RNA and protein were extracted from the cells (see below).

### RNA isolation and reverse transcriptional polymerase chain reaction

Total RNA was isolated with the Trizol reagent (Life Technologies, Gaithersburg, MD, United States). Agarose gel (1%) electrophoresis and spectrophotometric analysis ( $A_{260\text{ nm}}/A_{280\text{ nm}}$  ratio) were used to evaluate RNA quality and quantity. RNA was stored at -80 °C prior to use. First-strand cDNA was synthesized from 5 µg total RNA with random 6-mer primers and a Superscript II reverse transcriptional kit (Invitrogen). The reaction mixture was then diluted to 100 µL with water. Subsequently, 2.5 µL of this diluted cDNA mixture was used for polymerase chain reaction (PCR) amplification in a 25 µL reaction (final volume). PCR amplification of XAF1 was carried out using primers 5'-GAGCATGCAGAAGTCCTCGCT-3' (forward) and 5'-CCTGTTCACCTGCGACAGACATCT-3' (reverse). The primer set for XAF1 was designed to span intronic sequences between exons to exclude amplification of genomic DNA. A total of 32 cycles of amplification was performed for each reverse transcriptional polymerase chain reaction (RT-PCR) experiment. As an internal control, glyceraldehyde-3-phosphate dehydrogenase was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR reaction. Amplified products were analyzed on 1.5% agarose gels.





**Figure 1** X chromosome-linked inhibitor of apoptosis-associated factor 1 expression was silenced by DNA methylation. A: X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) expression was analyzed by semi-quantitative reverse transcriptional polymerase chain reaction before and after 5-aza-dc treatment (2 mol/L, 96 h) of the esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3). Methylation status of XAF1 CpG islands in esophageal cancer cell lines, esophageal normal mucosa, esophageal cancer tissue, and matched adjacent normal tissue. Primer efficiency was verified with a positive control (*in vitro* methylated DNA, IVD) and a negative control (normal blood lymphocyte DNA, NL). "U" indicates the presence of unmethylated alleles; "M" indicates the presence of methylated alleles; B1: Methylation of XAF1 in esophageal cancer cell lines (KYSE30, KYSE70, BIC1 and TE3); B2: Methylation of XAF1 in normal esophageal mucosa (NE1, NE2, NE3, NE4 and NE5); B3: Representative methylation-specific polymerase chain reaction (MSP) results for XAF1 in esophageal primary cancer tissue samples (EC); B4: Representative MSP results for XAF1 in esophageal matched adjacent normal tissue (EA). GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

### Methylation-specific PCR

Genomic DNA from all types of samples was prepared using the proteinase K method. After phenol/chloroform extraction, DNA was precipitated in ethanol, dissolved in low TE buffer, and stored at -20 °C. Genomic DNA from esophageal cancer, adjacent tissues, and cell lines was bisulfite modified as described before<sup>[18]</sup>. Methylation-specific PCR (MSP) was carried out using primers XAF1-ML: 5'-TTTGTAAGAAACGAAATTTAATCGA-3' and XAF1-MR: 5'-CCTACCTTAAAAACCCACGAT-3' and XAF1-UL: 5'-TTTGTAAGAAATGAAATTTAATTGA-3' and XAF1-UR: 5'-CTCCTACCTTAAAAACCCA-CAAT-3'<sup>[10]</sup>. Each MSP reaction included about 200 ng bisulfite-treated DNA, 25 pmol each primer, 100 pmol dNTPs, 2.5  $\mu$ L 10  $\times$  PCR buffer, and 1 U Taq Polymerase (Invitrogen) in a final reaction volume of 25  $\mu$ L. Cycle conditions were as follows: 95 °C for 10 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. MSP products were analyzed using 2% agarose gel electrophoresis.

### Immunohistochemistry

Immunohistochemistry was performed on 4- $\mu$ m-thick serial sections cut from paraffin blocks containing formaldehyde-fixed esophageal cancer tissue and paired adjacent tissue. After deparaffinization and rehydration, endogenous peroxidase activity was blocked for 30 min in methanol containing 0.3% hydrogen peroxide. Antigen retrieval was performed in target retrieval solution for 45 min at 96 °C, which was followed by a cooling-off period of 20 min. The primary rabbit antibody (anti-XAF1, 1:200; OriGene Technologies, MD, United States) was then incubated overnight at 4 °C. Then, the catalyzed sig-

nal amplification system (ZSGB Biotech., Beijing, China) was used to detect XAF1 staining.

### Protein preparation and Western blotting

KYSE30 cells were treated with 5-aza-dc (as described above), harvested, and lysed in ice-cold Tris buffer (20 mmol/L Tris, pH 7.5) containing 137 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid, 1% Triton X-100, 10% glycerol, 50 mmol/L NaF, 1 mmol/L dithiothreitol, and a protease inhibitor cocktail (Roche Applied Science). Cell lysate (35  $\mu$ g) was loaded into each lane, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted onto PVDF membranes (Hybond-P; Amersham, United States). After being blocked with 5% nonfat milk and 0.1% Tween-20 in TBS, the membranes were incubated with primary rabbit anti-XAF1 (1:1000; OriGene Technologies). Rabbit anti-actin (Beyotime Biotech., China) was used as a loading control. The blots were visualized using enhanced chemiluminescence (Pierce Bioscience, Rockford, IL, United States).

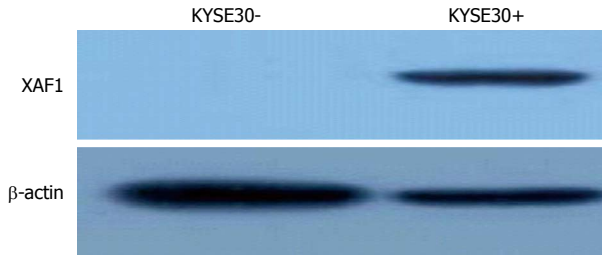
### Statistical analysis

Statistical analysis was carried out using the  $\chi^2$  test and Fisher's exact test. The relationship between methylation status and clinicopathological data was carried out using multiple logistic regression.  $P < 0.05$  was considered statistically significant.

## RESULTS

### XAF1 promoter methylation status in esophageal cancer

To ascertain whether the XAF1 promoter methylation status was associated with esophageal carcinogenesis,



**Figure 2** Western blotting analysis of X chromosome-linked inhibitor of apoptosis-associated factor 1 protein expression in the KYSE30 cell line before and after treatment with 2 mol/L 5-aza-deoxycytidine (+) for 96 h. XAF1: X chromosome-linked inhibitor of apoptosis-associated factor 1.

MSP was performed on nine cases of normal esophageal mucosa, 72 samples taken from patients with esophageal cancer, and 72 paired adjacent normal tissue samples. All nine cases of normal esophageal mucosa were unmethylated; 54 of 72 (75%) samples taken from patients with esophageal cancer were methylated; and 25 of 72 (34.7%) matched adjacent normal tissues were methylated ( $\chi^2 = 23.5840$ ,  $P = 0.000$ ; Figure 1B). These results suggest that methylation of the XAF1 promoter region is a potential early detection marker of esophageal cancer.

#### **XAF1 promoter methylation and XAF1 expression in esophageal cancer cell lines**

To determine whether XAF1 is a tumor suppressor in esophageal cancer tumorigenesis, XAF1 expression levels were detected with semi-quantitative RT-PCR and Western blotting. The mRNA level of XAF1 was detectable only in the TE3 cell line, and no expression was detected in the KYSE30, KYSE70 or BIC1 cell lines (Figure 1A). Protein expression was not observed in the KYSE30 cell line before treatment with 5-aza-dc (Figure 2). To examine if these findings were due to promoter region methylation of XAF1, the methylation status of XAF1 was analyzed in these cell lines with MSP. XAF1 was completely methylated in the KYSE30, KYSE70 and BIC1 cell lines and partially methylated in TE3 cells (Figure 1B).

To demonstrate further whether XAF1 expression was restored with promoter region methylation, these cell lines were treated with 5-aza-dc. As shown in Figure 1, XAF1 was expressed in KYSE30, KYSE70 and BIC1 cells after 5-aza-dc treatment, and we detected protein expression in KYSE30 after treatment with 5-aza-dc (Figure 2), suggesting that XAF1 expression was regulated by promoter region methylation in esophageal cancer.

#### **Correlation of XAF1 expression and methylation status in esophageal cancer tissue**

To explore the expression of XAF1, immunohistochemistry was performed on 32 cases of esophageal cancer and adjacent tissue paraffin samples. XAF1 expression was found in the nucleus and cytoplasm as previously reported<sup>[19,20]</sup>. XAF1 staining was found in 50% (16/32) of adjacent tissue samples and only in 25% (8/32) of cancer tissue samples ( $\chi^2 = 9.143$ ,  $P = 0.002$ ). Reduced expression of XAF1 was found in cancer tissues as compared

with the adjacent tissue samples (Figure 3).

We then analyzed the relationship between the methylation status and expression of XAF1 in 32 esophageal cancer samples. Twenty-four esophageal cancer samples (24/32, 75.00%) were methylated, and eight esophageal cancer tissue (8/32, 25.00%) were unmethylated. XAF1 staining was found in six samples (6/8, 75.00%) of unmethylated esophageal cancer tissue and two samples (2/24, 8.33%) of methylated esophageal cancer tissue. XAF1 staining was inversely correlated with XAF1 promoter region methylation (Fisher's exact test,  $P = 0.004$ ).

#### **Correlation of XAF1 methylation status with clinicopathological factors**

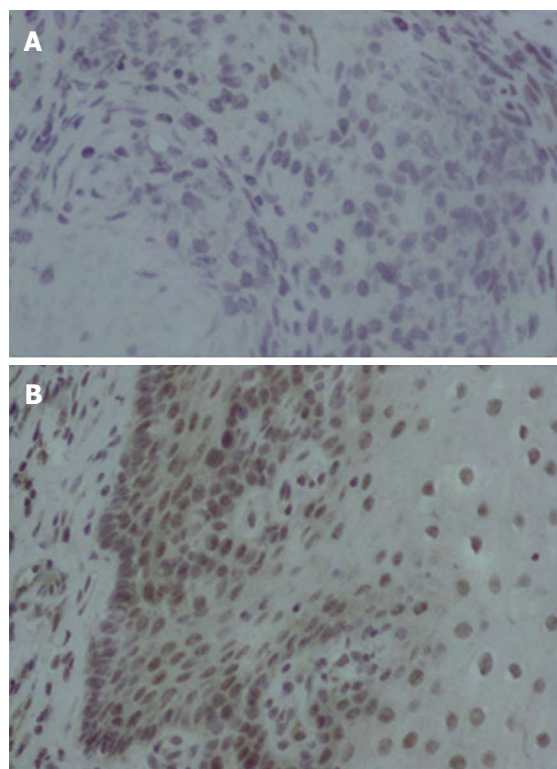
Regarding methylation status and clinicopathological data, we found no significant differences in sex, age, tumor size, tumor stage, or metastasis with respect to the methylation status of XAF1 for the 72 primary esophageal cancer patients (Table 1).

## **DISCUSSION**

The development of cancer is influenced by many factors and many genes and progresses through many stages<sup>[21]</sup>. In some ways, cancer is considered an epigenetic disease as well as a genetic disease. Epigenetics is the inheritance of information based on gene expression levels, whereas genetics refers to information transmitted according to the gene sequence. Epigenetic changes may lead to chromosomal instability, activation of parasitic endogenous sequences, loss of imprinting, illegitimate expression, aneuploidy, and mutations, and it may contribute to the transcriptional silencing of tumor suppressor genes<sup>[22]</sup>. In the human genome, CpG dinucleotides are inconsonantly distributed, resulting in CpG-rich regions<sup>[23]</sup>. The main epigenetic modification is aberrant CpG island methylation, which is tissue-specific but not species-specific. Methylation affects many pathways in cellular networks, such as the cell cycle and apoptosis<sup>[22]</sup>.

In life and death decisions at the cellular level, there is a balance between pro- and antiapoptotic factors, and a variety of pathological conditions such as cancer and autoimmune and neurodegenerative diseases can result from disruption of this balance<sup>[6]</sup>. Apoptosis is crucial for eliminating defective or potentially dangerous cells and provides a defense against malignant transformation and autoimmunity<sup>[11]</sup>. IAPs are a new family of intrinsic cell death proteins that work as endogenous caspase inhibitors and participate in cell cycle regulation and modulation of receptor-mediated signal transduction. A recent study has reported that the level of XIAP mRNA is relatively high in many human cancers, suggesting that XIAP is one of the most potent and versatile inhibitors of caspases and apoptosis<sup>[15]</sup>.

XAF1 has been previously identified as a binding partner of XIAP. In contrast to Smac/DIABLO and HtrA2, which promote caspase activation, XAF1 reverses XIAP-mediated inhibition of caspase-3 activity. Furthermore, XAF1 induces cell cycle arrest during G2/M phase and



**Figure 3** Immunohistochemistry analysis of X chromosome-linked inhibitor of apoptosis-associated factor 1 in esophageal cancer tissue and adjacent tissue. Esophageal cancer and adjacent normal tissue samples were immunohistologically analyzed with anti-X chromosome-linked inhibitor of apoptosis-associated factor 1 (XAF1) (1:200 dilution;  $\times 400$ ). A: XAF1 was not detected in esophageal cancer tissue; B: XAF1 was localized in the nucleus and cytoplasm in adjacent normal esophageal tissue.

mitotic catastrophe, and the restoration of XAF1 expression induces cancer cell apoptosis and inhibits tumor growth in many types of human cancers<sup>[24]</sup>. XAF1 is ubiquitously expressed in all normal adult and fetal tissues but is drastically decreased in many cancer cell lines<sup>[11]</sup>. Loss of XAF1 expression is associated with methylation in its promoter region in many cancers. For example, XAF1 is present at very low or undetectable levels in gastric cancer, colorectal cancer<sup>[15]</sup>, and cervical carcinoma<sup>[25]</sup>.

In this study, we found that XAF1 was frequently methylated in esophageal cancer. Moreover, expression of XAF1 was inversely correlated with its methylation status. XAF1 was methylated in three esophageal cancer cell lines and 54 samples of esophageal cancer tissue. XAF1 methylation resulted in loss of expression in esophageal cancer cell lines, and the expression of XAF1 was restored in KYSE30, KYSE70 and BIC1 cells after treatment with 5-aza-dc. Furthermore, we observed the expression of XAF1 in the methylated cell line KYSE30 after treatment with 5-aza-dc. The results indicated that promoter region methylation regulated the expression of XAF1. XAF1 was frequently methylated in esophageal cancer tissue (75%) but was methylated only in 34.7% of matched adjacent normal tissues and not at all in normal esophageal mucosa, indicating that promoter region methylation of XAF1 was likely to be related to

**Table 1** Clinicopathological characteristics and XAF1 methylation status of 40 patients with esophageal cancer  $n$  (%)

Clinical parameter	$n$	XAF1 methylation status		$P$ value ( $\chi^2$ test)
		Methylated $n = 30$ (75%)	Unmethylated $n = 10$ (25%)	
Age (yr)				
< 65	40	30 (75)	10 (25)	1.0
$\geq 65$	32	24 (75)	8 (25)	
Gender				
Male	53	40 (75.5)	13 (24.5)	0.8773
Female	19	14 (73.7)	5 (26.3)	
Tumor size (cm)				
< 5	48	33 (68.8)	15 (31.2)	0.0833
$\geq 5$	24	21 (87.5)	3 (12.5)	
Tumor stage				
I	12	6 (50.0)	6 (50.0)	0.081
II	21	17 (81.0)	4 (19.0)	
III	6	6 (100.0)	0 (0)	
IV	1	1 (100)	0 (0)	
Metastasis				
Negative	45	33 (73.3)	12 (26.7)	0.6733
Positive	27	21 (77.8)	6 (22.2)	

In this 72 patients, only 32 cases with tumor stage history.

esophageal carcinogenesis. In addition, XAF1 protein expression was decreased in cancer tissues as compared with adjacent normal samples, and low expression of XAF1 was significantly correlated with promoter region methylation. XAF1 expression and promoter region methylation status have been reported to be useful for identifying poorly differentiated cancer or patients with a poor disease outcome<sup>[16,17,26]</sup>.

XAF1 is frequently methylated in esophageal cancer, and XAF1 expression is regulated by promoter region methylation. The loss of XAF1 expression may play an important role in tumor growth, and methylation of XAF1 may serve as an early detection marker for esophageal cancer.

## COMMENTS

### Background

Esophageal cancer is the eighth most common cancer worldwide. The incidence of esophageal cancer has increased rapidly in the past 20 years. Esophageal squamous cell carcinoma (ESCC) is the main type of esophageal cancer. It is highly invasive, rapidly metastatic, and results in a poor postoperative quality of life. The mechanisms contributing to ESCC carcinogenesis are poorly understood. Recent studies showed that aberrant promoter DNA methylation contributes to gene silencing and may participate in the carcinogenesis of human cancer. X chromosome-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1) is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation. However, the relationship between the expression level of XAF1 and the methylation status of XAF1 in esophageal cancer has not been demonstrated.

### Research frontiers

XAF1 is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in human gastric cancer, colon cancer and urogenital malignancies because of aberrant DNA methylation. However, the relationship between expression level of XAF1 and methylation status of XAF1 in esophageal cancer has not been demonstrated. In this study, the authors demonstrated that expression level of XAF1 was inversely correlated with methylation status of XAF1, and XAF1 expression was regulated by



promoter region methylation.

### Innovations and breakthroughs

Recent reports have highlighted that the loss of XAF1 expression or downregulation of XAF1 expression may occur in different human cancers because of aberrant DNA methylation. This is the first study to report that XAF1 is also loss of expression because of aberrant DNA methylation in esophageal cancer.

### Applications

By understanding the relationship between loss of XAF1 expression and methylation status of XAF1 in esophageal cancer, and by inducing its expression with 5-aza-deoxycytidine, this study may represent a future strategy for therapeutic intervention in the treatment of patients with esophageal cancer.

### Terminology

Inhibitors of apoptosis (IAPs) are antiapoptotic factors in cancer cells that render cells resistant to apoptosis by inhibition of core death executioners, the caspases, or by neutralizing antagonists. In the IAP family, XIAP has been recognized as the most versatile caspase inhibitor. In many models of cancer, XIAP is overexpressed. XAF1 is one of the antagonists that has been identified as a mediator of XIAP by rescuing XIAP-suppressed caspase activity. XAF1 is a new candidate tumor suppressor gene. Recent studies have suggested that loss of XAF1 expression may occur in different human cancers because of aberrant DNA methylation.

### Peer review

The study is very interesting and throws light on future studies and confirms increased methylation of XAF1 in squamous cell carcinoma.

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## DNA-dependent activator of interferon-regulatory factors inhibits hepatitis B virus replication

Qi-Ying Chen, Ying-Hui Liu, Jian-Hua Li, Ze-Kun Wang, Jiang-Xia Liu, Zheng-Hong Yuan

Qi-Ying Chen, Jian-Hua Li, Jiang-Xia Liu, Zheng-Hong Yuan, Key Laboratory of Medical Molecular Virology, Ministry of Education and Ministry of Health, Shanghai Medical College, Fudan University, Shanghai 200032, China

Ying-Hui Liu, Ze-Kun Wang, Institutes of Biomedical Sciences, Fudan University, Shanghai 200032, China

**Author contributions:** Chen QY, Liu YH contributed equally to this work; Chen QY, Liu YH participated in the design of study, carried out the experiments and drafted the manuscript; Li JH participated in the design of study; Wang ZK and Liu JX performed the statistical analysis; and Yuan ZH designed the research and reviewed the drafts.

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**Correspondence to:** Zheng-Hong Yuan, MD, Professor, Key Laboratory of Medical Molecular Virology, Ministry of Education and Ministry of Health, Shanghai Medical College, Fudan University, 138 Yi Xue Yuan Road, Shanghai 200032, China. [zhyuan@shaphc.org](mailto:zhyuan@shaphc.org)

Telephone: +86-21-64161928 Fax: +86-21-64227201

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### Abstract

**AIM:** To investigate whether DNA-dependent activator of interferon-regulatory factors (DAI) inhibits hepatitis B virus (HBV) replication and what the mechanism is.

**METHODS:** After the human hepatoma cell line Huh7 was cotransfected with DAI and HBV expressing plasmid, viral protein (HBV surface antigen and HBV e antigen) secretion was detected by enzyme-linked immunosorbent assay, and HBV RNA was analyzed by real-time polymerase chain reaction and Northern blotting, and viral DNA replicative intermediates were examined by Southern blotting. Interferon regulatory factor 3 (IRF3) phosphorylation and nuclear translocation were analyzed *via* Western blotting and immunofluorescence

staining respectively. Nuclear factor- $\kappa$ B (NF- $\kappa$ B) activity induced by DAI was detected by immunofluorescence staining of P65 and dual luciferase reporter assay. Transwell co-culture experiment was performed in order to investigate whether the antiviral effects of DAI were dependent on the secreted cytokines.

**RESULTS:** Viral protein secretion was significantly reduced by 57% ( $P < 0.05$ ), and the level of total HBV RNA was reduced by 67% ( $P < 0.05$ ). The viral core particle-associated DNA was also dramatically down-regulated in DAI-expressing Huh7 cells. Analysis of involved signaling pathways revealed that activation of NF- $\kappa$ B signaling was essential for DAI to elicit antiviral response in Huh7 cells. When the NF- $\kappa$ B signaling pathway was blocked by a NF- $\kappa$ B signaling suppressor (I $\kappa$ B $\alpha$ -SR), the anti-HBV activity of DAI was remarkably abrogated. The inhibitory effect of DAI was independent of IRF3 signaling and secreted cytokines.

