

# World Journal of *Gastroenterology*

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## Staging colorectal cancer with the TNM 7<sup>th</sup>: The presumption of innocence when applying the M category

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### Abstract

One of the main changes of the current TNM-7 is the elimination of the category MX, since it has been a source of ambiguity and misinterpretation, especially by pathologists. Therefore the ultimate staging would be better performed by the patient's clinician who can classify the disease M0 (no distant metastasis) or M1 (presence of distant metastasis), having access to the completeness of data resulting from clinical examination, imaging workup and pathology report. However this important change doesn't take into account the diagnostic value and the challenge of small indeterminate visceral lesions encountered, in particular, during

radiological staging of patients with colorectal cancer. In this article the diagnosis of these lesions with multiple imaging modalities, their frequency, significance and relevance to staging and disease management are described in a multidisciplinary way. In particular the interplay between clinical, radiological and pathological staging, which are usually conducted independently, is discussed. The integrated approach shows that there are both advantages and disadvantages to abandoning the MX category. To avoid ambiguity arising both by applying and interpreting MX category for stage assigning, its abandoning seems reasonable. The recognition of the importance of small lesion characterization raises the need for applying a separate category; therefore a proposal for their categorization is put forward. By using the proposed categorization the lack of consideration for indeterminate visceral lesions with the current staging system will be overcome, also optimizing tailored follow-up.

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**Key words:** Colorectal cancer; Staging; Indeterminate lesions; Imaging; Metastases

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### INTRODUCTION

Stage IV colorectal cancer (CRC) is no longer considered a single entity<sup>[1]</sup> and after several proposals for stratifying it<sup>[2,3]</sup> the current TNM-7 subdivides the M1 category into M1a (metastasis confined to one organ: liver, lung,

ovary, non-regional lymph node(s) and M1b (metastasis in more than one organ or the peritoneum): accordingly stage IV is subdivided in IVA (Any T, Any N, M1a) and IVB (Any T, Any N, M1b)<sup>[4]</sup>. Population-based studies demonstrate that the prognosis for surgically resected stage IV disease is near identical for that of patients following potentially curative surgery for stage III disease<sup>[3]</sup>.

The prognosis and treatment options (including increasingly used multimodality approaches) within the M1 stages depend largely on the extent and distribution of the metastases. Therefore, the current staging will be able to take into account improvements that have been made in surgical techniques for resectable metastases, and the impact of modern chemotherapy on rendering initially unresectable CRC liver metastases operable, while at the same time distinguishing between patients with a chance of cure at presentation and those for whom only palliative treatment is possible<sup>[3]</sup>.

An other major change with the new TNM-7 is that the category “MX: Distant metastasis cannot be assessed” has been eliminated<sup>[4]</sup>.

In this report, the change with the TNM-7 is discussed in a multidisciplinary setting and the diagnostic significance of small indeterminate visceral lesions encountered during radiological staging of patients with colorectal cancer is presented.

The resulting problems, in particular the ambiguities for stage assigning when applying the MX category and the risk of inaccurate staging and follow-up planning because of its elimination, are discussed. A proposal is presented for the categorization of such small, indeterminate visceral lesions.

## CHANGE WITH THE TNM FROM THE PATHOLOGIST POINT OF VIEW

There are basically two reasons for MX elimination. Firstly the MX category has been a source of misinterpretation, especially by pathologists (*i.e.*, pMX) who “may assign MX, meaning that they cannot assess distant metastasis”<sup>[6]</sup>. This is a clinical, not pathological assessment. “If there are no obvious signs of metastasis, M0 or cM0 classifications are appropriate. In other words, once clinically examined, a patient is M0 until proven otherwise”<sup>[6]</sup> in analogy with the legal right Presumption of Innocence.

The second reason is that assigning the MX category prevents stage grouping by American cancer registries<sup>[6]</sup>.

The elimination of MX allows only two categories, M0 (cM0): no distant metastasis and M1 (cM1 or pM1): distant metastasis<sup>[4]</sup> utilizing imaging and/or pathological assessment<sup>[7]</sup>.