**CONCLUSION:** This study demonstrates that DAI can inhibit HBV replication and the inhibitory effect is associated with activation of NF- $\kappa$ B but independent of IRF3 and secreted cytokines.

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**Key words:** DNA-dependent activator of interferon regulatory factor; Antiviral activity; Hepatitis B virus; Nuclear factor- $\kappa$ B; Interferon regulatory factor-3

**Peer reviewer:** Ourania M Andrisani, PhD, Professor, B038 Hansen Bldg, Center for Cancer Research, Purdue University, West Lafayette, IN 47907, United States

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## INTRODUCTION

Hepatitis B virus (HBV) is a noncytopathic DNA virus which belongs to the *Hepadnaviridae* family. Infection of HBV results in acute or chronic hepatitis, liver failure, and hepatocellular carcinoma<sup>[1-2]</sup>. HBV clearance is usually associated with a multispecific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell response coordinated with an effective humoral immune component<sup>[3-5]</sup>. However, a growing body of evidence suggests that the innate immune response is important for limiting viral replication. Expression of key proteins in pattern recognition system, such as RNA sensor melanoma differentiation-associated gene-5, the caspase recruitment domain of retinoic acid inducible gene I and the adaptor protein, myeloid differentiation primary response protein 88 (MyD88), and interferon- $\beta$  promoter stimulator 1 (IPS-1) can activate innate immune response and inhibit HBV replication in human hepatocyte-derived cells<sup>[6,7]</sup>.

DNA-dependent activator of interferon-regulatory factor (DAI/DLM-1/ZBP1) is the first identified sensor of cytosolic dsDNA. Recent studies have demonstrated that DAI can initiate innate immune responses, including the induction of type I interferon (*IFN*) genes, independently of Toll-like receptor 9<sup>[8-10]</sup>. It was reported that herpes simplex virus 1 production was notably higher in DAI blocked L929 cells<sup>[10]</sup>. As DAI is highly expressed in the differentiated hepatocyte after interferon treatment<sup>[11]</sup>, it is hypothesized that DAI could be a protein possessing antiviral activity against HBV in human hepatocytes.

The aim of the present study was to determine whether DAI can inhibit HBV replication and what the underlying molecular mechanism is. We found that expression of DAI could inhibit HBV gene expression and replication noncytopathically in Huh7 cells. Further study revealed that activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling was essential for DAI to elicit antiviral responses, but this inhibitory effect is independent of cytokines' secretion.

## MATERIALS AND METHODS

### Cell culture and transfection

Huh7 and HEK 293T cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco). For plasmid transfections, we used Fugene reagent (Roche), according to the manufacturer's protocol. The transfection efficiency was normalized by detecting the expression of green fluorescent protein (GFP) after transfection of equal amount of plasmid GFP.

Transwell plate (Cat. No. 3450) was purchased from Corning Company (New York, the United States). The transwell chamber contains 0.4  $\mu$ m pore polyester membrane which is optimal for cell attachment and growth and is permeable for flow of liquids. The cytokines can transfer through the membrane freely while the cells are blocked.

### Plasmids and chemicals

pCAGGS-hemagglutinin (HA)-DAI encoding the whole transcript of DAI was kindly provided by Professor Tadaatsugu Taniguchi<sup>[9]</sup>. pHBV1.3 containing a 1.3-copy of the HBV genome was described previously. The plasmid pCMV-I $\kappa$ B $\alpha$ -SR expresses a repressor form of I $\kappa$ B $\alpha$  in which serines 32 and 36 were mutated to alanine<sup>[12]</sup>. The NF- $\kappa$ B-dependent luciferase reporter plasmid pNF- $\kappa$ B-Luc was obtained from Stratagene Corporation (La Jolla, CA, the United States). pIRF-3 and pIRF-3 $\Delta$ N were provided by John Hiscott<sup>[13]</sup>. The cytokines IFN- $\alpha$  and tumor growth factor (TGF)- $\alpha$  were purchased from R and D Company (Lorton, VA, the United States).

### Quantitative real-time polymerase chain reaction analysis of hepatitis B viral RNA

Total RNA was extracted directly using TRIzol reagent (Invitrogen), and reversely transcribed to cDNA using a complementary DNA (cDNA) synthesis kit (Fermentas) according to the manufacturer's instructions. The cDNA was mixed with SYBR Green polymerase chain reaction (PCR) Master Mix (Toyoba) and subjected to real-time PCR using the ABI PRISM 7500 (Applied Biosystems). Cellular glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA from the same cDNA was used as an internal control. The primers specific for HBV and GAPDH are available upon request. Forty cycles of PCR were performed with cycling conditions of 15 s at 95 °C, 20 s at 55 °C, 25 s at 72 °C, and 35 s at 79 °C to detect signal.

### Southern blotting analysis of viral DNA replicative intermediates

HBV DNA replicative intermediates in HBV core particles isolated from transfected Huh7 cells were analyzed using Southern blotting. The intracellular viral DNA was extracted as previously reported<sup>[14]</sup>.

The normalized viral DNA replicative intermediates were electrophoresed onto 1% agarose gel. Then DNA was blotted onto a positive nylon membrane (Roche) in 20  $\times$  SSC. After fixing at 120 °C for 30 min, the membrane was prehybridized for 1 h at 42 °C in ULTRAhyb hybridization solution, and then hybridized with full-length HBV DNA probes labeled with ( $\alpha$ -<sup>32</sup>P) deoxycytidine triphosphate (dCTP) by hexamer random labeling kit (Roche) under the same condition of prehybridization at 42 °C for 16 h. After stringent washing at 68 °C, signals were detected by autoradiography.

### Northern blotting analysis of total viral RNA

Total RNA was extracted directly from transfected cells using TRIzol reagent. Ten  $\mu$ g total cytoplasmic RNA was electrophoresed on 1% formaldehyde-agarose gel and then transferred to nylon membranes (Roche). Hybridization was undertaken as described above using ( $\alpha$ -<sup>32</sup>P) dCTP-labeled full-length HBV DNA probes. To normalize the total quantity of RNA loaded on each gel, blots

were stripped and rehybridized with ( $\alpha$ - $^{32}$ P) dCTP-labeled GAPDH probes.

### Western blotting analysis

Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted electrophoretically onto a nitrocellulose membrane (Whatman). The membrane was blocked with phosphate-buffered saline (PBS) containing 5% skim milk and then incubated overnight with 1:1000 anti-HA (Roche), 1:2000 anti-GFP (Sigma), 1:1000 anti-phospho-IRF3 (Cell signaling), 1:1000 anti-IRF3 (Santa Cruz), 1:10000 anti- $\beta$  actin antibody (Sigma), then washed three times in PBST (0.05% Tween 20 in PBS), and incubated with peroxidase conjugated secondary antibody (1:2000) for 1 h. After further washing with PBST, chemiluminescence detection was carried out using enhanced chemiluminescence detection reagents.

### Enzyme-linked immunosorbent assay

The HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) levels in the supernatants obtained from HBV transfected Huh7 cells were detected using a standard enzyme-linked immunosorbent assay (ELISA) (Sino-American Biotech). The assay was performed according to the manufacturer's protocol. All experiments were performed at least three times.

### Immunofluorescence staining

Huh7 cells were fixed with 3.5% paraformaldehyde for 10 min. The cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature and incubated in blocking buffer supplemented with 3% bovine serum albumin (Sigma) for 1 h at room temperature. Interferon regulatory factor 3 (IRF3) was detected by staining with rabbit anti-human IRF3 (1:200 dilution, Santa Cruz), followed by Cy3-coupled goat anti-rabbit IgG (1:500 dilution, Jackson Immunologicals). Flag-tagged IPS1 was detected by staining with mouse anti-Flag antibody (1:2000, Sigma), followed by Alexa488-coupled goat anti-mouse IgG (1:200, Jackson Immunologicals). P65 was detected by staining with diluted (1:100) rabbit anti-human P65 (cell signaling) and Cy3-coupled goat anti-rabbit IgG (1:500). The nuclei were counterstained with 10  $\mu$ g/mL 4',6'-diamidino-2-phenylindole (DAPI) (Sigma). After incubation with the secondary antibodies, the cells were visualized under a confocal laser scanning microscope.

### Dual-luciferase reporter assay

To measure report gene activation, 293T cells seeded into 24-well plates at density of  $1 \times 10^5$  cells/well were transiently transfected with NF- $\kappa$ B dependent luciferase reporter plasmid 100 ng pNF- $\kappa$ B-Luc, 10 ng renilla luciferase-HSV thymidine kinase promoter (expressing Renilla luciferase, Promega) together with pCAGGS-HA-DAI or the empty vector pCAGGS-HA at the indicated amount. The cells were lysed and analyzed for firefly luciferase and renilla luciferase activity (Promega). The results were

reported as the normalized mean  $\pm$  SD.

### Statistical analysis

Results were reported as means  $\pm$  SD. *T* tests were applied for comparisons between groups; and *P* < 0.05 was considered statistically significant.

## RESULTS

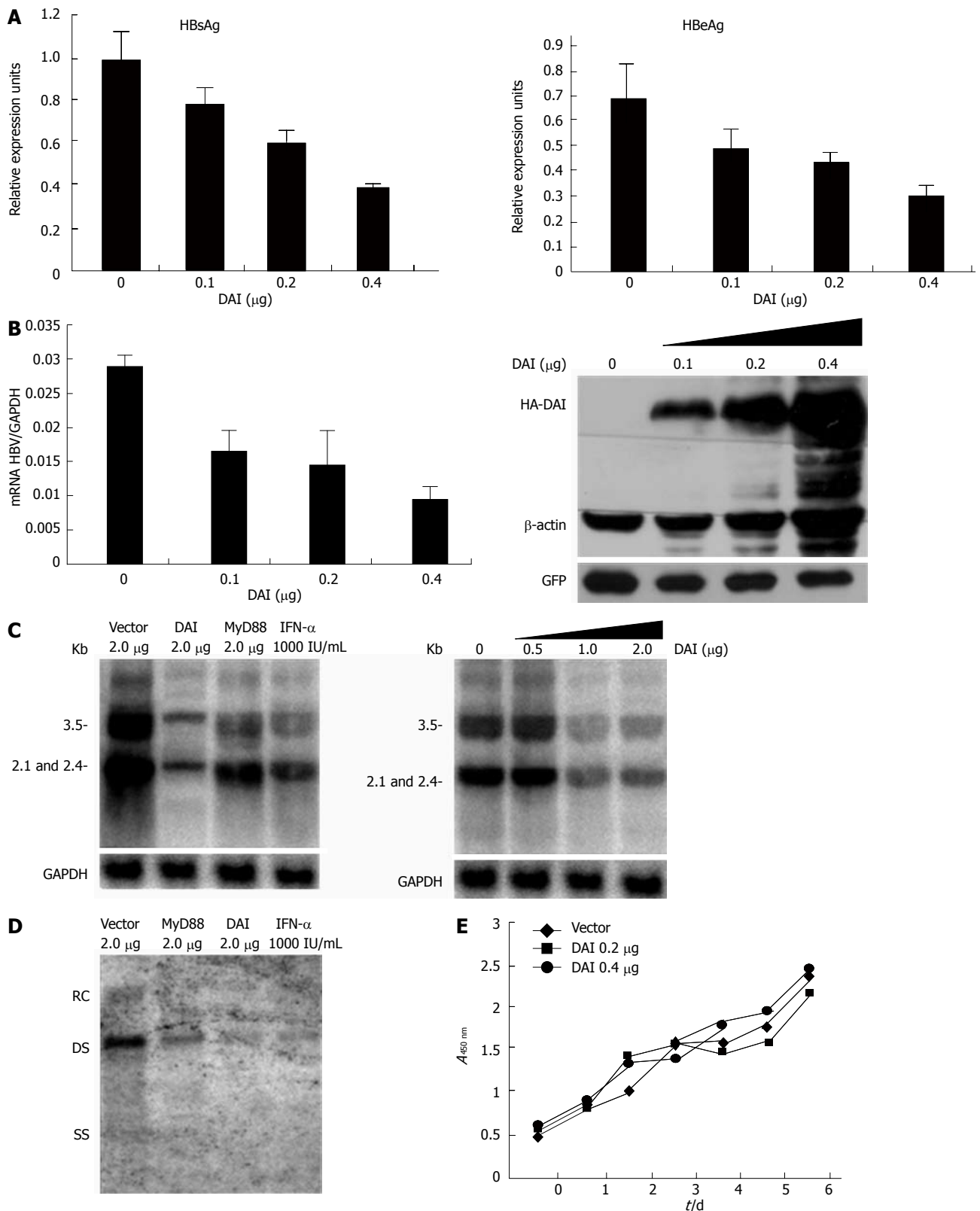
### DAI inhibits HBV replication in the human hepatoma Huh7 cells

To investigate the antiviral activity of DAI against HBV, we firstly examined the effect of DAI on the synthesis of HBV proteins. HBV-replicating plasmid HBV1.3 was co-transfected with either empty vector or HA-DAI into Huh7 cells. Supernatants were collected and HBsAg and HBeAg were analyzed by standard ELISA immunoassay. Compared with the control, the secretion of HBsAg was reduced by 17%, 33% and 57% and secretion of HBeAg was reduced by 25%, 34% and 57% when the increasing amount of DAI was transfected (Figure 1A). In order to study the inhibitory effect of DAI on HBV RNA transcription, the HBV RNA level was examined by quantitative real-time PCR. Results showed that HBV RNA level was also decreased by 44%, 51%, and 67% with an increased level of DAI expression. Expression of DAI in Huh7 cells was monitored by Western blotting (Figure 1B). To further investigate the effect of DAI on HBV viral RNA transcription, Northern blotting analysis was employed. As MyD88 has been reported as interferon inducible protein which can inhibit HBV replication<sup>[6,7]</sup>, MyD88 and 1000 IU/mL IFN- $\alpha$  treatment were included as positive controls. As shown in Figure 1C, expression of DAI dramatically reduced HBV RNA level. To investigate the influence of DAI on HBV replication, Southern blotting was performed to analyze the viral DNA replicative intermediates which were extracted from core particles. As shown in Figure 1D, the HBV core particle-associated DNA was significantly reduced. These results suggested that viral genome replication, viral RNA transcription and viral protein expression were all downregulated by DAI.

To exclude the possibility that the reduction of HBV RNA and DNA in Huh7 cells was due to cell death induced by DAI, the growth of DAI-expressing Huh7 cells was examined by cell counting assay for 6 d. Results demonstrated that DAI did not obviously affect cell growth (Figure 1E). Taken together, DAI can inhibit HBV gene expression and replication noncytopathically in Huh7 cells.

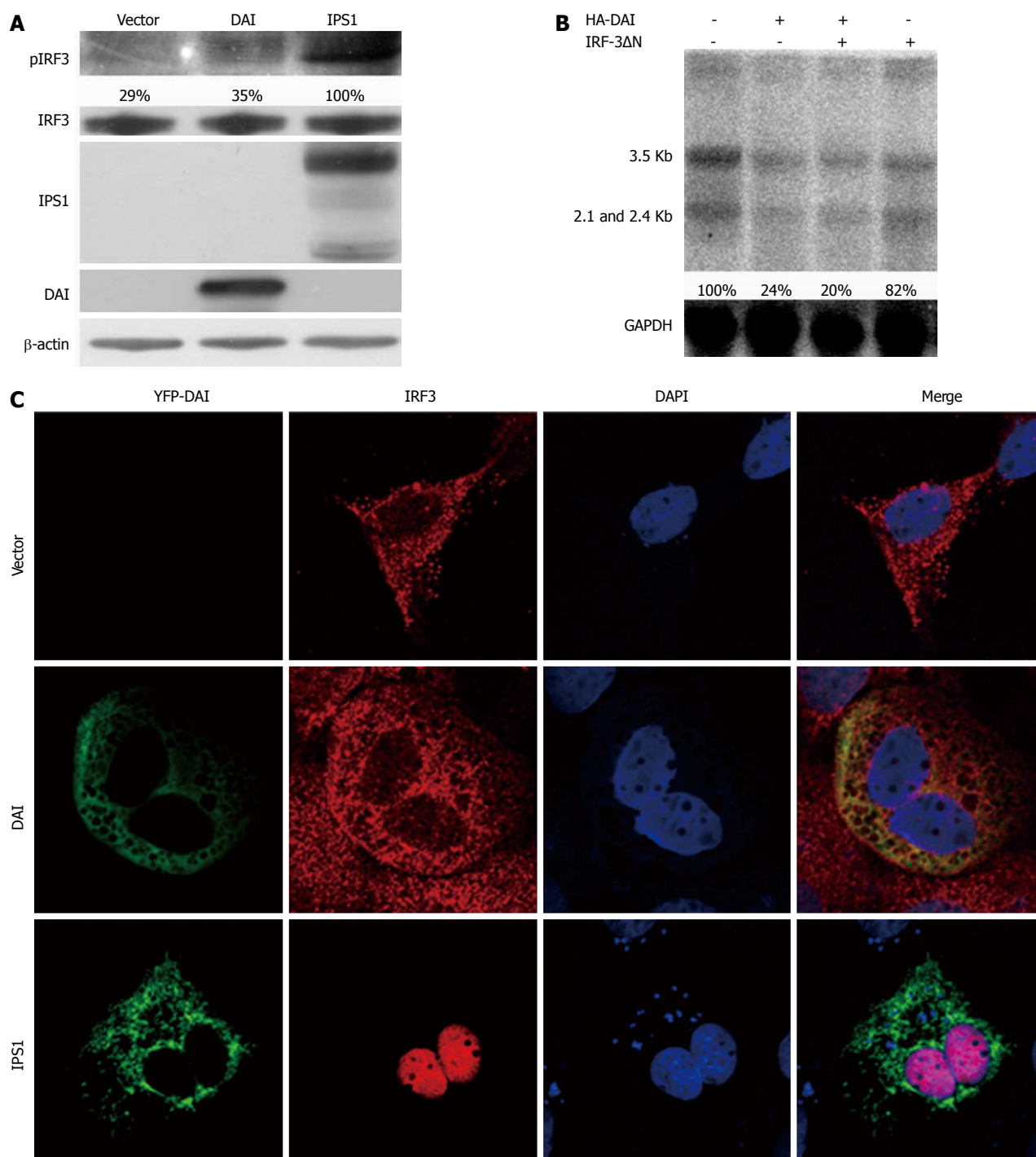
### IRF3 signaling pathway is not required for inhibition of HBV by DAI

The activation of innate immune system by DAI was through IRF3 or NF- $\kappa$ B mediated signaling pathways<sup>[10]</sup>. To investigate the possible effect of the pathways DAI on it, we firstly examined the activation of IRF3 after overexpression of DAI. IPS1, which can activate IRF-3 sig-



**Figure 1** Expression of DNA-dependent activator of interferon-regulatory factors in Huh7 cells can suppress hepatitis B virus replication. A: ELISA analysis of HBV protein synthesis. GFP was transfected to monitor transfection efficiency; B: Real-time PCR analysis of HBV RNA. Huh7 cells were cotransfected with pHBV1.3 and different doses of hemagglutinin (HA)-DAI. Total RNA was extracted 48 h after transfection and HBV RNA was examined by real-time PCR; C: Northern blotting analysis of HBV RNA; Huh7 cells were cotransfected with pHBV1.3 and control DNA or MyD88 and HA-DAI. 1000 IU/mL IFN- $\alpha$  was added 12 h after transfection, and 48 h later, total RNA was extracted for Northern blotting hybridization. The positions of the HBV 3.5-, 2.4- and 2.1-kb RNA were indicated; D: Southern blotting analysis of HBV core particle associated DNA. Huh7 cells were treated as in C. HBV core particle associated DNA was analyzed 48 h later. Southern blotting was performed to detect HBV DNA as described. The positions of relaxed circular (RC), double stranded (DS) and single stranded (SS) DNAs were indicated; E: Effect of DAI on cell growth. Cell number was counted by adding cell counting kit-8 at 1, 2, 3, 4, 5, 6 d after transfection. DAI: DNA-dependent activator of interferon-regulatory factors; HBV: Hepatitis B virus; ELISA: Enzyme-linked immunosorbent assay; PCR: Polymerase chain reaction; IFN: Interferon; GFP: Green fluorescent protein; HBsAg: Hepatitis B virus surface antigen; HBeAg: Hepatitis B virus e antigen; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; MyD88: Myeloid differentiation primary response protein 88.

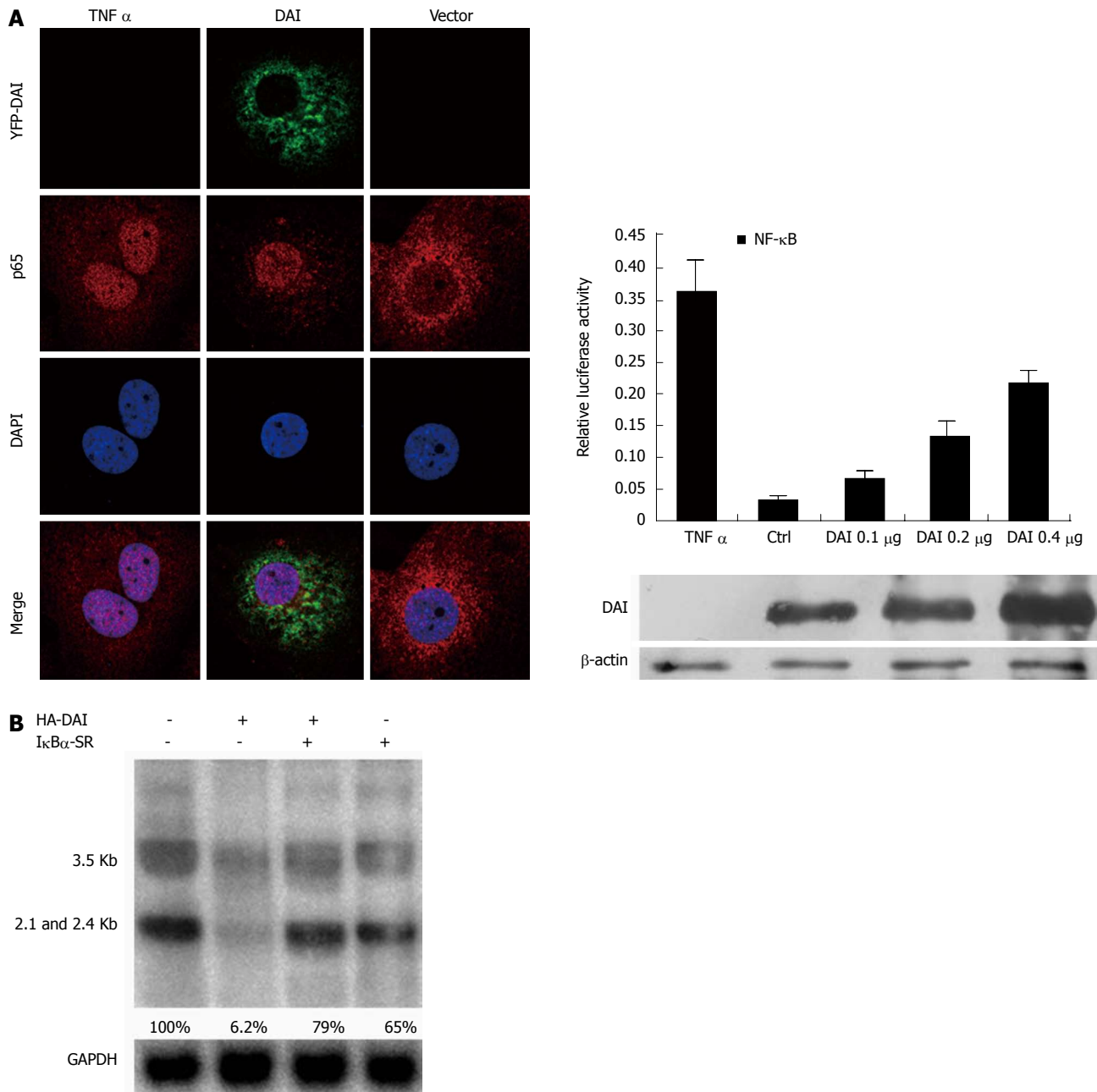




**Figure 2** Interferon regulatory factor 3 signaling pathway is not required for inhibition of hepatitis B virus by DNA-dependent activator of interferon-regulatory factors. A: DAI did not induce IRF-3 phosphorylation. Control DNA, hemagglutinin (HA)-DAI or Flag-interferon- $\beta$  promoter stimulator 1 (IPS1) was transfected into Huh7 cells. Forty-eight hours later, the phosphorylated form of IRF-3 was analyzed by Western blotting; B: Blockage of IRF-3 signaling did not affect inhibitory effect of DAI on HBV replication. Northern blotting assay was performed as shown in Figure 1C; C: Expression of DAI could not induce IRF-3 nuclear translocation. Cells were harvested 48 h after transfection and IRF-3 was stained as described. DAI: DNA-dependent activator of interferon-regulatory factors; IRF: Interferon regulatory factor; IRF-3 $\Delta$ N: IRF-3 dominant negative plasmid; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6'-diamidino-2-phenylindole; YFP: Yellow fluorescent protein.

naling pathway, was set as positive control<sup>[15]</sup>. The results showed that DAI cannot induce the phosphorylation of IRF-3 (Figure 2A). Furthermore, as shown in Figure 2C, nuclear translocation of IRF-3 was not observed after DAI expression. These results indicated that DAI cannot activate IRF-3. To further confirm that DAI-mediated inhibition of HBV replication is not associated with IRF-3,

an IRF-3 dominant negative plasmid (IRF-3 $\Delta$ N), in which the DNA binding domain was removed to express the repressor form of IRF-3, was used<sup>[13]</sup>. Results suggested that when the IRF-3 pathway was blocked by IRF-3 $\Delta$ N, the inhibitory effect of DAI on HBV replication was not affected (Figure 2B). Taken together, inhibition of HBV replication by DAI was not associated with acti-



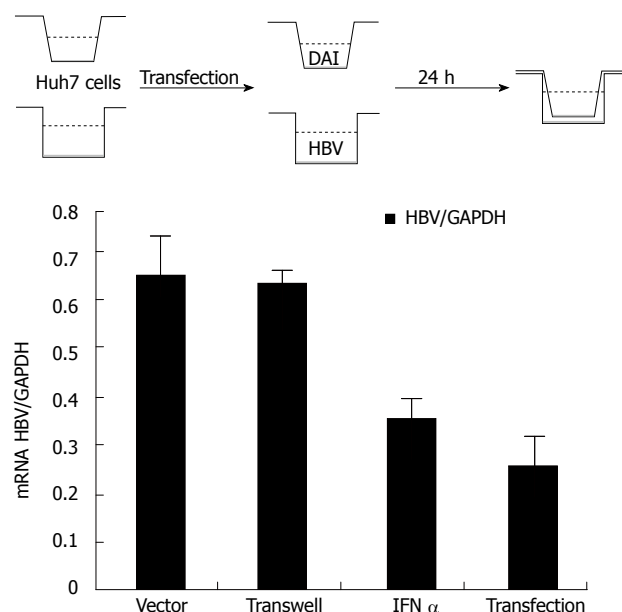
**Figure 3** Inhibition of hepatitis B virus by DNA-dependent activator of interferon-regulatory factors depends on activation of nuclear factor- $\kappa$ B. A: NF- $\kappa$ B activity was induced by dependent activator of interferon-regulatory factors (DAI). Forty-eight hours after transfection, NF- $\kappa$ B (p65) was stained as described. NF- $\kappa$ B dependent luciferase reporter plasmid pNF- $\kappa$ B-Luc was co-transfected with control DNA or different doses of hemagglutinin (HA)-DAI into 293T cells. Renilla luciferase-herpes simplex virus thymidine kinase promoter was transfected to monitor the transfection efficiency; B: Blockage of NF- $\kappa$ B activation abolished DAI-mediated suppression of hepatitis B virus (HBV) replication. Forty-eight hours after transfection, the levels of hepatitis B e antigen and hepatitis B surface antigen were examined by enzyme-linked immunosorbent assay. HBV RNA was determined by Northern blotting hybridization. NF- $\kappa$ B: Nuclear factor- $\kappa$ B; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; DAPI: 4',6'-diamidino-2-phenylindole; YFP: Yellow fluorescent protein.

vation of IRF3 pathway.

### Inhibition of HBV replication by DAI depends on activation of NF- $\kappa$ B

In addition to IRF-3 signaling pathway, NF- $\kappa$ B is another key pathway induced by DAI to activate antiviral innate immunity. In most cells, NF- $\kappa$ B signaling pathway complexes are inactive, residing primarily in the cytoplasm in a complex with the inhibitory I $\kappa$ B proteins. Once activated, NF- $\kappa$ B is released from the I $\kappa$ B $\alpha$  and translocated

to nucleus, where it binds to specific  $\kappa$ B sequences in the promoter or enhancer regions to induce the expression of multiple target genes<sup>[14,16]</sup>. To investigate if NF- $\kappa$ B was activated by DAI, we firstly examined the translocation of NF- $\kappa$ B p65. Yellow fluorescent protein-DAI and control DNA was transfected into Huh7 cells and TNF- $\alpha$  treatment was included as positive control. As expected, nuclear translocation of p65 was observed in both DAI-expressing and TNF- $\alpha$ -treated cells. Furthermore, a NF- $\kappa$ B-dependent luciferase reporter plasmid (pNF- $\kappa$ B-Luc)



**Figure 4** Inhibiting hepatitis B virus replication by DNA-dependent activator of interferon-regulatory factors is an intracellular event. Transwell co-culture experiment was performed: Huh7 cells were seeded in both 6-well plates (below) and transwells (top). In transwell co-culture group, pHBV1.3 was transfected into the cells in 6-well plates while hemagglutinin (HA)-DNA-dependent activator of interferon-regulatory factors (DAI) was transfected into the cells in transwells. Twenty-four hours after the transfection, the cells in 6-well plates and transwells were co-cultured; in the direct cotransfection groups, pHBV1.3 and control DNA or HA-DAI were cotransfected into cells in 6-well plates; in interferon (IFN)- $\alpha$  treatment group, pHBV1.3 was transfected into the cells in 6-well plates, 1000 IU/mL IFN- $\alpha$  was added 12 h later. Seventy-two hours after transfection, all the cells were harvested and hepatitis B virus (HBV) RNA was determined by real-time polymerase chain reaction. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

was used to detect NF- $\kappa$ B activity after overexpression of DAI. Results showed that DAI increased the NF- $\kappa$ B-dependent luciferase activity in a dose dependent manner (Figure 3A), suggesting that DAI can induce the activation of NF- $\kappa$ B signaling pathway.

To further confirm that suppression of HBV replication by DAI was NF- $\kappa$ B-dependent, we used a NF- $\kappa$ B signaling suppressor I $\kappa$ B $\alpha$ -SR, in which Ser32 and Ser36 residues critical for phosphorylation are replaced by alanine<sup>[12]</sup>. As shown in Figure 3B, the inhibition of HBV RNA by DAI was reversed in the presence of I $\kappa$ B $\alpha$ -SR.

In conclusion, these data demonstrated that the activation of NF- $\kappa$ B signaling pathway played an indispensable role in DAI-mediated suppression of HBV replication.

#### Inhibition of HBV by DAI is independent of secreted cytokines

After NF- $\kappa$ B was activated, IFNs, chemokines and other pro-inflammatory cytokines could be induced, which might be directly involved in inhibiting viral infection<sup>[17]</sup>. To investigate whether the observed antiviral effects of DAI were dependent on the secreted cytokines, transwell co-culture experiments were conducted. The IFN- $\alpha$  treatment was set as positive control. We found that the

obvious inhibitory effects of DAI on HBV replication could be observed in the HBV and HA-DAI directly co-transfected cells, but not in transwell co-cultured Huh7 cells (Figure 4), indicating a secreted cytokine-independent mechanism of inhibiting HBV replication by DAI.

The above results suggested that the observed inhibition of HBV replication by DAI was most likely due to some inducible intracellular factor(s), rather than secreted cytokines.

## DISCUSSION

After viral infection, innate immune receptors can detect the invading virus and subsequently initiate the synthesis of IFN and protective cellular genes to directly limit viral replication<sup>[18,19]</sup>. DAI is the first identified sensor of cytosolic dsDNA, which elicits innate immune responses and induces type I IFN to control viral replication. In this study, we found that DAI could inhibit HBV replication in Huh7 cells, and further study revealed that NF- $\kappa$ B signaling pathway was essential for this inhibition.

NF- $\kappa$ B signaling pathway plays pivotal roles in mediating inflammation, immune responses to pathogen infections, proliferation, apoptosis, and other cellular activities. The activation of NF- $\kappa$ B presents different results for different viruses. Some viruses activate NF- $\kappa$ B pathway to improve their transcription and replication<sup>[20]</sup>. Under other conditions, activation of NF- $\kappa$ B can repress viral replication. For example, NF- $\kappa$ B activation can mediate inhibition of human cytomegalovirus replication<sup>[21]</sup>. Rotavirus could antagonize cellular antiviral responses by inhibiting the nuclear accumulation of NF- $\kappa$ B<sup>[22]</sup>. As far as HBV is concerned, on one hand, viral replication itself can activate NF- $\kappa$ B, and on the other hand, upregulation of NF- $\kappa$ B by some host cytokines' stimulation has shown to be an inhibitory factor. For example, TNF- $\alpha$  could inhibit HBV replication by activating NF- $\kappa$ B signaling<sup>[23]</sup>. Besides, activation of NF- $\kappa$ B is also required for MyD88, IPS-1 and TRIF to elicit antiviral response to limit HBV replication<sup>[23]</sup>. In this study, we also found that DAI inhibited HBV replication *via* activating NF- $\kappa$ B signaling. Therefore, we speculated that the NF- $\kappa$ B signaling might be a common pathway for host to inhibit the replication of HBV and other DNA viruses.

The activation of NF- $\kappa$ B is associated with increased transcription of genes encoding chemokines, such as IL-8, MCP-1, cytokines such as IL-6, TNF- $\alpha$ , IFNs, adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule-1), enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis<sup>[24-26]</sup>. These molecules are important components of the innate immune response to invading microorganisms. When further exploring the mechanisms responsible for suppression of viral replication after NF- $\kappa$ B activation, we speculated that cytokines, especially type I interferon, may be the direct effector to inhibit HBV replication. However, we did not detect the induction of IFN in DAI-



expressing cells. By transwell experiment, we found that the secreted cytokines were not required for the inhibition of HBV replication by DAI (Figure 4). Interestingly, some components of pattern-recognition receptor system, such as MyD88, IPS-1 and TRIF, could also control HBV replication in a cytokine independent manner<sup>[24]</sup>. It is possible that there is a common strategy to inhibit HBV by these functional proteins. In addition, the antiviral factor downstream of NF- $\kappa$ B induced by DAI is worthy to be further explored.

In summary, this study demonstrates that DAI is a cellular antiviral protein. When expressed in Huh7 cells, DAI activated NF- $\kappa$ B but not IRF-3 signaling to suppress HBV replication. This inhibitory effect is independent of secreted cytokines. The findings could potentially lead to the development of novel therapies that induce the host cytoplasmic antiviral protein to control HBV infections.

## COMMENTS

### Background

The hepatitis B virus (HBV) is a DNA virus that replicates its genome via an RNA intermediate using reverse transcription. Chronic infection with this virus can result in cirrhosis and hepatocellular carcinoma. Nowadays, more and more evidence suggests that the innate immune response is important for limiting viral replication. Pattern recognition receptors play a pivotal role in host innate immune responses against microbial infection. DNA-dependent activator of interferon-regulatory factor (DAI/DLM-1/ZBP1) is a potent activator of immune responses during infection or tissue damage.

### Research frontiers

Expression of key proteins in pattern recognition system, such as RNA sensor melanoma differentiation-associated gene-5, the caspase recruitment domain of retinoic acid inducible gene 1 and the adaptor protein, and myeloid differentiation primary response protein88 can activate innate immune response and inhibit HBV replication in human hepatocyte-derived cells. DAI is the first identified sensor of cytosolic dsDNA. Recent studies have demonstrated that DAI can initiate innate immune responses, including the induction of type I interferon genes, independently of Toll-like receptor 9. The authors hypothesize that DAI could be a protein possessing antiviral activity against HBV replication.

### Innovations and breakthroughs

The aim of the present study was to determine whether DAI can inhibit HBV replication and what the underlying molecular mechanism is. The authors found that expression of DAI could inhibit HBV gene expression and replication non-cytopathically in Huh7 cells. Further study revealed that activation of Nuclear factor- $\kappa$ B signaling was essential for DAI to elicit antiviral responses, but this inhibitory effect was independent of cytokines' secretion.

### Applications

The study could potentially lead to the development of novel therapies that induce the host cytoplasmic antiviral protein to control HBV infections.

### Peer review

The study describing the inhibitory role of DAI on HBV replication is generally well-performed and the results support the conclusions reached. The manuscript is well-written.

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## Double balloon enteroscopy in the old: Experience from China

Qiong He, Qiang Zhang, Jian-Dong Li, Ya-Dong Wang, Tian-Mo Wan, Zhen-Yu Chen, De-Shou Pan, Jian-Qun Cai, Si-De Liu, Bing Xiao, Ya-Li Zhang, Bo Jiang, Yang Bai, Fa-Chao Zhi

Qiong He, Qiang Zhang, Guangdong Provincial Key Laboratory of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China  
Jian-Dong Li, Department of Epidemiology, School of Public Health and Tropical Medicine, Southern Medical University, Guangzhou 510515, Guangdong Province, China

Ya-Dong Wang, Tian-Mo Wan, Zhen-Yu Chen, De-Shou Pan, Jian-Qun Cai, Si-De Liu, Bing Xiao, Ya-Li Zhang, Bo Jiang, Yang Bai, Fa-Chao Zhi, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China

**Author contributions:** He Q and Zhang Q performed this study; He Q wrote the paper; Li JD participated in data analysis; endoscopic procedures were performed by Wang YD, Wan TM, Chen ZY, Pan DS, Cai JQ, Liu SD, Xiao B, ZhangYL, Jiang B, Bai Y and Zhi FC; and Zhi FC and Bai Y designed the study.

**Correspondence to:** Fa-Chao Zhi, MD, Department of Gastroenterology, Nanfang Hospital, Southern Medical University, Guangzhou 510515, Guangdong Province, China. [zfc@fimmu.com](mailto:zfc@fimmu.com)

Telephone: +86-20-61641532 Fax: +86-20-61641532

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sequently analyzed. The mean age was  $69.63 \pm 3.89$  years (range 65-84), 34 were males. Indications for DBE were melena/hematochezia (36 cases), abdominal pain (15 cases), diarrhea (3 cases), stool change (1 case), weight loss (1 case), vomiting (2 cases), and debilitation (1 case). The average duration of symptoms was  $33.34 \pm 64.24$  mo. Twenty-seven patients suffered from age-related diseases. Severe complications were not found during and after DBE. Comparison between systolic and diastolic blood pressure before and after DBE was statistically significant (mean  $\pm$  SD,  $P < 0.01$ ,  $P < 0.05$ , respectively). Small bowel pathologies were found by DBE in 35 patients, definite diagnoses were made in 31 cases, and detection rate and diagnostic yield for DBE were 68.6% and 60.8%, respectively.

**CONCLUSION:** DBE is a safe and effective method for gastrointestinal examination in the aged population. Aging alone is not a risk factor for elderly patients with suspicious gastrointestinal diseases and thorough preparation prior to the DBE procedure should be made for individuals with multiple diseases especially cardiovascular disorders.

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### Abstract

**AIM:** To evaluate the safety, efficacy and management of double balloon enteroscopy (DBE) carried out in those aged individuals with suspicious small intestine diseases.

**METHODS:** DBE is a wonderful invention of the past decade and is widely used as an examination tool for the gastrointestinal tract. From January 2003 to July 2011, data from patients who were  $\geq 65$  years old and underwent DBE examination in the Nanfang Hospital were included in a retrospective analysis.

**RESULTS:** Fifty-nine individuals were found and sub-

**Key words:** Double balloon enteroscopy; Capsule endoscopy; Small bowel diseases; Multiple systematic diseases

**Peer reviewer:** H Choi, Professor, Department of Internal Medicine, Incheon St. Mary's Hospital, The Catholic University of Korea College of Medicine, 665 Bupyeong Dong, Incheon 403720, South Korea

He Q, Zhang Q, Li JD, Wang YD, Wan TM, Chen ZY, Pan DS, Cai JQ, Liu SD, Xiao B, Zhang YL, Jiang B, Bai Y, Zhi FC. Double balloon enteroscopy in the old: Experience from China. *World J Gastroenterol* 2012; 18(22): 2859-2866 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2859.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2859>

## INTRODUCTION

As the world population is aging, age-related diseases continue to increase and utilization of endoscopic techniques is rising<sup>[1]</sup>, but the feasibility, safety, and effectiveness of endoscopic use for the elderly are still unknown, and there are still concerns about the use of clinical endoscopy in the elderly. Double balloon enteroscopy (DBE) is a wonderful invention in the past decade<sup>[2]</sup> and widely used as an examination tool in the gastrointestinal tract, which has the double advantages of being useful for diagnosis and interventional management. Use of DBE has mainly been in the small bowel in the past few years<sup>[3-10]</sup>.

Compared to esophagogastroduodenoscopy (EGD), colonoscopy, and push endoscopy, clinical endoscopists are usually uncertain or perplexed about whether elderly individuals who are suspect for small bowel disorders could endure and be investigated by DBE with prolonged duration examination. There are a number of clinical studies regarding DBE performed in adults<sup>[11-15]</sup> and clinical trials using DBE performed in children<sup>[16-18]</sup>, but little data concerning DBE exclusively used in the aged population. Therefore, the safety, efficacy, diagnosis and therapeutic management of DBE carried out in those aged subjects were assessed.

## MATERIALS AND METHODS

Inclusion criteria were senile patients who were  $\geq 65$  years old and underwent DBE from January 2003 to July 2011 at Department of Gastroenterology, Nanfang Hospital (a tertiary center and university hospital), Southern Medical University, Guangzhou, China. The records of all patients were included in a retrospective analysis. This work was approved by Institutional Review Board of Nangfang hospital, Southern Medical University.

All procedures were performed by DBE with no obvious absolute contraindications and carried out after written informed consent from patients and/or their guardians. A low residue and liquid diet were required and colored foods were avoided as far as possible at least one day prior to the test. All patients finished a bowel cleansing preparation by ingesting a 1.8-2 L polyethylene-glycol solution followed by overnight fasting (if the test was carried out in the morning), at least 6 h prior to the start of the procedure.

The Fujinon system (EN-450P5/20, Fuji Photo Optical Incorporated Company, Fujinon Inc, Japan) was used. This system consists of an endoscope at a working length of 200 cm with an outer diameter of 8.5 mm, and a flexible overtube 140 cm in length and 12 mm in outer diameter. By inflating the overtube balloon enough to grip the intestinal wall, the endoscope can be inserted further without forming redundant loops in the small bowel, and then the overtube can in turn be inserted while the endoscope balloon is inflated. DBE could be moved back and forth in a controlled manner by experienced endoscopists

and an assistant to produce observations of the bowel.