This goes against the TNM rule of assigning the X category, since the proper use of X is to denote the absence or uncertainty of assigning a given category<sup>[8]</sup> but importantly these are two different situations.

A similar clinical-pathological context is encountered

when applying the R classification (R for residual as descriptor of “tumour remaining in the patient” after primary surgical resection): the assignment of the R classification must be performed “by a designated individual who has access to the complete data”<sup>[9]</sup>.

Despite extensive clinical assessment of treatment results and careful pathologic examination, in some cases the presence of residual tumour cannot be assessed: the RX category applies<sup>[9,10]</sup>.

If the pathologist hasn't access to the preoperative clinico-radiological work-up, he/she can do only a limited staging (pT and pN), in the dark about these clinical data.

As an accurate pathological examination is the prerequisite for a prognostic staging and a tailored patient treatment, the enhanced search for additional prognostic factors applies in particular cases (problems with resection margins assessment; involvement of the peritoneum and/or adjacent structures or organs; suspicion for vascular invasion; few lymph nodes recovered; pericolic tumour deposits)<sup>[11,12]</sup>.

In this setting, patient characteristics, treatment-related features and disease extension as evaluated both during surgery and preoperatively are all relevant information.

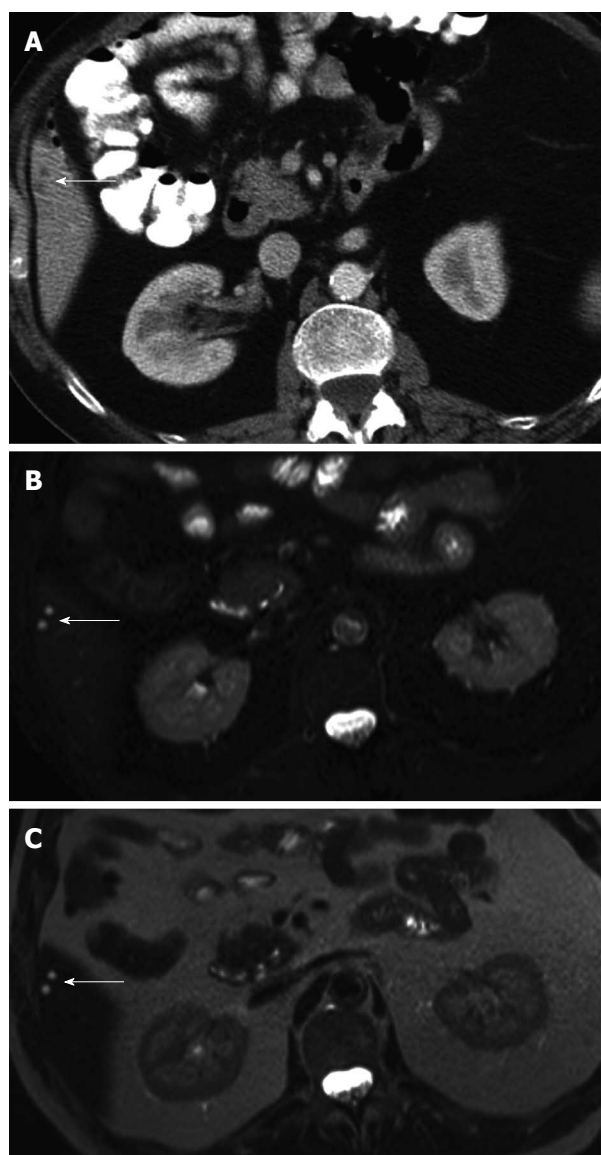
In other words, the stage grouping, as well the risk assessment of patients belonging to the same stage, are better performed in the multidisciplinary setting than considered in isolation.