Preparations and evaluation before and during the DBE procedure were as follows: (1) comprehensive assessment was carried out combining medical history and physical examination of the patients, individuals with suspected small bowel lesions and without absolute contraindications were included in the DBE investigation; (2) all subjects without other systematic diseases were evaluated prior to DBE operation, including vital signs (body temperature, blood pressure, pulse, respiration); hemoglobin (if this was abnormally low, it had to be corrected to a value  $> 80$  g/L); electrolytes in serum (these also had to be corrected to normal range if abnormal levels were detected); electrocardiograph, chest X-ray, abdominal ultrasound were performed as regular examinations. Combined advice from other medical professionals was considered if unusual manifestations were found and the trans-oral or anal route or a combination of the two approaches was taken into account; (3) patients receiving pharmacotherapy for other known diseases were asked to discontinue administration the day before starting the test (steroid hormones, nonsteroidal anti-inflammatory drugs, and anticoagulant drugs had to be discontinued for at least a week). Experts in other professional departments, including anesthesiologists, were invited to participate in diagnosis and treatment of patients who suffered from concomitant diseases. The risk of DBE manipulation was decided in combination with endoscopists. Cases who were at extremely high risk were required to be treated by medical means in stable conditions if DBE was necessary, according to the suggestions of other professionals; (4) endoscopists, clinicians, anesthesiologists, and endoscopic nurses jointly participated in the DBE process. Concurrently, real-time monitoring equipment, resuscitative devices and necessary drugs were always ready for use in case of emergency. DBE was implemented in the operating room with full equipped medical measures being used when it was necessary; (5) antegrade, retrograde or a combination of the two approaches was performed with or without intervention under conscious or deep sedation, or general anesthesia (antegrade approaches generally included mechanical ventilation); and (6) specific management was individualized on the basis of different conditions in distinct patients before and during DBE procedure.

Observations were followed after DBE exploration, as described above, related laboratory parameters, and serious complications were monitored and managed accordingly.

### Statistical analysis

Statistical analysis was performed using SPSS version 17.0 for Windows software. Continuous data were represented as means, mean  $\pm$  SD or range and categorical variables were expressed as frequency or percentages. Student's test was used to compare continuous variables. The  $\chi^2$  test or Fisher exact probability test were used to compare differences in categorical variables examined.

Table 1 Characteristics of included elderly patients (*n* = 59)

Features	
Gender (M/F)	34/25
Age (yr, mean $\pm$ SD, range)	69.63 $\pm$ 3.89 (65-84)
Age group (yr)	
$\geq 65$	58
$\geq 80$	1
Complaints	
Melena/hematochezia	36
Abdominal pain	15
Diarrhea	3
Vomiting	2
Weight loss	1
Stool change	1
Debilitation	1
Duration of symptoms (mo, mean $\pm$ SD, range)	33.34 $\pm$ 64.24 (0.10- 324.00)
Other medical examination	
Esophagogastroduodenoscopy	52
Colonoscopy	50
Computed tomography	14
Barium study	8
Digital subtraction angiography	4
Magnetic resonance imaging	3
Mekel's scan	3
Bone marrow aspiration	3
Position-emission tomography	1
Capsule endoscopy	20
Other diseases	27
Hypertension	19
Coronary diseases	2
Hypertension+ coronary diseases	1
Hypertension+ chronic bronchitis	1
Diabetes mellitus	2
Chronic bronchitis	1
Blood systemic diseases	1
Blood transfusion (Y/N)	29/30
Hemoglobin level (g/L) (mean $\pm$ SD, range)	96.00 $\pm$ 26.40 (39.00-160.00)
Prior surgery (Y/N)	19/40
Abdominal operation	15
Thoracic operation	1
Other operation	3

M/F: Male/female; Y/N: Yes/no.

McNemar's  $\chi^2$  test was used in comparison of diagnosis between capsule endoscopy (CE) and DBE. Agreement analysis between CE and DBE was assessed by the kappa statistic.  $P < 0.05$  (two-sided) was considered statistically significant.

## RESULTS

### Characteristics of patients

Fifty-nine individuals who were aged  $\geq 65$  years were found and subsequently analyzed; only one patient was  $\geq 80$  years. The mean age was 69.63  $\pm$  3.89 years (range 65-84), 34 were males. Indications for DBE were melena/hematochezia (36 cases), abdominal pain (15 cases), diarrhea (3 cases), stool change (1 case), weight loss (1 case), vomiting (2 cases), and debilitation (1 case). The average duration of symptoms was 33.34  $\pm$  64.24 mo (range 0.10-324.00 mo). Prior blood transfusion had been

Table 2 Capsule endoscopy *vs* double balloon enteroscopy for examination of gastrointestinal tract in this study<sup>a</sup> (*n* = 19)

CE Findings	DBE Findings		Total
	Positive	Negative	
Positive	5	1	6
Negative	9	4	13
Total	14	5	19

CE: Capsule endoscopy; DBE: Double balloon enteroscopy. <sup>a</sup> $P = 0.021$ ;  $\kappa = 0.10$ .

performed at least once in 29 subjects. Almost half the patients (27 cases) suffered from age-related diseases, including cardiovascular diseases, respiratory diseases, cardiopulmonary sickness, endocrine illnesses, *etc.* Hypertension and coronary disease were the main cardiovascular diseases and the most common respiratory illness was chronic bronchitis. Anticoagulant drugs were used in 1 case and 19 individuals had a prior surgical procedure. The mean hemoglobin level in plasma at initial examination was 96.00  $\pm$  26.40 g/L (range 39.00-160.00 g/L). The demographic information was listed in Table 1.

Twenty individuals had prior CE investigation. Inspection time between CE and DBE was within 1-13 d of the DBE procedure. One patient did not complete the entire CE process because the CE battery ran out. CE was successfully discharged through the anus. The remaining patients accomplished examinations without any complications. Abnormalities were seen in 17 patients, clear diagnoses were established in 6. Comparison between CE and DBE was given in Table 2.

### Safety and efficacy of DBE

In this review, the mean levels of systolic and diastolic blood pressure in patients before the DBE procedure were 130.49  $\pm$  17.19 mmHg (range 98-171 mmHg), 76.56  $\pm$  10.70 mmHg (range 55-105 mmHg), respectively. Low levels of hemoglobin and abnormal levels of electrolytes were all corrected prior to DBE; heart rate remained in the normal range. The mean level of oxygen saturation before the test was 99.15%  $\pm$  1.54 % (range 92%-100%).

All patients received atropine prior to the DBE procedure. Administration of benzodiazepines such as diazepam or midazolam, meperidine or fentanyl, rocuronium, and propofol were used for sedation, induction and maintenance of narcosis through injection; real-time blood pressure control for patients undergoing DBE was maintained according to the distinct conditions of different patients; drug use was under real-time monitoring by electrocardiography, and measurement of transdermal oxygen saturation during the intervention process and carried out by professional anesthetists.

All patients completed the DBE procedures whether a peroral, peranal or combination approach was chosen. Severe complications were not found during and after DBE. Only a few patients complained of slight discomfort after DBE, and the symptoms soon disappeared



**Table 3** Aged patients with small bowel pathologies examined by double balloon enteroscopy in this study (*n* = 51)

Findings	<i>n</i> = 51
Location	
Duodenum	9
Jejunum	14
Ileum	7
Cecum	1
Multiple segments of small bowel	4
Final diagnoses	
Primary or metastatic tumors	15
Diverticula	7
Single ulcer	5
Angiectasis	4
Erosions	2
Angioma	1
Lymphangiectasis	1
Lymphangioma	1
Single stenosis	1
Crohn's disease	1
Functional gastrointestinal diseases	13

without medical treatment. The average levels of systolic and diastolic blood pressure in patients after DBE were  $124.15 \pm 17.18$  mmHg (range 88-170 mmHg) and  $72.34 \pm 9.88$  mmHg (range 50-92 mmHg), respectively. The mean level of oxygen saturation after the test was  $99.76\% \pm 0.47\%$  (range 98%-100%). There was a statistically significant change in systolic and diastolic blood pressure before and after DBE examination.

### Diagnosis of gastrointestinal pathologies via DBE

Fifty-nine cases underwent 81 DBE procedures, including 27 performed by the antegrade approach, 10 by the retrograde approach, and 22 by combining the two approaches. Total enteroscopy combining the 2 approaches which could scrutinise the whole small bowel was achieved in 12 patients. The mean insertion depth was  $278.37 \pm 102.68$  cm (from the pylorus to the furthest distance, performed by antegrade DBE), and  $305.00 \pm 97.72$  cm (from the ileum valve to the furthest distance, performed by retrograde DBE) respectively; the mean total procedure time was  $112.61 \pm 39.32$  min (antegrade DBE) and  $119.50 \pm 37.52$  min, respectively. Twenty subjects received endoscopic biopsy and definite positive findings were made in 3 individuals. Lesions detected in the gastrointestinal tract were found in 42 patients and the diagnosis yield was 64.4% (38/59). Twenty-three individuals underwent surgical procedures and one person underwent intra-operative enteroscopy. All lesions in final diagnoses were found in the stomach, small intestine and other organs.

Gastric lesions in 8 patients diagnosed by DBE were excluded from having any small bowel pathology. Pathologies were found by DBE in 35 cases, definite diagnoses were made in 31, and the detection rate and diagnostic yield were 68.63% and 60.78%, respectively. Lesions examined were in the duodenum (9 cases), jejunum (14 cases), ileum (7 cases), cecum (1 case) and multiple sections

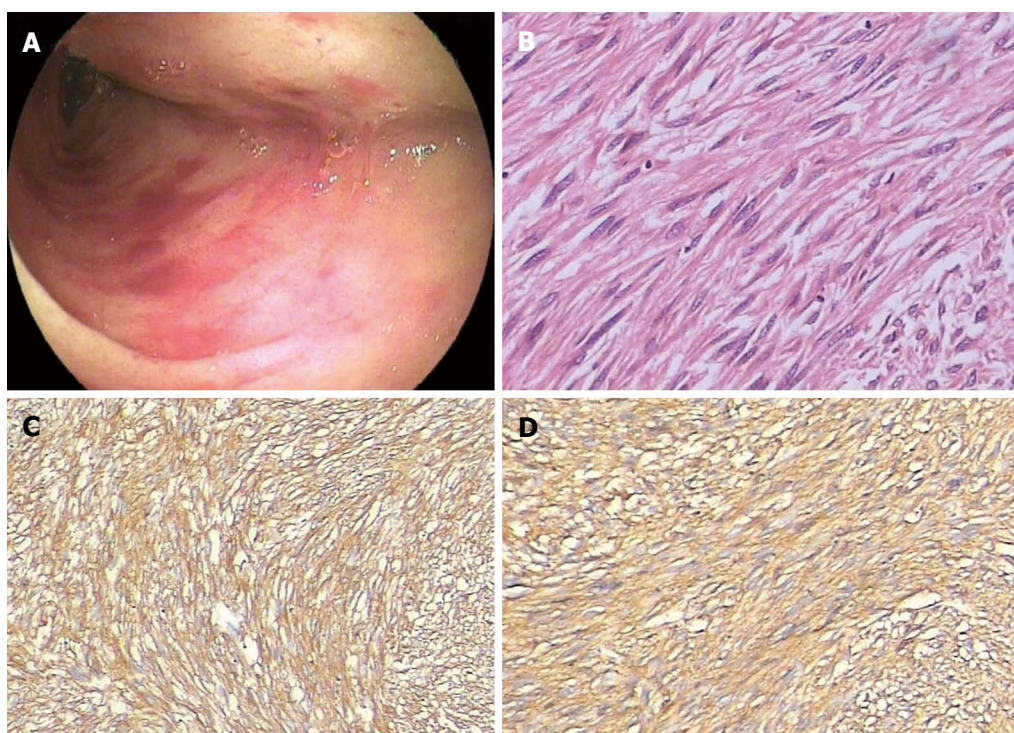
of small intestine (4 cases). Final diagnoses with lesions in gastrointestinal tract were found to be in the stomach of 8 patients, duodenum of 8 patients, jejunum of 14 patients, ileum of 7 patients, small bowel as multiple segmental lesions of 4 patients, the cecum of 1 patient, and other locations in 3 patients. Negative diagnoses were determined by DBE in 20 patients whose symptoms were melena/hematochezia in 10 individuals, abdominal pain (7 cases), diarrhea (2 cases), and weight loss (1 case). Twenty-seven DBE procedures incorporated 7 oral routines, 6 anal routines and 7 combined routines. Lesions were found in 4 subjects, final diagnoses were 1 case with metastatic lung cancer, 1 with metastatic liver cell carcinoma, 1 with gastrointestinal stromal tumor, 2 with pancreatic carcinoma, 2 with intestinal adenocarcinoma and others with gastrointestinal functional diseases (Table 3; Figures 1-3).

## DISCUSSION

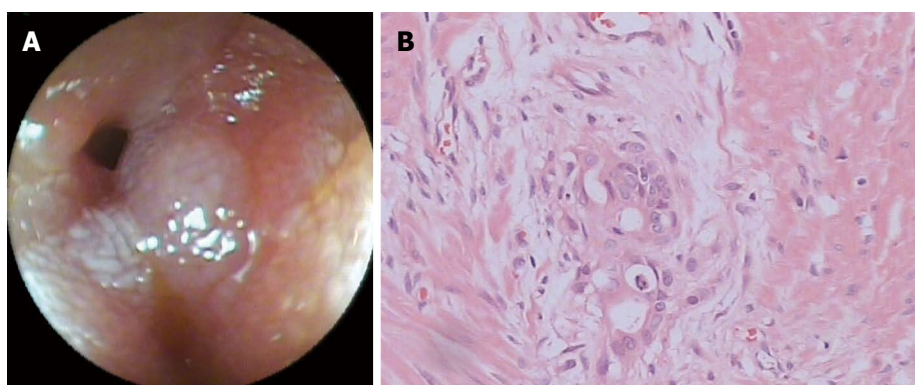
With advances in society and development of medical science, aging of the world's population is an inevitable trend. According to the latest demographic data, released on August 16, 2011 in Beijing, the China Committee on Aging's Office "2010 Annual Statistical Bulletin of China Aging Development" showed that in developing countries as China, 180 million of the population were aged  $\geq 60$  years and 120 million were aged  $\geq 65$  years. An increasingly aging population is bound to be accompanied by rises in age-related disorders. Gastrointestinal diseases are very common in children, adults and the elderly; consequently, the frequency of use of gastrointestinal endoscopy which can visualize the digestive tract is rising sharply.

Traditional and conventional upper gastrointestinal endoscopy and colonoscopy could scrutinise only the proximal small bowel and distal ileum owing to their limitations of length; the mid gut which spans the stomach and the colon is the longest part of the intestinal tract and could not be directly observed for examination, diagnosis and even intervention. Even if traditional techniques such as push enteroscopy, barium meal and advanced methods such as computed tomography (CT), magnetic resonance imaging, positron emission tomography could make correct diagnoses of gastrointestinal diseases, their limitations, such as length, and the difficulty of smaller lesions, make therapeutic management impossible, especially in the small bowel. That fact, coupled with unspecific clinical symptoms presented by small bowel diseases, misdiagnosis, missed lesions, delayed diagnosis and treatment usually promote poor prognosis and increase mortality. Over the past decade, there have been two significant inventions, namely CE and DBE, which have been applied in practice and revolutionized gastrointestinal diseases, particularly in the small intestine. Screening and/or diagnosis of patients with intestinal illnesses have been greatly improved by CE and DBE<sup>[19-22]</sup>.

With aging, the deterioration of physiological function in various organs also gradually becomes clear and



**Figure 1** Gastrointestinal stromal tumor in ileum was diagnosed by retrograde double balloon enteroscopy in a 73 year-old male patient (A), and confirmed by histopathology (B, hematoxylin and eosin) and immunohistochemistry (C:CD117; D:CD34).



**Figure 2** A 74 year-old male with adenocarcinoma was diagnosed using trans-oral double balloon enteroscopy. Tumor and stricture of the small bowel were found (A) and verified through pathology (B, HE). HE: Hematoxylin and eosin.

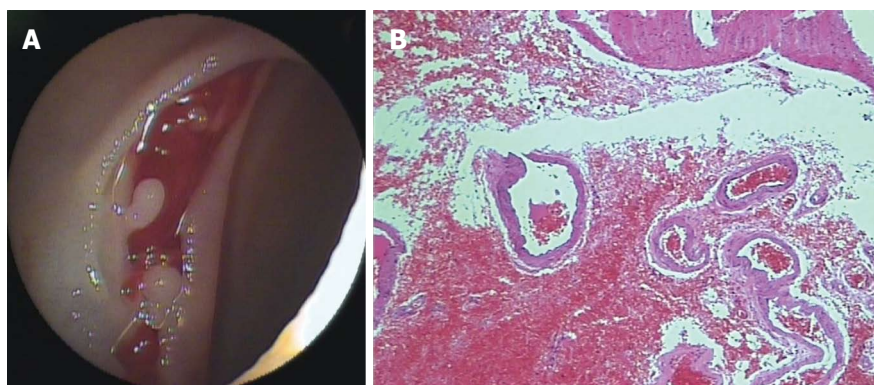
increases in age-related systemic diseases such as cardiovascular diseases, lung diseases, malignant tumors, *etc.* A number of clinical studies showed that EGD, colonoscopy, and endoscopic retrograde cholangiopancreatography were safe and effective methods for use in the aged<sup>[1,23-26]</sup>. CE was considered a non-invasive method for screening the gastrointestinal tract<sup>[6,27,28]</sup>; nevertheless, the lower specificity<sup>[29,30]</sup> and inability of its use for therapeutic management<sup>[31]</sup> were regarded as its shortcomings.

Plentiful studies confirmed excellent safety, effectiveness and appreciable benefits for adults investigated by DBE, and these results were reported in published literature. Diagnosis, treatment and safety of DBE for children who were aged  $\geq 3$  years were also demonstrated in clinical trials<sup>[16,17,32]</sup>. In contrast to other endoscopic meth-

ods, DBE with prolonged performance which is used in the elderly is usually performed more meticulously by clinicians and endoscopists due to safety concerns. This is partly because intervention costs significantly rise and, if necessary, anesthesia itself may also have moderate or severe side effects<sup>[1]</sup>. Clinicians are likely to choose non-invasive and relatively safe methods to investigate gastrointestinal pathogenesis that have given rise to various manifestations; patients are also likely to undergo a number of medical examinations as well but unclear diagnosis is obtained.

Patients with heart- or lung-related diseases for DBE must be taken into detailed consideration; insufficient preparations for those with poor health status are hazardous during and after DBE investigation. In this ret-





**Figure 3** A 66 year-old individual with diverticulum accompanied by bleeding in the jejunum was diagnosed using antegrade double balloon enteroscopy (A) and affirmed by histopathology (B).

rospective review, all patients completed the entire DBE procedure and serious adverse effects were not reported. Degraded function of aged organs makes the body have higher sensitivity and poorer tolerance for drug administration, if coexisting diseases are present; sedatives and anesthetic drugs were used in the process of DBE much less in adults. It is important to highlight that close attention must be paid during anesthesia, because intervention by DBE and/or combining with drug use may result in serious consequences or even death. Frequent anesthesia-related complications such as hypotension, desaturation and apnea were not found in our group. A slight decrease in blood pressure after DBE exploration was found and could be attributed to after effects of drug use during the procedure on account of lowering blood pressure and poor metabolism of the elderly. Complications such as perforation<sup>[33]</sup>, pancreatitis<sup>[11,34,35]</sup>, and intussusception resulting from the use of DBE described in previous studies<sup>[36]</sup> were not found in our study.

The results in this study suggested that the average age of the participants was approximately 70 years old which was considered a high-risk age associated with various diseases. Age-related disorders, the majority of which were cardiopulmonary diseases, were also found in our series. All patients complained of obvious discomfort and the most frequent symptoms were gastrointestinal bleeding and abdominal pain. A higher diagnostic yield for gastrointestinal diseases than usual was achieved and the diagnostic yield of small bowel diseases was 60.78%. Missed lesions are unavoidable by total enteroscopy in patients with prior abdominal surgery and where single routine insertion by DBE is not readily advanced. Final diagnoses of tumor, gastrointestinal functional disorders and intestinal diverticula were common in our study and this is different from results in western countries. Based on the above results, we maintain that DBE is a safe and effective method for gastrointestinal examination in the aged population. Aging alone is not a risk factor for elderly patients with suspicious gastrointestinal diseases and thorough preparations prior to the DBE procedure should be made for individuals with multiple systematic diseases, especially cardiopulmonary disorders.

A problem was revealed in this study in that the non-normative examination flow of DBE was used for small bowel evaluation at an early stage since DBE was first introduced in our unit in 2003. DBE was used in a few patients followed by combining negative EGD or negative colonoscopy with other medical examinations such as barium enema or abdominal CT rather than combining negative EGD with negative colonoscopy. The practical problems were corrected at a later stage and a series of normative flow for balloon-assisted enteroscopy have been established and all DBE procedures are carried out in patients with negative EGD and colonoscopy in our unit. Our study is not novel but has clinical significance and interest. The limitations of small sample size, retrospective analysis, non-randomized selection of elderly patients, and few patients aged  $\geq 80$  years were found in our study. Prospective, large-scale and blinded randomized trials are expected to be used in future clinical studies.

## COMMENTS

### Background

As the world population is aging, age-related diseases continue to increase and utilization of endoscopic techniques is rising, but few studies about the feasibility, safety, and effectiveness of double balloon enteroscopy (DBE) for the elderly have been performed, and there is still concern about its use in the elderly.

### Research frontiers

The invention of balloon assisted enteroscopy was first reported in 2001 by Yamamoto. A growing number of studies were chiefly focused on research of whole small bowel diseases, in order to facilitate earlier diagnoses and intervention in disorders of the mid gut. This study investigated the value of DBE for examination of an older population with suspected small bowel disease.

### Innovations and breakthroughs

Although the prevalence of small intestine diseases is not as high as that of colon disease, its manifestations are always unspecific and can even be fatal because of delayed diagnosis. The present study suggests that a higher proportion of the Chinese elderly population has tumors in the small bowel or functional gastrointestinal diseases. The results show that DBE is a safe and effective method in the older population.

### Applications

DBE-based examination can be used safely and effectively in older patients for diagnosis and treatment of small bowel diseases.

### Peer review

This retrospective study of DBE in the old is of significant interest in clinical practice. The safety and efficacy of DBE confirmed in this study may result in

wider use of balloon-assisted enteroscopy for exploration of the small intestine when older patients are suspected of having small bowel disorders.

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## Intervention of Mirtazapine on gemcitabine-induced mild cachexia in nude mice with pancreatic carcinoma xenografts

Shu-Man Jiang, Jian-Hua Wu, Lin Jia

Shu-Man Jiang, Jian-Hua Wu, Lin Jia, Department of Gastroenterology, Guangzhou Nan Sha Center Hospital, Guangzhou First Municipal People's Hospital Affiliated with the Guangzhou Medical College, Guangzhou 510180, Guangdong Province, China

**Author contributions:** Jia L, Jiang SM, and Wu JH designed the study; Wu JH performed the experiments; Jiang SM, Wu JH, and Jia L analyzed the data; Jiang SM and Jia L wrote the paper. **Correspondence to:** Dr. Lin Jia, Department of Gastroenterology, Guangzhou Nan Sha Center Hospital, Guangzhou First Municipal People's Hospital Affiliated with the Guangzhou Medical College, No. 1 Panfu Road, Guangzhou 510180, Guangdong Province, China. [jialin@medmail.com.cn](mailto:jialin@medmail.com.cn)

Telephone: +86-20-81628678 Fax: +86-20-81628809

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### Abstract

**AIM:** To investigate the effect of Mirtazapine on tumor growth, food intake, body weight, and nutritional status in gemcitabine-induced mild cachexia.

**METHODS:** Fourteen mice with subcutaneous xenografts of a pancreatic cancer cell line (SW1990) were randomly divided into Mirtazapine and control groups. Either Mirtazapine (10 mg/kg) or saline solution was orally fed to the mice every day after tumor implantation. A model of mild cachexia was then established in both groups by intraperitoneal injection of Gemcitabine (50 mg/kg) 10 d, 13 d, and 16 d after tumor implantation. Tumor size, food intake, body weight, and nutritional status were measured during the experiment. All mice were sacrificed at day 28.

**RESULTS:** (1) After 7 d of gemcitabine administration, body-weight losses of 5%-7% which suggested mild cachexia were measured; (2) No significant difference in tumor size was detected between the Mirtazapine and control groups ( $P > 0.05$ ); and (3) During the entire experimental period, food intake and body weight were

slightly greater for the Mirtazapine group compared with controls (although these differences were not statistically significant). After 21 d, mice in the Mirtazapine group consumed significantly more food than control mice ( $3.95 \pm 0.14$  g vs  $3.54 \pm 0.10$  g,  $P = 0.004$ ). After 25 d, mice in the Mirtazapine group were also significantly heavier than control mice ( $17.24 \pm 0.53$  g vs  $18.05 \pm 0.68$  g,  $P = 0.014$ ).

**CONCLUSION:** Mild cachexia model was successfully established by gemcitabine in pancreatic tumor-bearing mice. Mirtazapine can improve gemcitabine-induced mild cachexia in pancreatic tumor-bearing mice. It was believed to provide a potential therapeutic perspective for further studies on cachexia.

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**Key words:** Pancreatic carcinoma; Cachexia; Mirtazapine; Gemcitabine; Antidepressant

**Peer reviewers:** Ji Kon Ryu, Professor, Department of Internal Medicine, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-744, South Korea; Dr. Joseph Cullen, University of Iowa, Iowa City, IO 52242, United States; Shoichiro Sumi, Associate Professor, Department of Organ Reconstruction, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin-Kawara-cho, Sakyo-ku, Kyoto 606-8507, Japan

Jiang SM, Wu JH, Jia L. Intervention of Mirtazapine on gemcitabine-induced mild cachexia in nude mice with pancreatic carcinoma xenografts. *World J Gastroenterol* 2012; 18(22): 2867-2871 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2867.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2867>

### INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies as the overall survival rate remains 4% for all stages and

ances<sup>[1]</sup>. Clinical studies have established gemcitabine as the standard treatment for advanced pancreatic cancer. These studies have demonstrated significant clinical benefits (including improved survival) from gemcitabine<sup>[2-4]</sup>. Unfortunately, there are serious side effects associated with this anti-cancer drug that can adversely affect the patient's quality of life. These include nausea, vomiting, dyspepsia, weight loss, and cachexia.

Cachexia is characterized by major metabolic abnormalities and maladaptations. Often, food/energy intake is reduced, resting energy expenditure is increased, and catabolism is accelerated<sup>[5]</sup>. Cachexia is associated with anorexia, fat- and muscle-tissue wasting, and a progressive deterioration in the quality of life<sup>[6]</sup>. As many as 80% of all patients with cancer develop cachexia before death, and in over 20% of these patients, cachexia is the primary cause of death<sup>[7-9]</sup>. For patients with advanced-stage cancer, 80% suffer from cancer-associated anorexia/cachexia syndrome<sup>[10]</sup>.

Mirtazapine represents a new class of antidepressant drugs. It is a noradrenergic and specific serotonergic antidepressant, which stimulates 5-hydroxytryptamine (5-HT) 1 receptors, but blocks serotonin 5-HT<sub>2/3</sub> and histamine H<sub>1</sub> receptors<sup>[11]</sup>. We have previously shown that 78% of patients with pancreatic cancer also suffer from clinical depression<sup>[12]</sup>. This figure jumps to 92.3% for patients undergoing chemotherapy. Mirtazapine has the potential to increase appetite and stimulate body-weight gains in these patients<sup>[13-15]</sup>. Using a mouse model for pancreatic cancer, we previously demonstrated that Mirtazapine increases food intake, enhances body weight, and improves the nutritional status of mice with cancer<sup>[16]</sup>.

A 5% loss of body mass suggests an advanced to mild form of cachexia, whereas losses > 10% suggest severe cachexia<sup>[17,18]</sup>. Early interventions that treat mild cachexia often lead to substantial quality-of-life benefits for cancer patients. For the study reported herein, we therefore established a mouse model of chemotherapy-induced mild cachexia using gemcitabine and measured the effects of Mirtazapine monotherapy on tumor growth, food intake, body weight, and general nutritional status. These experiments were performed using nude mice implanted with human pancreatic cancer cells.

## MATERIALS AND METHODS

### Drugs and reagents

Mirtazapine was kindly provided by Organon (Oss, The Netherlands). Gemcitabine was purchased from Eli Lilly and Co. (Indianapolis, IN, United States). RPMI1640 and fetal bovine serum were purchased from Gibco (Grand Island, NY, United States).

### Animals

BALB/c *nu/nu* mice were purchased from the Experimental Animal Center, Guangzhou University of Chinese Medicine. Approval for these studies was acquired from the animal-care committee (license number: SCXK 2008-0020). Mice were bred and maintained under patho-

gen-free conditions in the Animal Center of Sun Yat-Sen University. Mice were housed 4-5 per cage under standard conditions, i.e., 22 ± 1 °C, ad libitum access to water and standard rat chow, and a 12-h light/dark cycle. Experiments were performed using mice that were 4-6 wk old and 20-22 g.

### Pancreatic cancer cell lines and culture conditions

The human pancreatic cancer cell line SW1990 was the kind gift of the Second Affiliated Hospital of Sun Yat-Sen University. Cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). Monolayer cultures were maintained in culture flasks and incubated under 50 mL/L CO<sub>2</sub> and 950 mL/L O<sub>2</sub> at 37 °C. Trypsinization was stopped with medium that contained 10% FBS. Cells were then washed once in serum-free medium and resuspended in Hanks' balanced salt solution. Only single-cell suspensions that displayed greater than 90% viability were used for injections.

### Establishment of a chemotherapy-induced cachexia model using gemcitabine

The subcutaneous pancreatic cancer model was established using the methods of Jia *et al.*<sup>[19]</sup> with slight modifications. To produce the SW1990 tumor, 3 × 10<sup>6</sup> cells (in 0.2 mL) were inoculated subcutaneously into the right flank of each nude mouse. Tumor sizes were measured *via* calliper. When the subcutaneous solid tumors reached approximately 1 cm in diameter, they were aseptically removed from the donor animals. Macroscopically visible necrotic tissue was cut away, and the remaining healthy tumor tissue was cut with scissors into pieces that were approximately 1 mm<sup>3</sup>. The tumor pieces were placed into Hanks' balanced salt solution that contained 100 units/mL penicillin and 100 mg/mL streptomycin. A small incision was then made through the right dorsal flank of each nude mouse and a piece of tumor was implanted beneath its skin. Chemotherapy-induced mild cachexia was established by intraperitoneal injection of 50 mg/kg gemcitabine on days 10, 13, and 16 after tumor implantation.

### Mirtazapine administration

Fourteen mice were randomly assigned to either the Mirtazapine or the control group (7 mice per group). After tumor implantation, Mirtazapine (10 mg/kg) was orally fed to the Mirtazapine group once per day. Normal saline solution was fed to the control group. Chemotherapy-induced mild cachexia was then established in both groups, as described above. Animals were sacrificed after 28 d.

### Measurements

The transplanted tumor sizes were measured using a Vernier caliper every fourth day, and tumor volume (*V*) was calculated as:  $V = w^2 \times l / 2$ , where *w* is the width and *l* is the length of the tumor<sup>[20]</sup>. Mice were sacrificed on day 28 after tumor transplantation, and the tumors were then removed. Both the tumors and the mice carcasses were then weighed. During the experiment, body weight was

Table 1 Effect of Mirtazapine on body weight and nutritional status

Nutritional status	Group	Baseline	5 d	9 d	13 d	17 d	21 d	25 d	28 d
Body weight (g)	Control	21.64 ± 0.96	21.51 ± 0.89	21.20 ± 0.73	20.86 ± 0.71	20.15 ± 0.67	18.70 ± 0.58	17.24 ± 0.53	16.04 ± 0.66
	Mirtazapine	21.75 ± 0.75	21.69 ± 0.79	21.59 ± 0.75	21.14 ± 0.52	20.50 ± 0.50	19.13 ± 0.55	18.05 ± 0.68	16.89 ± 0.73
	<i>P</i> value	0.783	0.654	0.282	0.386	0.266	0.151	0.014	0.017
Subcutaneous fat (mm)	Control	0.74 ± 0.14	0.72 ± 0.14	0.70 ± 0.13	0.69 ± 0.09	0.66 ± 0.09	0.60 ± 0.10	0.50 ± 0.10	0.41 ± 0.07
	Mirtazapine	0.71 ± 0.12	0.70 ± 0.11	0.70 ± 0.10	0.69 ± 0.10	0.68 ± 0.10	0.63 ± 0.10	0.52 ± 0.09	0.44 ± 0.08
	<i>P</i> value	0.691	0.773	0.928	0.938	0.845	0.697	0.708	0.548
Arm circumference (mm)	Control	19.45 ± 1.21	19.03 ± 0.81	18.29 ± 0.88	17.24 ± 0.52	16.00 ± 0.62	15.13 ± 0.68	13.76 ± 0.65	13.06 ± 0.52
	Mirtazapine	19.52 ± 0.88	19.20 ± 0.73	18.51 ± 0.65	17.58 ± 0.67	16.27 ± 0.67	15.31 ± 0.68	14.04 ± 0.54	13.25 ± 0.53
	<i>P</i> value	0.901	0.694	0.619	0.323	0.482	0.621	0.424	0.566

Table 2 Effects of Mirtazapine on tumor size, pancreatic tumor weight and food intake

Effects on tumor size (mL)							
Group	5 d	9 d	13 d	17 d	21 d	25 d	28 d
Control group	37.67 ± 7.57	62.59 ± 24.06	105.39 ± 19.92	174.77 ± 15.16	258.54 ± 20.98	365.58 ± 17.63	472.62 ± 13.03
Mirtazapine group	37.12 ± 10.55	58.09 ± 26.00	100.21 ± 21.87	171.57 ± 16.94	246.88 ± 25.64	357.80 ± 15.75	466.95 ± 14.21
<i>P</i> value	0.926	0.748	0.638	0.726	0.376	0.411	0.454
Effects on pancreatic tumor weight (g)							
Group	Mice body weight (Tumor-bearing) (g)		Mice body weight (Tumor removed) (g)		Tumor weight (g)	<i>P</i> value	
Control group	16.04 ± 0.66		15.61 ± 0.59		0.42 ± 0.09	0.35	
Mirtazapine group	16.89 ± 0.73		16.51 ± 0.63		0.37 ± 0.11		
Effects on food intake							
Group	Basic line	7 d	14 d	21 d	28 d		
Control group	5.15 ± 0.12	5.13 ± 0.13	4.21 ± 0.10	3.54 ± 0.10	3.02 ± 0.16		
Mirtazapine group	5.17 ± 0.15	5.19 ± 0.14	4.41 ± 0.16	3.95 ± 0.14	3.57 ± 0.11		
<i>P</i> value	0.917	0.544	0.054	0.004	0.003		

measured every fourth day. Food intake was expressed as daily consumption in grams per animal weekly. The rate of weight loss was calculated as: weight loss (%) =  $(1 - \text{body weight}_{\text{timepoint}} / \text{body weight}_{\text{base-line}}) \times 100\%$ . A weight loss > 5% suggested the development of mild cachexia<sup>[17,18]</sup>. Abdominal skin-fold and arm diameter were measured using the caliper every third day. Subcutaneous fat was calculated as: subcutaneous fat (mm) = skin-fold thickness × 0.5. Arm circumference was calculated as: arm circumference (mm) = diameter × 3.14.

### Statistical analysis

Statistical analyses were performed using SPSS 13.0 for Windows. Data were expressed as mean ± SD, and were compared using one-way analysis of variance and the Student-Newman-Keuls test for multiple comparisons between groups. Tumor inhibition rates were compared using the  $\chi^2$  test. Differences were considered statistically significant for  $P < 0.05$  using two-tailed tests.

## RESULTS

### Establishment of a model for chemotherapy-induced mild cachexia using gemcitabine

Seven days after the first gemcitabine injection, body weight in the control group had declined from 21.64 ± 0.96 g to 20.15 ± 0.67 g (a 6.89% decrease) (Table 1). A similar decline was measured for the Mirtazapine group: 21.75 ± 0.75 g to 20.50 ± 0.50 g (a 5.75% decrease). A mild form of cachexia had therefore been established, indicating that the administration of gemcitabine (50

mg/kg) for 1 wk could induce mild cachexia in mice that carried a pancreatic tumor.

### Effect of Mirtazapine on tumor growth

As the experiment progressed, small increases in the sizes of the tumors were regularly measured (Table 2). Similar rates of tumor growth were evident in both the Mirtazapine and control groups, however, statistically significant differences in tumor size were never detected between the two groups.

Tumor weight was measured at 28 d, immediately after the mice were sacrificed. Again, a statistically significant difference between the Mirtazapine and control groups concerning tumor weight was not detected ( $0.37 \pm 0.11$  g *vs*  $0.42 \pm 0.09$  g,  $P > 0.05$ ). It was indicated that gemcitabine had inhibitory effect on pancreatic cancer growth, which could not be apparently strengthened by Mirtazapine.

### Effect of Mirtazapine on daily food intake

Following the administration of gemcitabine, daily food intake gradually declined in both groups (Table 2). By day 21 of the experiment, however, mice given Mirtazapine were eating more food than did the controls ( $3.54 \pm 0.10$  g *vs*  $3.95 \pm 0.14$  g,  $P < 0.01$ ). This effect was also seen at the end of the experiment (day 28) ( $3.02 \pm 0.16$  g *vs*  $3.57 \pm 0.11$  g,  $P < 0.01$ ), demonstrating that Mirtazapine can slow the reduction in food intake caused by chemotherapy.

### Effect of Mirtazapine on body weight and nutritional status

At the beginning of the study, mice from the two groups



had similar average body weights ( $P > 0.05$ ). Throughout the course of the experiment, mice in both groups exhibited a gradual decrease in body weight (Table 1). During initial stages of the experiment, the control group seemed to lose slightly more weight than did the Mirtazapine group, although these differences were not statistically significant ( $P > 0.05$ ). At day 25, however, mice fed Mirtazapine were significantly heavier than control mice ( $18.05 \pm 0.68$  g *vs*  $17.24 \pm 0.53$  g,  $P = 0.014$ ). This phenomenon was also seen at 28 d suggesting that early Mirtazapine interventions can ameliorate the weight loss that is typically associated with chemotherapy (e.g., gemcitabine).

Subcutaneous fat and arm circumference were also measured for the two groups of mice (Table 1). For both groups these measured parameters gradually decreased during the course of the experiment. The data suggest that slower reductions were taking place in the Mirtazapine group (compared with the control group), but statistically significant differences were not detected ( $P > 0.05$ ).

## DISCUSSION

Cachexia is a disease process that develops in numerous chronic and end-stage pathologies. Clinical manifestations of cachexia include weight loss, anorexia, fatigue, muscle wasting, aesthesia, anemia, and edema. Particularly strong correlations between cachexia and solid tumors of the upper gastrointestinal tract have been described. It is estimated that 83% of pancreatic cancer patients suffer from cachexia during the course of their disease<sup>[17]</sup>. In addition, patients with pancreatic cancer have the highest incidence of weight loss (83%-87%), with about 30% reporting a weight loss of  $> 10\%$ <sup>[21,22]</sup>.

To experimentally dissect cachexia, a variety of cachexia models have been established. Murine colon-26 adenocarcinoma cells, Yoshida ascites hepatoma (AH-130) ascites hepatoma cells, and several other cachexigenic cell lines (JHU012, JHU022, and MAC1) have been used to establish different cachexia models<sup>[23-25]</sup>. The administration of chemotherapy drugs, however, has only rarely been used to generate mild cachexia. Gemcitabine is an extremely effective chemotherapy agent that inhibits the growth of cancerous tumors. Unfortunately, this drug is also associated with a number of adverse side effects, which include nausea, vomiting, loss of appetite, weight loss, and cachexia<sup>[26,27]</sup>. For the study reported herein, we injected nude mice with Gemcitabine to induce cachexia. Significant weight loss was observed in these animals, suggesting that a model for mild cachexia had been established.

Potential treatments for cancer cachexia (e.g., megestrol acetate, testosterone, growth hormone, or ghrelin) have been the subject of intense research recently. One study suggested that progestogens (e.g., megestrol acetate, or medroxyprogesterone) should be preferentially used to treat anorexia in patients with cancer because of toxic side effects associated with corticosteroids<sup>[17]</sup>. Several studies have demonstrated that ghrelin, which is a peptide found in both the brain and gut and stimulates food intake, may ameliorate cancer cachexia<sup>[9,28]</sup>. To date, however, there are

no consistent opinions or guidelines that support the use of one therapeutic agent over another.

Mirtazapine represents a novel antidepressant and has been shown to cure insomnia, depression, and anxiety, but it is unclear if it also stimulates appetite and improves the nutritional status of patients<sup>[29-32]</sup>. We have previously shown that a 6-wk administration of Mirtazapine in a murine model of pancreatic cancer increased food intake and body weight by 16.39% and 8.39%, respectively. These nutritional improvements were significantly better than was seen with other antidepressants<sup>[16]</sup>. Our previous work had also shown that gemcitabine induces cachexia. In the current study, therefore, we investigated whether an early Mirtazapine intervention could ameliorate the mild cachexia associated with gemcitabine (50 mg/kg) administration in mice that bear pancreatic tumors. Mirtazapine significantly improved both food intake and body weight of these mice, although losses in subcutaneous fat and skeletal muscle were not slowed. The reason for these fat and muscle losses may be that Mirtazapine does not affect lipolysis or the adenosine triphosphate/ubiquitin/proteasome system, both of which are upregulated in cachexia. In the future, therefore, it will be important to determine the dose-effect relationship between Mirtazapine and chemotherapy-induced cachexia. We will also test whether enhanced nutritional improvements can be obtained *via* combinatorial treatments that include Mirtazapine and megestrol acetate or ghrelin.

In summary, for the first time, chemotherapy-induced mild cachexia has been established in a murine model of pancreatic cancer using gemcitabine. In addition, our results demonstrate that early administration of Mirtazapine may represent an effective treatment for both chemotherapy- and cancer-related cachexia. Future experiments that use larger groups of animals and long-term courses of treatment will be necessary to confirm these findings. Clinical tests in cancer patients are also needed.

## COMMENTS

### Background

Gemcitabine is commonly used to treat pancreatic cancer. This anti-cancer drug, however, is often associated with side effects (e.g., nausea, vomiting, dyspepsia, weight loss, and cachexia) that adversely affect the patient's quality of life. Mirtazapine may increase appetite and weight gain, thereby ameliorating cachexia in this context.

### Research frontiers

Mirtazapine can significantly increase the food intake, enhance the body weight and improve the nutritional state in a pancreatic cancer mouse model in the authors' previous researches. In this study, the authors do the advanced research to find whether Mirtazapine could also show positive effects on improvement of appetite loss and weight loss which were the main features of cachexia.

### Innovations and breakthroughs

The research shows that Mirtazapine improved gemcitabine-induced mild cachexia in mice that bear pancreatic tumors.

### Applications

Mirtazapine represents a new class of antidepressant that may also positively affect appetite and weight gain. These results suggest novel therapeutic applications for Mirtazapine, and will help direct future studies concerning cachexia.

### Peer review

This is an interesting research that the authors utilized gemcitabine to induce

mild cachexia in mice with pancreatic tumors and found that Mirtazapine improved the symptoms associated with mild cachexia. These findings identify a novel means of treating cachexia, although additional experiments, both in the lab and in the clinic, are necessary before this strategy can be widely applied to cancer patients.

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## Bleeding duodenal hemangioma: Morphological changes and endoscopic mucosal resection

Noriko Nishiyama, Hirohito Mori, Hideki Kobara, Shintarou Fujihara, Takako Nomura, Mitsuyoshi Kobayashi, Tsutomu Masaki

Noriko Nishiyama, Hirohito Mori, Hideki Kobara, Shintarou Fujihara, Takako Nomura, Mitsuyoshi Kobayashi, Tsutomu Masaki, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa Medical University, 1750-1 Miki, Kita, Kagawa 761-0793, Japan

**Author contributions:** Nishiyama N led the study and wrote the manuscript, and Mori H, Kobara H, Fujihara S, Nomura T, Kobayashi M and Masaki T researched the case reports; all of the authors contributed significantly and have read and approved the final version of the manuscript.

**Correspondence to:** Noriko Nishiyama, MD, Department of Gastroenterology and Neurology, Faculty of Medicine, Kagawa Medical University, 1750-1 Miki, Kita, Kagawa 761-0793, Japan. [n-nori@med.kagawa-u.ac.jp](mailto:n-nori@med.kagawa-u.ac.jp)

Telephone: +81-87-8912156 Fax: +81-87-8912158

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### Abstract

Recently, the development of endoscopic procedures has increased the availability of minimally invasive treatments; however, there have been few case reports of duodenal hemangioma treated by endoscopic mucosal resection. The present report describes a case of duodenal hemangioma that showed various endoscopic changes over time and was treated by endoscopic mucosal resection. An 80-year-old woman presented with tarry stools and a loss of appetite. An examination of her blood revealed severe anemia, and her hemoglobin level was 4.2 g/dL. An emergency upper gastrointestinal endoscopy was performed. A red, protrusive, semipedunculated tumor (approximately 20 mm in diameter) with spontaneous bleeding on its surface was found in the superior duodenal angle. Given the semipedunculated appearance of the tumor, it was suspected to be an epithelial tumor with a differential diagnosis of hyperplastic polyp. The biopsy results suggested a telangiectatic hemangioma. Because this le-

sion was considered to be responsible for her anemia, endoscopic mucosal resection was performed for diagnostic and treatment purposes after informed consent was obtained. A histopathological examination of the resected specimen revealed dilated and proliferated capillary lumens of various sizes, which confirmed the final diagnosis of duodenal hemangioma. Neither anemia nor tumor recurrence has been observed since the endoscopic mucosal resection (approximately 1 year). Duodenal hemangiomas can be treated endoscopically provided that sufficient consideration is given to all of the possible treatment strategies. Interestingly, duodenal hemangiomas show morphological changes that are influenced by various factors, such as mechanical stimuli.

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**Key words:** Duodenal hemangioma; Endoscopic mucosal resection; Gastrointestinal bleeding; Morphological changes; Capillary hemangioma

**Peer reviewers:** Naoki Ishii, MD, Department of Gastroenterology, St. Luke's In, 9-1 Akashi-cho, Chuo-ku, Tokyo 104-8560, Japan; Dr. Peter Draganov, MD, University of Florida, 1600 SW Archer Rd., Room HD 602, PO Box 100214, Gainesville, FL 32610, United States

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### INTRODUCTION

Vascular lesions of the duodenum, including hemangiomas, are rare causes of gastrointestinal bleeding. Indeed,

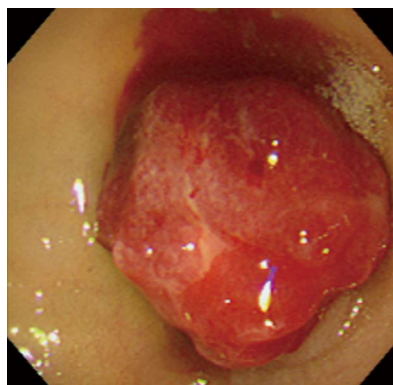


there have been very few reports of hemangioma in the duodenum. Endoscopically, hemangiomas have various morphological features. The present case is the first report of a duodenal hemangioma that showed various morphological features at the time of endoscopy. Although duodenal hemangioma is treated by surgical duodenectomy, the use of less-invasive procedures, such as endoscopic mucosal resection (EMR), has increased. The present study found that EMR is an effective treatment for hemorrhagic duodenal hemangioma. In addition, the present study summarized previous case reports of duodenal hemangioma.

## CASE REPORT

An 80-year-old woman presented with tarry stools and a loss of appetite. She had undergone laparoscopic cholecystectomy for acute cholecystitis when she was in her seventies. She had experienced a loss of appetite and anemic symptoms since approximately March 10, 2008, but she did not seek medical attention. She noticed a tarry stool on March 15 and experienced pallor of the face and astasia on March 18, which was the day she presented at the emergency department of Sakaide municipal hospital. The physical findings upon admission included a body height of 148 cm, a body weight of 45 kg, clear sensorium, a body temperature of 36.6 °C, a blood pressure of 116/56 mmHg, a pulse rate of 84 bpm, and an oxygen saturation of 100%. The palpebral conjunctiva was noted to be anemic. The abdomen was flat and soft with no spontaneous pain or tenderness. An examination of the blood at the time of admission revealed significant anemia with a hemoglobin level of 4.2 g/dL. In addition, her albumin level was decreased to 3.3 g/dL. Platelet counts, coagulation tests, and renal/hepatic function tests were all normal. An emergency upper endoscopy was performed on admission, but no abnormalities were found in the esophagus or the stomach. A red, semipedunculated tumor (20 mm in diameter) with spontaneous bleeding was found in the superior duodenal angle (SDA) (Figure 1). This patient had received an upper endoscopy at another hospital 9 mo earlier and had undergone biopsy after a blue, nonpedunculated, submucosal tumor-like lesion was found in SDA (Figure 2). Because there was no active bleeding during the endoscopic observation, biopsies were obtained from the basal and apical portions of the protruding lesion for diagnostic purposes. Although the pathological examination of the biopsy samples only identified necrotic material in the basal sample, we observed a dense proliferation of capillaries in the apical sample, which resulted in a diagnosis of suspected capillary hemangioma. Contrast-enhanced computed tomography (CT) revealed a slightly enhanced tumor (approximately 20 mm in diameter) in the SDA. Interestingly, we did not observe the presence of thick supplying blood vessels or an extramural extension of the tumor.

Based on the upper endoscopic findings and the



**Figure 1** A red protruding lesion (20 mm in diameter) with spontaneous bleeding was found in the superior duodenal angle.



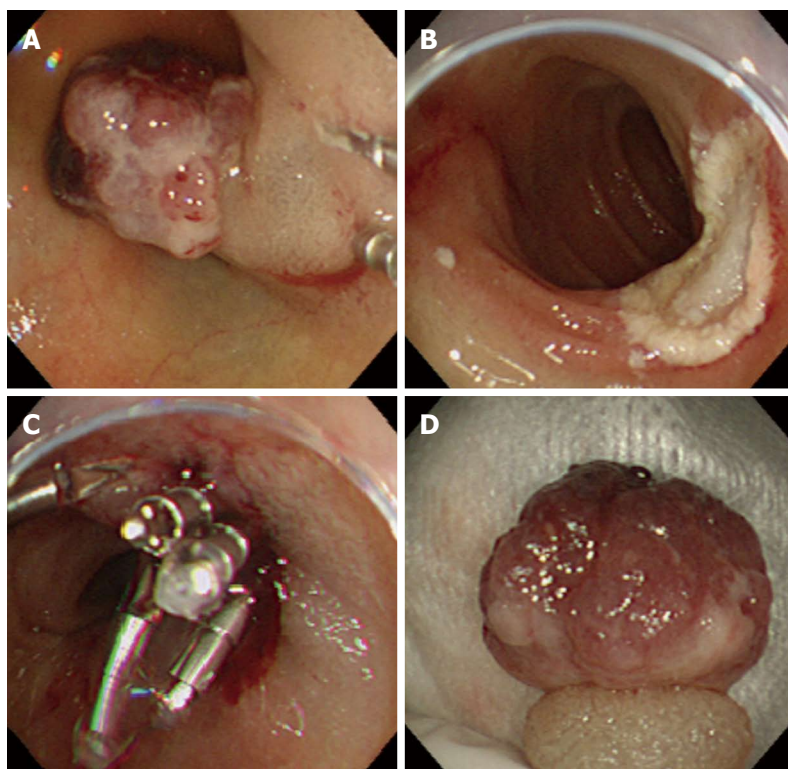
**Figure 2** A blue, nonpedunculated, submucosal tumor-like lesion. The tumor is almost entirely covered by mucosa.

results of the pathological examination, a duodenal hemangioma was considered to be the source of the bleeding. With conservative therapy, the hemoglobin level increased to 7 g/dL, and hemostasis was achieved.

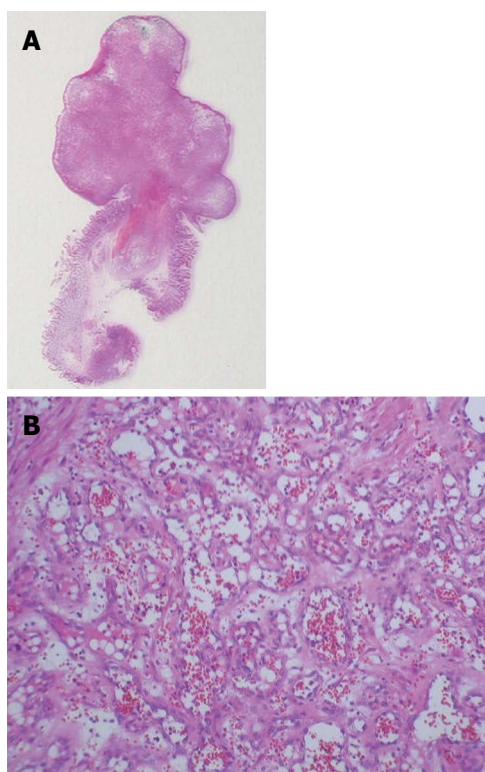
Because of the possibility of recurrent bleeding, EMR was performed to remove the hemangioma (Figure 3A-D). The top of the tumor was semipedunculated and irregular. First, the endothelial tumor was distinguished, but the biopsy showed the presence of a hemangioma, and the CT revealed an absence of thick supplying blood vessels. Additionally, the patient was of advanced aged and suffered from dementia. Also, the patient's family did not want her to undergo surgery. Moreover, the duodenal mucosa was sufficiently elevated following a local injection of saline solution; thus, EMR was performed. If histology of the resected specimen had revealed malignant potential, then surgery would have been performed.

We performed another upper endoscopy 3 d after the EMR, and hemostasis was confirmed. The patient had a favorable postoperative course and was discharged from the hospital without complications 1 wk after the EMR. Histopathological examination revealed the proliferation of endothelial cells lining the lumens of capillaries of various sizes and confirmed the diagnosis of capillary hemangioma (Figure 4A and B).





**Figure 3 Duodenal hemangioma and endoscopic mucosal resection.** A: EMR was performed after the mucosa was sufficiently elevated by local injection; B: The mucosal defect; C: The mucosal defect was closed using the clipping technique, and the procedure was completed; D: A resected specimen obtained by EMR. EMR: Endoscopic mucosal resection.



**Figure 4 Pathological specimen.** A: A magnified pathological image of the resected specimen (10 ×); B: A magnified pathological image of the central part of the 20 mm × 12 mm tumor, which shows proliferation of various sized capillary lumens (100 ×).

## DISCUSSION

Hemangiomas only account for approximately 0.3% of all gastrointestinal tumors<sup>[1]</sup>. A search for Japanese and foreign case reports of duodenal hemangioma between 1978 and 2008 through the Japana Centra Revuo Medicina and MEDLINE only identified 22 reports, including the present case, which indicated a very low incidence of the disease (Table 1)<sup>[2-12]</sup>. We conducted a clinicopathological review of the identified Japanese case reports, including the present case. The mean age of the patients was 55 years, and the ages ranged from 2 years to 85 years. The chief complaints in the Japanese cases included melena/anemic symptoms in 82% (14 of 17) of the cases and obstructive symptoms in 1 case. Interestingly, there were only 2 asymptomatic cases. Nader *et al.* reported that only 30% of hemangioma cases were asymptomatic. Gastrointestinal hemorrhage/anemia was observed in 73.2% of the hemangioma cases, and obstructive symptoms were observed in 12.8% of the cases<sup>[13]</sup>. Tumors were slightly more commonly in the descending portion (8 of 17 cases, 48%) of the duodenum compared with the other portions of the duodenum. In terms of size, cavernous hemangiomas tend to be larger than capillary hemangiomas (11-80 mm *vs* 8-20 mm, respectively). Interestingly, cavernous hemangiomas accounted for the majority of the reported hemangioma cases. Indeed, cavernous hemangioma was found in 11 cases, capillary hemangioma was observed in 4 cases, and mixed capillary hemangioma and multiple

Table 1 Summary of the case reports

Author	Year	Patient age	Chief complaint	Location in duodenum	size (mm)	Morphology	Pathology	Treatment
1 Koga	1978	65	Anemia	1-2nd	1 × 1	Unknown	Multiple hemangiomas	Surgical resection
2 Ikeda <i>et al</i> <sup>[6]</sup>	1980	33	Tarry stool, shock	2nd	Unknown	SMT-like	Cavernous	Surgical resection
3 Kawamura	1982	77	Anemia	3rd	80 × 30	SMT-like	Cavernous	Surgical resection
4 Furuya	1987	72	Tarry stool, anemia	2nd	19 × 14	SMT-like	Cavernous	Endoscopic polypectomy
5 Tadokoro	1991	55	Massive melena	2nd	10 × 14	SMT-like	Capillary	Endoscopic polypectomy
6 Amau	1991	20	Melena	2nd	Several lesions	SMT-like	Capillary	Local ethanol injection alone
7 Hata	1992	69	Hematemesis	1st	14 × 9	SMT-like	Cavernous	Surgical resection
8 Fujikawa <i>et al</i> <sup>[7]</sup>	1996	52	Anemia	4th	20 × 10	SMT-like	Cavernous	Surgical resection
9 Maeda <i>et al</i> <sup>[2]</sup>	2000	85	Anemia	3rd	11 × 8	SMT-like	Capillary/venous	Surgical resection
10 Terui	2002	7	Intestinal obstruction	3rd	11 × 7 × 3	SMT-like	Cavernous	Surgical resection
11 Inoue	2002	40	Health check-up	3rd	Unknown	SMT-like	Cavernous	Surgical resection
12 Oikawa	2005	53	Melena	3rd	30	SMT-like	Cavernous	Surgical resection
13 Taniguchi	2006	53	Melena	3rd	30	SMT-like	Cavernous	Surgical resection
14 Kakinuma <i>et al</i> <sup>[3]</sup>	2007	63	Melena	4th	50	SMT-like	Cavernous	Surgical resection
15 Yamashita <i>et al</i> <sup>[4]</sup>	2008	70	Tarry stool	3rd	20	SMT-like	Cavernous	Unknown
16 Sakamoto <i>et al</i> <sup>[5]</sup>	2008	54	Health check-up	3rd	15	SMT-like	Unknown	Follow-up
17 Nishiyama (present case)	2008	83	Tarry stool, shock	1st	20 × 12	Polypous	Capillary	Endoscopic mucosal resection
18 Bibao <i>et al</i> <sup>[8]</sup>	1989	61	Anemia	2nd	Unknown	SMT-like	Unknown	Embolization (catheter)
19 Lee <i>et al</i> <sup>[10]</sup>	1993	31	Melena	2nd	40	Unknown	Cavernous	Surgical resection
20 Chattopadhyay <i>et al</i> <sup>[11]</sup>	2002	2	Vomiting	4th	100	SMT-like	Unknown	Surgical resection
21 Devadason <i>et al</i> <sup>[12]</sup>	2007	4	None	1-2nd	Unknown	SMT-like	Capillary	Follow-up

SMT: Submucosal tumor.

phlebectasia were observed in 1 case.

Characteristic endoscopic findings included color ranging from blue to dark red and a structure that was easily deformed by compression. Although duodenal hemangiomas are morphologically classified as submucosal tumorlike, diffusely infiltrating, or polypous lesions, all of the reported cases except for the present case have been classified as submucosal tumor-like lesions. Interestingly, the present case had a polypous appearance.

Because hemangiomas show variable endoscopic findings in terms of size, shape, and color, examinations and pretreatment information are required for the diagnosis. These examinations include contrast-enhanced CT and magnetic resonance imaging to determine the location of the tumor and the blood stream, endoscopic ultrasonography to determine the extent of the lesion, selective angiography, and duodenal X-rays. The final diagnosis is generally made based on the results of the pathological examination of the resected specimens.

Kaijser *et al*<sup>[14]</sup> pathologically classified duodenal hemangiomas into 4 types: type I, multiple phlebectasia; type II, cavernous hemangioma of diffuse and localized polypous types; type III, simple capillary hemangioma; and type IV, angiomatosis with a gastrointestinal lesion. In terms of frequency, type II hemangiomas account for the majority (55%) of all cases, but type II and a mixture of types II and III are almost comparable in frequency. The present case was classified as a type II lesion of the localized polypous type.

Although duodenal hemangiomas are conventionally treated surgically (i.e., partial intestinal resection), less invasive treatment strategies have recently been reported, including endoscopic polypectomy, EMR, local ethanol injection therapy, catheter embolization therapy, and endoscopic sclerotherapy. Among the reported cases, surgery was performed in 11 cases, polypectomy was performed in 2 cases, and EMR was performed in 1 case (i.e., the present case). Although no formal criteria have been established for the indications of EMR, patients meeting the following three criteria are considered eligible for EMR: (1) patients with a lesion in the endoscopically accessible SDA or descending portion of the duodenum; (2) patients with a 25-mm or smaller polypous lesion classified as type II, III, or IV according to Yamada's classification<sup>[15]</sup>; and (3) patients without a large blood vessel in the lesion (determined using contrast-enhanced abdominal CT or abdominal angiography).

We chose EMR in the present case because the patient had a 20-mm polypous lesion in the SDA, which is an endoscopically accessible region, and we did not detect a large supplying vessel on the contrast-enhanced abdominal CT. Surgical treatment was likely selected in many of the previous cases because the lesions were typically in the descending portion of the duodenum. Because the surgical resection of a duodenal lesion tends to cause excessive surgical stress, endoscopic or catheter-based treatments may be considered as an option after careful consideration. It should be noted, however, that

duodenal blood vessels have thinner walls compared with blood vessels in other parts of the intestine. Furthermore, it is difficult to manipulate an endoscope in the duodenum, which has 2 curved sections. In addition, if a resected specimen falls into the anal side of the intestine, it may be difficult to retrieve. Thus, the indication for treatment should be carefully considered.

When the patient from the present study presented at our hospital, the tumor was exposed on the mucosal surface and had increased in size. Hemangiomas can grow into various shapes according to the influence of several factors, such as thrombi, fibrosis, calcification, infection, peristalsis, and mechanical stimuli. Although the literature search did not identify any case reports of duodenal hemangiomas characterized by increasing size, the lesion in the present case appeared to have initially developed as a submucosal tumor. Interestingly, mucosal bleeding and ulceration caused by mechanical stimuli, such as biopsy and contact with food, led to the tumor being exposed to the lumen and resulted in its deformation into a polypous lesion.

We experienced a rare case of duodenal hemangioma in the present patient, and duodenal hemangioma in general can be treated by an appropriately selected, less invasive treatment strategy. The present case provided a valuable experience that enabled us to understand the morphological changes that hemangiomas can display over time.

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## Duodenal variceal bleeding after balloon-occluded retrograde transverse obliteration: Treatment with transjugular intrahepatic portosystemic shunt

Min Joung Kim, Byoung Kuk Jang, Woo Jin Chung, Jae Seok Hwang, Young Hwan Kim

Min Joung Kim, Byoung Kuk Jang, Woo Jin Chung, Jae Seok Hwang, Department of Gastroenterology, Keimyung University School of Medicine, Dongsan Medical Center, 194 Dongsan-dong, Jung-gu, Daegu 700-712, South Korea

Young Hwan Kim, Department of Diagnostic Radiology, Keimyung University School of Medicine, Dongsan Medical Center, 194 Dongsan-dong, Jung-gu, Daegu 700-712, South Korea

Author contributions: Kim MJ and Jang BK contributed equally to this work; Hwang JS and Chung WJ provided clinical advice; Kim YH performed the procedure; Jang BK, Kim MJ and Kim YH designed the case report; and Kim MJ wrote the paper.

Correspondence to: Byoung Kuk Jang, MD, PhD, Department of Gastroenterology, Keimyung University School of Medicine, Dongsan Medical Center, 194 Dongsan-dong, Jung-gu, Daegu 700-712, South Korea. [jangha106@dsmc.or.kr](mailto:jangha106@dsmc.or.kr)

Telephone: +82-53-2507088 Fax: +82-53-2507088

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and complete obliteration of duodenal varices, but multinodular hepatocellular carcinoma had developed. He died of hepatic failure 28 mo after TIPS.

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**Key words:** Duodenal variceal bleeding; Balloon occluded retrograde transvenous obliteration; Transjugular intrahepatic portosystemic shunt

**Peer reviewer:** Valentin Hans Fuhrmann, Associate Professor, Internal Medicine 3, Department of Gastroenterology and Hepatology, Medical University Vienna, Waehringer Guertel 18-20, Vienna A-1090, Austria

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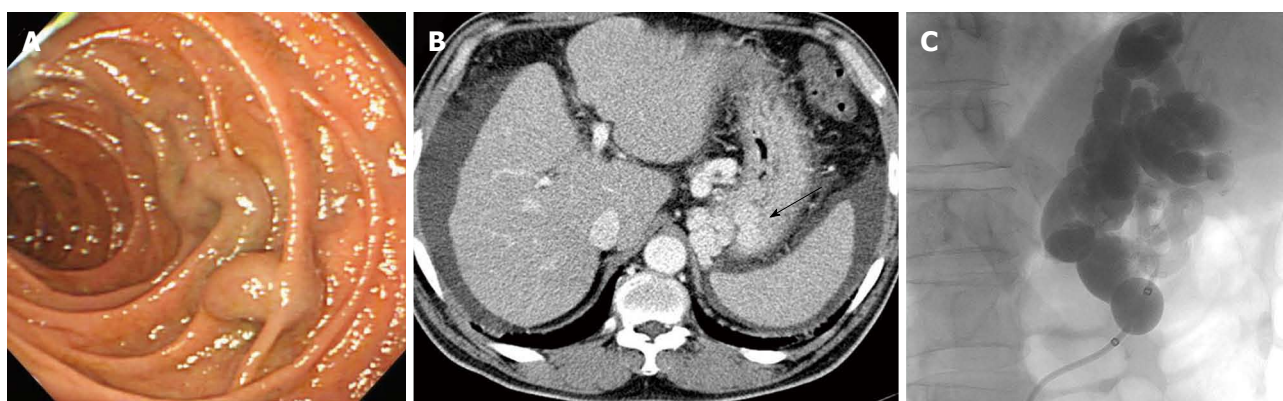
### Abstract

We report a case of duodenal varix bleeding as a long term complication of balloon occluded retrograde transvenous obliteration (BRTO), which was successfully treated with a transjugular intrahepatic portosystemic shunt (TIPS). A 57-year-old man was admitted to the emergency room suffering from melena. He had undergone BRTO to treat gastric varix bleeding 5 mo before admission. Endoscopy and a computed tomography (CT) scan showed complete obliteration of the gastric varix, but the nodular varices in the second portion of the duodenum expanded after BRTO, and spurting blood was seen. TIPS was performed for treatment of duodenal variceal bleeding, because attempts at endoscopic varix ligation were unsuccessful. The post-operative course was uneventful and the patient was discharged without complications. A follow up CT scan obtained 21 mo after TIPS revealed a patent TIPS tract

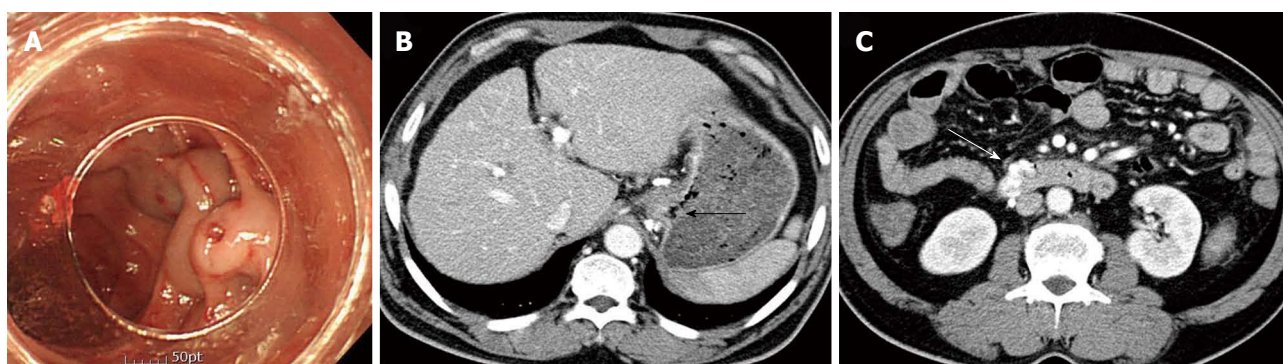
### INTRODUCTION

Balloon-occluded retrograde transvenous obliteration (BRTO) is minimally invasive and effective in patients with gastric variceal bleeding<sup>[1-3]</sup>. Although BRTO achieves excellent prevention of recurrent bleeding with few complications, its long-term efficacy and safety have not been fully evaluated. In particular, aggravation of esophageal varices and portal hypertension have been proposed as serious complications of BRTO. There have also been reports that BRTO may aggravate esophageal varices<sup>[2,6-8]</sup>. However, there are no reports concerning the deterioration of pre-existing duodenal varices by BRTO. Here, we describe our clinical experience of successfully treating bleeding duodenal varices with a transjugular-intrahepatic portosys-





**Figure 1** Endoscopic variceal ligation was performed to control variceal bleeding. A: Tiny nodular varices with no bleeding were found at the duodenum second portion; B: Enhanced computed tomography scan showing large gastric varices (arrow); C: Balloon occluded retrograde transvenous obliteration was successfully performed.



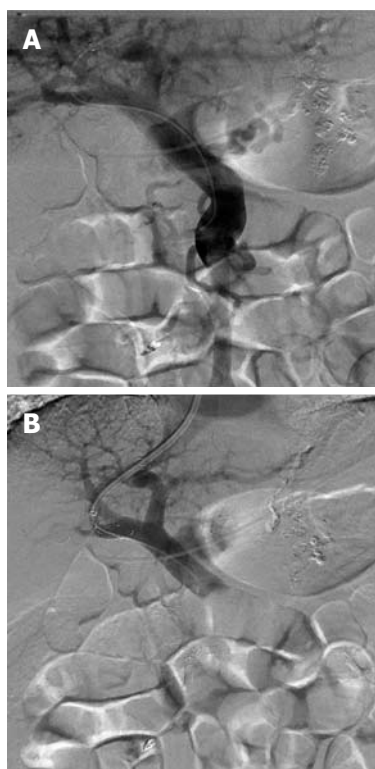
**Figure 2** An emergency endoscopic examination revealed a small, linear esophageal varices without evidence of recent bleeding. A: In the second portion of the duodenum, nodular varices expanded before balloon occluded retrograde transvenous obliteration and presented with hematocystic spots; B, C: Enhanced computed tomography scan obtained 5 mo after balloon occluded retrograde transvenous obliteration revealing complete obliteration of gastric varices (black arrow); however, duodenal varices were aggravated (white arrow).

temic shunt (TIPS) and transcatheter embolization.

## CASE REPORT

A 57-year-old man was admitted with a history of melena and intermittent hematochezia. He was hepatitis B virus-positive and had liver cirrhosis. Five mo before admission, an emergency endoscopic examination revealed gastric varices with active bleeding. Endoscopic variceal ligation (EVL) was performed to control variceal bleeding. A tiny nodular varix without bleeding was found in the second portion of the duodenum at that time (Figure 1A). Computed tomography (CT) showed dilated gastric varices with a gastroduodenal shunt (Figure 1B). BRTO was performed to prevent recurrent gastric variceal bleeding (Figure 1C). The patient did not have any further bleeding events until our examination. At the time of admission, his blood pressure was 112/56 mmHg, heart rate was 115/min, and respiratory rate was 24/min with symptomatic orthostatic hypotension. No abnormalities were noticed on cardiac and respiratory examinations, except tachycardia. Laboratory tests performed on admission revealed a hemoglobin of 5.9 g/dL, a hematocrit of 17.4%, and a thrombocyte count of  $1.6 \times 10^4$ /mL. The

total serum protein and albumin level were 5.5 g/dL and 2.7 g/dL, respectively. Other laboratory data included total bilirubin (0.7 mg/dL) and aspartate aminotransferase [AST: 321 IU/L (normal < 40 IU)]. An emergency endoscopic examination revealed a small, linear esophageal varix without evidence of recent bleeding. In the second portion of the duodenum, the nodular varices had expanded after BRTO and hematocystic spots with fresh blood were seen (Figure 2A). A CT scan showed complete obliteration of gastric varices, but the duodenal varices were aggravated (Figure 2B and C). EVL was done at that site, but the bleeding continued. Therefore, a TIPS was created according to standard procedures. Portography during the TIPS operation revealed a large duodenal varix originating from the superior mesenteric vein and draining into the gonadal vein (Figure 3A). The TIPS tract was created and the vein feeding the duodenal varix was embolized using three metallic coils. The portosystemic gradient was 30 mmHg, which decreased to 12 mmHg after TIPS, and portography showed the disappearance of duodenal variceal flow (Figure 3B). The postoperative course was uneventful. The patient was discharged without complications, and he remained in a stable condition. A follow-up CT scan obtained 21

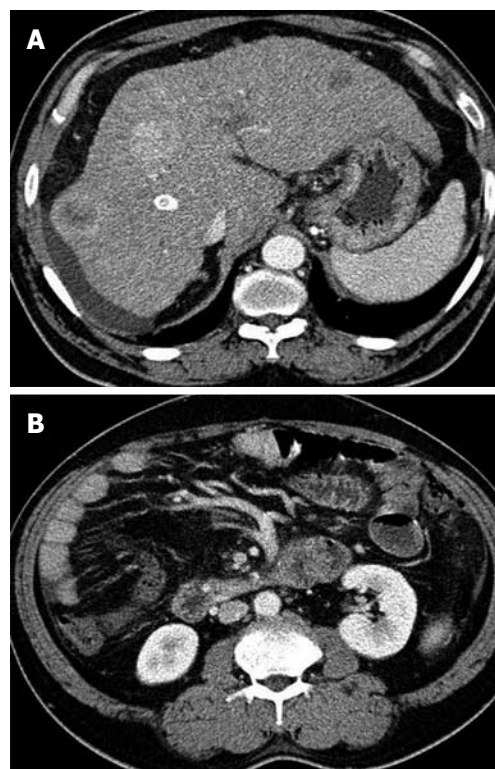


**Figure 3** Transjugular intrahepatic portosystemic shunt was created according to standard procedures. A: Portogram showing duodenal variceal flow; B: After transjugular intrahepatic portosystemic shunt and coil embolization of duodenal varices, variceal flow disappeared.

mo after TIPS revealed a patent TIPS tract and complete obliteration of duodenal varices; however, multinodular hepatocellular carcinoma had developed (Figure 4A and B). He died of hepatic failure 28 mo after TIPS.

## DISCUSSION

Interventional treatments for variceal bleeding can be roughly classified into two types: Shunt occlusion and shunt creation. BRTO is representative of shunt occlusion therapies, and has been widely performed in Korea and Japan since it was introduced by Kanakawa in the mid-1990s<sup>[4]</sup>. It is an interventional radiological technique designed specifically for the treatment of gastric fundal varices with gastorenal shunt. The principle of BRTO is to eradicate the gastric fundal varices by injecting sclerosant into the varices retrogradely with the balloon inflated in the draining veins, after insertion *via* the systemic circulation from the femoral or jugular vein. The sclerosant for BRTO is 5% ethanolamine oleate with iopamidol, which injures the endothelial cells of varicose veins and induces thrombosis in the varices. This technique controls gastric variceal bleeding effectively, with success rates of 76% to 92%<sup>[3]</sup>. Satisfactory results have been reported for managing variceal bleeding with improved liver function and hepatic encephalopathy<sup>[1,3,5,9,10]</sup>. Theoretically, shunt-obliterating therapies such as BRTO could increase portal blood flow and pressure, and thereafter



**Figure 4** A follow-up computed tomography scan was obtained 21 mo after transjugular intrahepatic portosystemic shunt. A, B: Enhanced computed tomography scan shows a still patent transjugular portosystemic shunt tract and complete improvement of duodenal varices. Note the appearance of multinodular hepatocellular carcinomas in the liver.

increase the bleeding risk from other varices, except sclerosed varices. Therefore, aggravation of esophageal varices is a serious complication of BRTO<sup>[2,6-8]</sup>. A long-term consequence of BRTO is the development or worsening of esophageal varices (EV) resulting from increased portal pressure, which has been reported in 10% to 66% of procedures. In a recent study, worsening of EV was observed after BRTO, with aggravation rates of 27%, 58%, and 66% at 1 years, 3 years, and 5 years, respectively<sup>[2]</sup>. However, shunt creation therapy, such as TIPS, can decompress the portal pressure, and therefore can be used in the treatment of most complications of portal hypertension, except hepatic encephalopathy. These include acute esophageal variceal bleeding refractory to medical therapy, recurrent esophageal variceal bleeding, gastric variceal bleeding, ectopic variceal bleeding, portal hypertensive gastropathy, Budd-Chiari syndrome, refractory ascites, refractory hepatic hydrothorax, hepatorenal syndrome, hypersplenism, and pancreatic arteriovenous malformation<sup>[11]</sup>. Nevertheless, TIPS has two main drawbacks. One is aggravation of hepatic encephalopathy and the other is deterioration of hepatic function. Therefore, doing both BRTO and TIPS should compensate for each other's weak points during treatment of variceal bleeding, because the two therapies are complementary.

Duodenal varices can develop in patients diagnosed with portal hypertension secondary to liver cirrhosis,

portal vein thrombosis, schistosomiasis, chronic pancreatitis, and, rarely, pancreatic cancer. Although the bleeding is often severe and fatal, no definitive treatments or guidelines for bleeding duodenal varices have been established<sup>[12,13]</sup>. Several studies of endoscopic therapies, such as band ligation (BL) or sclerotherapy, have reported variable success rates<sup>[13]</sup>. However, BL cannot ligate duodenal varices larger than 15 mm<sup>[12]</sup>. In addition, BL does not completely disrupt the blood flow within varices, and the potential for recurrent bleeding remains<sup>[12]</sup>. Sclerotherapy has technical difficulties, including a non-ideal approach to the vessels when varices are located in the second portion of the duodenum<sup>[13]</sup>.

The use of radiological interventions provides an alternative method for treating patients presenting with bleeding duodenal varices (DV). Several cases concerning the use of TIPS or BRTO for controlling duodenal variceal bleeding have been reported. Akazawa *et al.*<sup>[12]</sup> reported a case of duodenal variceal bleeding managed with BRTO. In our patient, the duodenal variceal bleeding caused by BRTO could not be managed endoscopically. As a result, we performed TIPS and transcatheter embolization. In conclusion, BRTO is believed to be effective for controlling gastric variceal bleeding. However, it may increase the risk of coexisting EV, DV, and ectopic variceal bleeding. Using TIPS in combination with embolization, we successfully treated a patient with DV bleeding.

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## Adenocarcinoma arising from intrahepatic heterotopic pancreas: A case report and literature review

Mao-Lin Yan, Yao-Dong Wang, Yi-Feng Tian, Ying Lin

Mao-Lin Yan, Yao-Dong Wang, Yi-Feng Tian, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Provincial Clinical College of Fujian Medical University, Fuzhou 350001, Fujian Province, China

Ying Lin, Department of Pathology, Fujian Provincial Hospital, Provincial Clinical College of Fujian Medical University, Fuzhou 350001, Fujian Province, China

**Author contributions:** Yan ML, Wang YD and Tian YF performed the surgery and took care of the patient; Yan ML wrote and revised manuscript; Yan ML and Lin Y organized the patient's data and figures; all authors have read and approved the manuscript.

**Correspondence to:** Yao-Dong Wang, MD, Department of Hepatobiliary Surgery, Fujian Provincial Hospital, Provincial Clinical College of Fujian Medical University, Fuzhou 350001, Fujian Province, China. [chenhuiwyd@yahoo.com.cn](mailto:chenhuiwyd@yahoo.com.cn)

Telephone: +86-591-87552554 Fax: +86-591-87552554

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Sant Pau, Av. Sant Antoni M Claret 167, 08025 Barcelona, Spain

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### INTRODUCTION

The heterotopic pancreas is defined as aberrant pancreatic tissue without vascular, neural or anatomic continuity with the normal pancreas<sup>[1]</sup>. The incidence of heterotopic pancreas in autopsy studies is approximately 0.5%-13.7%<sup>[2]</sup>. Heterotopic pancreatic tissues can be found anywhere in the gastrointestinal tract and the predilection site is the stomach, duodenum and jejunum<sup>[3]</sup>. Unusual sites include the biliary system and liver<sup>[4]</sup>. Carcinoma within heterotopic pancreatic tissues is rare, and about 31 well-documented cases have been reported in the literature<sup>[5]</sup>. We present a case with malignant transformation in heterotopic pancreas of the intra-hepatic biliary tract. To our knowledge, no case of adenocarcinoma arising in the intrahepatic heterotopic pancreas has been described previously.

### CASE REPORT

A 45-year-old woman who had been healthy underwent a routine medical check-up in our hospital. The physical examination was normal. Laboratory tests revealed elevated values of  $\gamma$  glutamyl transpeptidase, but normal aspartate aminotransferase and alanine aminotransferase. The tumor markers including  $\alpha$  fetoprotein, carcinoembryonic antigen, CA125 and CA-19-9 were all within normal ranges. Hepatitis virus markers were negative. Ultrasound showed the dilatation of the bile ducts of left

### Abstract

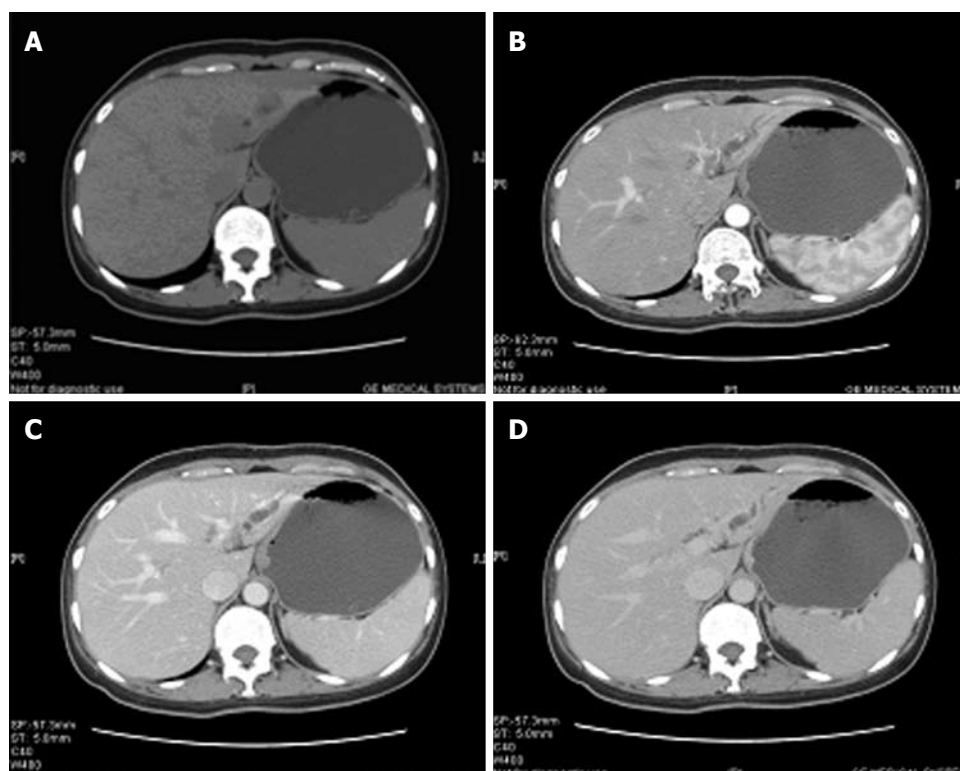
Heterotopic pancreas is mostly found incidentally, and adenocarcinoma arising from heterotopic pancreas appears to be extremely rare. A case of a 46-year-old woman with adenocarcinoma arising from intrahepatic heterotopic pancreas is reported herein. Computed tomography demonstrated a mass located in the bile duct of the left hepatic lobe. Pathological examination revealed a moderately differentiated adenocarcinoma arising from intrahepatic heterotopic pancreas with nerve infiltration. This may be the first reported case of adenocarcinoma arising from intrahepatic heterotopic pancreas.

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**Key words:** Heterotopic pancreas; Liver; Biliary tract; Adenocarcinoma

**Peer reviewer:** Dr. Antoni Farre, Hospital de la Santa Creu i





**Figure 1** Computed tomography scan shows the dilatation of the bile ducts of left hepatic lobe and a mass located in the bile duct of left hepatic lobe. A-D: Computed tomography scan of abdomen showing dilatation of the bile ducts of left hepatic lobe and a mass located in the bile duct of left hepatic lobe near the hepatic hilus.

hepatic lobe. Abdominal computed tomography (CT) scan revealed a mass located in the bile duct of left hepatic lobe near the hepatic hilus, which had no enhancement in arterial and portal venous, but clear enhancement in delayed phase (Figure 1). Dilatation of the bile ducts of left hepatic lobe was observed up to the periphery of the liver. CT scan and ultrasound failed to demonstrate any pancreatic lesion. A radical resection was performed under the clinical impression of cholangiocarcinoma. She underwent left hepatic and caudate lobectomy and resection of the gallbladder. Postoperative recovery was uneventful.

Gross examination revealed a firm, gray, nodular mass measuring 3.0 cm × 2.0 cm × 2.0 cm, which was located in the bile duct of left hepatic lobe near the hepatic hilus and had a uniform cut surface without hemorrhage or necrosis. The bile duct of left hepatic lobe was also dilated with a maximum circumference of 0.9 cm. Histologically, the pancreatic tissue consisted of acinar cells and centroacinar cells, and ductal elements were often situated around the acini (Figure 2A). The acinar cells contained eosinophilic granules (Figure 2B). The histological diagnosis was consistent with a moderately differentiated adenocarcinoma arising from intrahepatic heterotopic pancreas (Figure 2C and D).

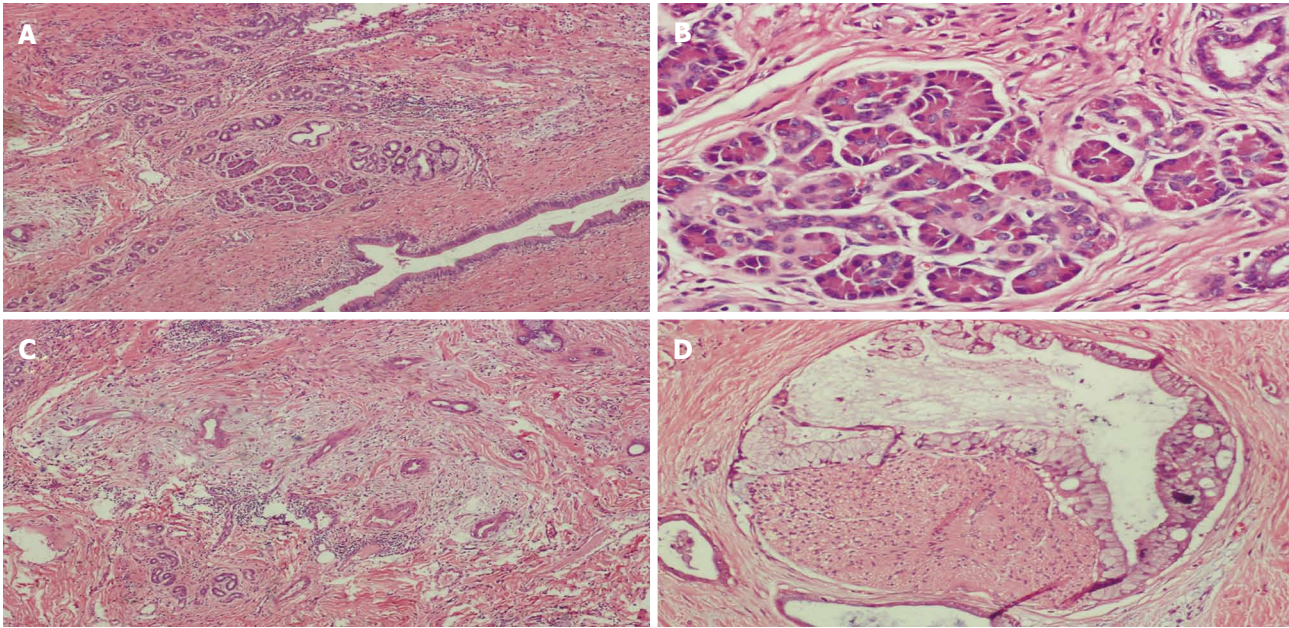
## DISCUSSION

Heterotopic pancreas was firstly described in 1727 by

Jean Schultz and lacked anatomic or vascular connection with the main body of the pancreas. Although pancreatic heterotopia can be found virtually anywhere along the gastrointestinal tract, the most frequent sites are the stomach (27.5%), duodenum (25.5%), and jejunum (15.9%)<sup>[3]</sup>. It is also observed less frequently in the esophagus, spleen, gallbladder, biliary tract, liver and lung. Up to now, only eight cases of intrahepatic heterotopic pancreas have been reported, including a case of insular carcinoma probably arising from intrahepatic heterotopic pancreas<sup>[6]</sup>, a case of hepatic and extrahepatic choledochal cysts<sup>[7]</sup>, a case presenting as hepatic mass<sup>[8]</sup>, a case of primary cholesterol hepatolithiasis<sup>[9,10]</sup>, a case found in autopsy liver specimens<sup>[11]</sup>, a case of cirrhosis<sup>[12]</sup>, and a case of Caroli's disease<sup>[13]</sup>.

In 1909, Heinrich *et al.*<sup>[14]</sup> classified heterotopic pancreas into three types. Type I is defined by the presence of ducts, acini and endocrine islets similar to those seen in normal pancreatic tissues. Type II contains a large number of acini, a few ducts and no islets. Type III is characterized by the presence of numerous ducts, a few acini, and no islets. The histological classification of the ectopic pancreatic tissue in the present case is type II, consisting of acini and a few ductal components.

Heterotopic pancreas is often found incidentally during surgery or endoscopy or clinically silent and benign. Malignant transformation in heterotopic pancreas is extremely rare. Thirty-one well-documented cases of carcinoma arising in a heterotopic pancreas were reviewed by



**Figure 2** Histopathology shows the heterotopic pancreas and ductal adenocarcinoma arising from heterotopic pancreas. A: Histopathology showing the pancreatic tissue consisting of acinar cells and ductal elements [hematoxylin and eosin (HE),  $\times 4$ ]; B: Histopathology showing the acinar cells containing eosinophilic granules (HE,  $\times 10$ ); C: The solid area showing ductal adenocarcinoma arising from heterotopic pancreas with nerve infiltration (HE,  $\times 4$ ); D: The solid area showing ductal adenocarcinoma arising from heterotopic pancreas with nerve infiltration (HE,  $\times 20$ ).

Goodarzi<sup>[5]</sup>. The results showed that most tumors in the heterotopic pancreatic tissues occurred in the stomach and most of them were adenocarcinomas. The present case is the first case of adenocarcinoma arising from the intrahepatic heterotopic pancreas. Guillou *et al.*<sup>[15]</sup> suggested that three criteria should be met for a carcinoma to be diagnosed as arising from the heterotopic pancreas. First, the tumor must be within or near the ectopic pancreatic tissue. Second, transition between pancreatic tissue and carcinoma must be observed. Third, the nonneoplastic pancreatic tissue should comprise acini, epithelial and ductal structures. The present case satisfied these three criteria.

A potential confusion is a primary intrahepatic cholangiocarcinoma with unrelated pancreatic heterotopia of the liver, which may happen if the heterotopic pancreas is completely replaced by the adenocarcinoma or if the presence of pancreatic tissue can not be confirmed due to sampling. Furthermore, primary intrahepatic cholangiocarcinoma is associated with intrahepatic bile duct stones and chronic infection in most cases. Zhang *et al.*<sup>[16]</sup> also demonstrated that the adenocarcinoma can arise from heterotopic pancreas. Extensive sampling of the specimen and a high index of suspicion for carcinoma arising from heterotopic pancreas can avoid this diagnostic confusion.

In conclusion, malignant transformation in heterotopic pancreas is extremely rare. Extensive sampling of the specimen and a high index of suspicion for carcinoma arising from heterotopic pancreas can provide accurate diagnosis.

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## Colorectal cancer screening behavior and willingness

Stefano Pontone

Stefano Pontone, Department of Surgical Sciences, "Sapienza" University of Rome, V.le Regina Elena 324, 00161 Rome, Italy  
Author contributions: Pontone S wrote this letter.

Correspondence to: Stefano Pontone, PhD, MD, Department of Surgical Sciences, "Sapienza" University of Rome, V.le Regina Elena 324, 00161 Rome,

Italy. [stefano.pontone@uniroma1.it](mailto:stefano.pontone@uniroma1.it)

Telephone: +39-6-49972446 Fax: +39-6-49972446

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**Peer reviewers:** Andrada Seicean, MD, PhD, University of Medicine and Pharmacy Cluj-Napoca, 15, Closca street, 400039 Cluj-Napoca, Romania; Akio Inui, MD, PhD, Professor, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan

Pontone S. Colorectal cancer screening behavior and willingness. *World J Gastroenterol* 2012; 18(22): 2885-2886 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i22/2885.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i22.2885>

### Abstract

The outpatient-based study by Deng *et al* [*World J Gastroenterol* 2011 July 14; 17(26): 3133-3139] on the factors that may influence the colorectal cancer (CRC) screening feasibility, encouraged our curiosity. Establishing a simple method for quickly assessing the educational level of patients and modulating a questionnaire for each type of patient, may be an effective protocol to increase the people participation, mainly in countries where sufficient medical resources and financial support are lacking. In fact, the knowledge directly affects the feasibility when screening is offered. Patient educational level influences the understanding of the knowledge and the screening method. This factor may affect patient's priority level on the study participation, the understanding of questions, and the motivation to complete the questionnaire and, consequently, the screening success. Recent studies have found a relationship between high educational level and CRC screening participation, and emphasized the questionnaire ineffectiveness in the illiterate people. Although the questionnaire is an excellent method for this kind of evaluation, physician's contribution could be the most important factor associated with the screening method. Thus, further studies should be conducted to explore the compliance of patients with low educational level and to look for the best solutions for their enrollment.

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### TO THE EDITOR

We read with great interest the study by Deng *et al*<sup>[1]</sup> on the factors that may influence the colorectal cancer (CRC) screening feasibility [*World J Gastroenterol* 2011 July 14; 17(26): 3133-3139]. We strongly agree with the authors about the importance of CRC screening knowledge. Indeed, this study shows that a better knowledge leads patients to choose the most accurate test. However, although the questionnaire is an excellent method for this kind of evaluation, bias could affect the results. Thus, the educational level plays a key role in the understanding of questions and the response accuracy. On this regard, we noted that only 6.9% of patients had a low educational level. This factor may affect the patient's priority level on the study participation, the understanding of questions, the motivation to complete the questionnaire and, consequently, the screening success. In order to get a true picture of the screening results, the percentage of patients with low educational levels should be similar to the other two educational classes of patients. Thus, it would be interesting to know the educational class distribution in sections of "not responding" (14.25%) and "unfilled" (28 patients). In a recent study, Garcia *et al*<sup>[2]</sup> found a relationship between high educational level and CRC screening participation. In the illiterate people, the questionnaire had a reduced efficacy. In addition, von Wagner *et al*<sup>[3]</sup> demonstrated that lower



health literacy had a direct impact on information-seeking. For these reasons, a variation of the questionnaire design would be useful based on the literacy level<sup>[4]</sup>.

On the other hand, the study of Deng *et al*<sup>[1]</sup> is associated to a series of studies that emphasize the impact of socioeconomic and cultural factors on the CRC screening feasibility<sup>[5,6]</sup>. Sung *et al*<sup>[7]</sup>'s study, which refers to an "ostrich" strategy of the Chinese population, asserts that in a society as the Chinese one, where the public knowledge of CRC is poor, a physician's recommendation is the most important factor associated with the acceptance of CRC screening. Even though in this study<sup>[7]</sup>, the percentage of respondents with a low educational level is significantly low (17.5%). In my opinion, the physician's recommendation, becomes more significant, especially in the population that requires more assurances to be convinced. Thus, this finding is true for various communities worldwide<sup>[8-10]</sup>. In fact, people with low educational level are less willing to the interview participation.

In conclusion, the protocol used in the study by Deng *et al*<sup>[1]</sup>, is correct to get a greater adherence to the CRC screening. In this regard, it would be appropriate to study, even in multi-centers, the difference in terms of adherence to the CRC screening among people who would accept physician's recommendation or other kinds of communications.

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**Amin Ibrahim Amin, FRCS**, Department of Surgery, Queen Margaret Hospital, Dunfermline, Fife KY12 0SU, United Kingdom

**Dr. Chin Wee Ang, MD, Clinical Research Associate**, Division of Surgery and Oncology, University of Liverpool, 5th Floor UCD, Duncan Building, Daulby Street, Liverpool, L69 3GA, United Kingdom

**Dr. Herwig R Cerwenka, Professor**, Department of Surgery, Medical University of Graz, Auenbruggerplatz 29, A-8036 Graz, Austria

**Salvatore Gruttadauria, MD, Assistant Professor**, Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

**Maria Concepción Gutiérrez-Ruiz, PhD**, Department of Health Sciences, Universidad Autónoma Metropolitana-Iztapalapa, DCBS, Av San Rafael Atlixco 186, Colonia Vicentina, México DF 09340, México

**Imran Hassan, MD, Assistant Professor**, Department of Surgery, SIU School of Medicine, 701 North Rutledge, PO Box 19638, Springfield, IL 62794, United States

**Dr. Kok Sun Ho**, Department of Colorectal Surgery, Singapore General Hospital, Outram Road, Singapore 169608, Singapore

**Beata Jolanta Jabłońska, MD, PhD**, Department of Digestive Tract Surgery, University Hospital of Medical University of Silesia, Medyków 14 St., 40-752 Katowice, Poland

**Won Ho Kim, MD, Professor**, Department of Internal Medicine, Yonsei University College of Medicine, 134 Shinchon-dong Seodaemun-ku, Seoul 120-752, Korea

**Dr. Patricia F Lalor, PhD**, Liver Research Laboratory, Room 537 Institute of Biomedical Research, Division of Medical Science, University of Birmingham, Birmingham B15 2TT, United Kingdom

**Yu-Yuan Li, Professor**, Department of Gastroenterology, First Municipal People's Hospital of Guangzhou, 1 Panfu Road, Guangzhou 510180, Guangdong Province, China

**Dr. Kaye M Reid Lombardo, FACS, MD, Assistant Professor**, Department of Surgery, Mayo Clinic, 200 First St. SW, Rochester, MN

55905, United States

**Shin Maeda, Professor, Chairman**, Department of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan

**Jukka-Pekka Mecklin, MD, PhD, Professor, Surgeon-in-Chief**, Department of Surgery, Jyväskylä Central Hospital, 40620 Jyväskylä, Finland

**Dr. Luca Morelli, MD, UO**, Anatomy and Histology, Ospedale S. Chiara, largo Medaglie d'Oro 9, 38100 Trento, Italy

**Alan C Moss, MD, FACP, Assistant Professor of Medicine, Director** of Translational Research, Center for Inflammatory Bowel Disease, Beth Israel Deaconess Medical Center, Harvard Medical School, Rose 1/East, 330 Brookline Ave, Boston, MA 02215, United States

**Yuji Naito, Professor**, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-8566, Japan

**Patrick O'Dwyer, MB, BCh, BAO, FRCS (I), MCh, FRCS (Glasg)**, University Department of Surgery, Western Infirmary, Glasgow, G11 6NT, United Kingdom

**Paolo R Salvalaggio, MD, PhD, MBA**, Hospital Israelita Albert Einstein, Serviço de Transplantes, Av. Albert Einstein 627, Morumbi, 05652-900 Sao Paulo, SP, Brasil

**Ala Sharara, MD, FACP, AGAF, Professor of Medicine, Head**, Division of Gastroenterology, American University of Beirut Medical Center, Consulting Professor, Duke University Medical Center, PO Box 11-0236, Riad El Solh 110 72020, Beirut, Lebanon

**Masayuki Sho, MD, PhD, Professor**, Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan

**Dr. Wei-Dong Tong, MD, PhD, Associate Professor**, Daping hospital, Third Military Medical University, Chongqing 400042, China

**Omar Vergara-Fernandez, MD**, Departments of Surgery, National Institute for Medical Sciences and Nutrition Salvador Zubirán, Vasco de Quiroga No. 15, Col. Sección XVI. Deleg. Tlalpan, CP 14000, México

**Marco Vivarelli, MD, Assistant Professor**, Department of Surgery and Transplantation, University of Bologna, S. Orsola Hospital, 40123 Bologna, Italy

**Satoshi Yamagiwa, MD, PhD**, Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata 951-8510, Japan



## MEETINGS

### Events Calendar 2012

January 13-15, 2012  
Asian Pacific *Helicobacter pylori*  
Meeting 2012  
Kuala Lumpur, Malaysia

January 19-21, 2012  
American Society of Clinical  
Oncology 2012 Gastrointestinal  
Cancers Symposium  
San Francisco, CA 3000,  
United States

January 19-21, 2012  
2012 Gastrointestinal Cancers  
Symposium  
San Francisco, CA 94103,  
United States

January 20-21, 2012  
American Gastroenterological  
Association Clinical Congress of  
Gastroenterology and Hepatology  
Miami Beach, FL 33141,  
United States

February 3, 2012  
The Future of Obesity Treatment  
London, United Kingdom

February 16-17, 2012  
4th United Kingdom Swallowing  
Research Group Conference  
London, United Kingdom

February 23, 2012  
Management of Barretts  
Oesophagus: Everything you need  
to know  
Cambridge, United Kingdom

February 24-27, 2012  
Canadian Digestive Diseases Week  
2012  
Montreal, Canada

March 1-3, 2012  
International Conference on  
Nutrition and Growth 2012  
Paris, France

March 7-10, 2012  
Society of American Gastrointestinal  
and Endoscopic Surgeons Annual  
Meeting  
San Diego, CA 92121, United States

March 12-14, 2012  
World Congress on  
Gastroenterology and Urology  
Omaha, NE 68197, United States

March 17-20, 2012  
Mayo Clinic Gastroenterology and  
Hepatology  
Orlando, FL 32808, United States

March 26-27, 2012  
26th Annual New Treatments in  
Chronic Liver Disease  
San Diego, CA 92121, United States

March 30-April 2, 2012  
Mayo Clinic Gastroenterology and  
Hepatology  
San Antonio, TX 78249,  
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March 31-April 1, 2012  
27th Annual New Treatments in  
Chronic Liver Disease  
San Diego, CA 92121, United States

April 8-10, 2012  
9th International Symposium on  
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April 13-15, 2012  
Asian Oncology Summit 2012  
Singapore, Singapore

April 15-17, 2012  
European Multidisciplinary  
Colorectal Cancer Congress 2012  
Prague, Czech

April 18-20, 2012  
The International Liver Congress  
2012  
Barcelona, Spain

April 19-21, 2012  
Internal Medicine 2012  
New Orleans, LA 70166,  
United States

April 20-22, 2012  
Diffuse Small Bowel and Liver  
Diseases  
Melbourne, Australia

April 22-24, 2012  
EUROSON 2012 EFSUMB Annual

Meeting  
Madrid, Spain

April 28, 2012  
Issues in Pediatric Oncology  
Kiev, Ukraine

May 3-5, 2012  
9th Congress of The Jordanian  
Society of Gastroenterology  
Amman, Jordan

May 7-10, 2012  
Digestive Diseases Week  
Chicago, IL 60601, United States

May 17-21, 2012  
2012 ASCRS Annual Meeting-  
American Society of Colon and  
Rectal Surgeons  
Hollywood, FL 1300, United States

May 18-19, 2012  
Pancreas Club Meeting  
San Diego, CA 92101, United States

May 18-23, 2012  
SGNA: Society of Gastroenterology  
Nurses and Associates Annual  
Course  
Phoenix, AZ 85001, United States

May 19-22, 2012  
2012-Digestive Disease Week  
San Diego, CA 92121, United States

June 2-6, 2012  
American Society of Colon and  
Rectal Surgeons Annual Meeting  
San Antonio, TX 78249,  
United States

June 18-21, 2012  
Pancreatic Cancer: Progress and  
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Lake Tahoe, NV 89101, United States

July 25-26, 2012  
PancreasFest 2012  
Pittsburgh, PA 15260, United States

September 1-4, 2012  
OESO 11th World Conference  
Como, Italy

September 6-8, 2012  
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Neurogastroenterology and Motility  
Meeting  
Bologna, Italy

September 7-9, 2012  
The Viral Hepatitis Congress  
Frankfurt, Germany

September 8-9, 2012  
New Advances in Inflammatory  
Bowel Disease  
La Jolla, CA 92093, United States

September 8-9, 2012  
Florida Gastroenterologic Society  
2012 Annual Meeting  
Boca Raton, FL 33498, United States

September 15-16, 2012  
Current Problems of  
Gastroenterology and Abdominal  
Surgery  
Kiev, Ukraine

September 20-22, 2012  
1st World Congress on Controversies  
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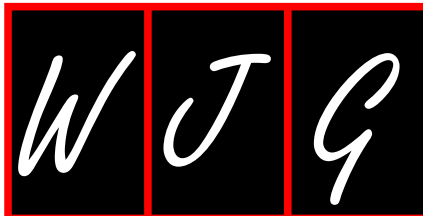
October 19-24, 2012  
American College of  
Gastroenterology 77th Annual  
Scientific Meeting and Postgraduate  
Course  
Las Vegas, NV 89085, United States

November 3-4, 2012  
Modern Technologies in  
Diagnosis and Treatment of  
Gastroenterological Patients  
Dnepropetrovsk, Ukraine

November 4-8, 2012  
The Liver Meeting  
San Francisco, CA 94101,  
United States

November 9-13, 2012  
American Association for the Study  
of Liver Diseases  
Boston, MA 02298, United States

December 1-4, 2012  
Advances in Inflammatory Bowel  
Diseases  
Hollywood, FL 33028, United States



## GENERAL INFORMATION

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### Editorial office

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Editorial Department: Room 903, Building D,  
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No. 62 Dongsihuan Zhonglu,  
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homogeneous data can be averaged. Standard deviations are preferred to standard errors. Give the number of observations and subjects (*n*). Losses in observations, such as drop-outs from the study should be reported; (4) Values such as ED50, LD50, IC50 should have their 95% confidence limits calculated and compared by weighted probit analysis (Bliss and Finney); and (5) The word 'significantly' should be replaced by its synonyms (if it indicates extent) or the *P* value (if it indicates statistical significance).

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- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunolog-



ic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

#### In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

#### Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 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]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

- 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 dis-

eases. *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>

#### Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean  $\pm$  SD or mean  $\pm$  SE.

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Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *ν* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4  $\pm$  2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5  $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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### Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

### Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindI*, *BamHI*, *Kpn I*, etc.

Biology: *H. pylori*, *E. coli*, etc.

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