## RADIOLOGICAL STAGING

The major task of radiological staging is the exclusion (cM0) or detection (cM1) of metastases, particularly in the liver and/or the lung. The most frequently used imaging modalities for staging of CRC cancer patients are ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET)/CT (PET/CT)<sup>[13]</sup>. In the past 10 years, important advances have been made within all four techniques. While some (CT and PET/CT) will give patient specific information regarding abnormal masses and increased tissue metabolic activity throughout the body, others (MRI) will yield organ (liver) specific information and allow characterization of specific abnormalities to differentiate benign from malignant lesions. However, no single modality will diagnose all metastases, and the optimal imaging strategy to classify cM0 or cM1 depends on the clinical context, the organ site investigated, and the individual aims of oncologic care.

With the introduction of multidetector row CT (MDCT) scanning, CT imaging will continue to play the dominant role in the radiological staging of CRC patients<sup>[14]</sup>. Improved MDCT technologies have resulted in increased CT detection of small (< 1 cm) indeterminate lesions in the lung and/or liver (in around 10%-40% of CT CRC staging examinations)<sup>[15-20]</sup>.

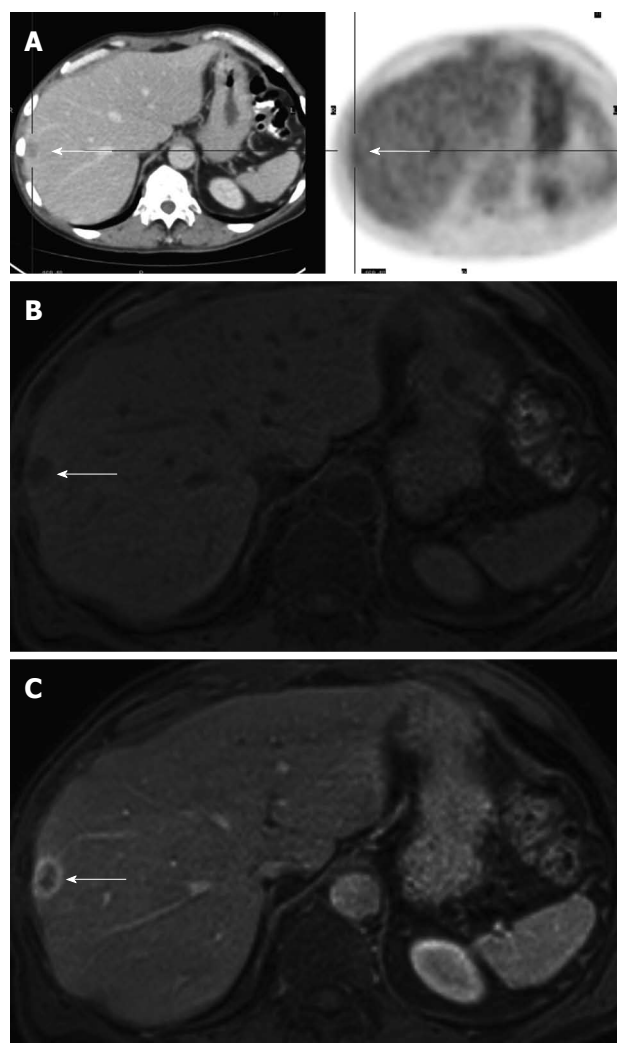




**Figure 1** Example of a small lesion which proved to be benign after follow-up. A: Two small (< 10 mm) indeterminate focal liver lesions (white arrow) on contrast-enhanced computed tomography; B: Corresponding magnetic resonance imaging (MRI), images showing a T2w Turbo Spin Echo sequence with fat suppression. Two clearly hyperintense focal liver lesions (white arrow) are displayed compatible with simple liver cysts; C: Corresponding MRI images showing a T2w Turbo Spin Echo sequence without fat suppression. Two clearly hyperintense focal liver lesions (white arrow) are displayed compatible with simple liver cysts.

A major drawback of MDCT is that its ability to detect small lesions has outstripped its ability to characterize them. Although most tiny lung and/or liver lesions detected on CT staging of CRC patients are benign (Figure 1), 10%-20% of CT-indeterminate lung and/or liver lesions do develop into definite metastases (Figure 2)<sup>[17,18,20,21]</sup>. The M stage of CRC patients with CT-indeterminate lung and/or liver lesions, therefore, would require the category MX ("X" meaning uncertainty).

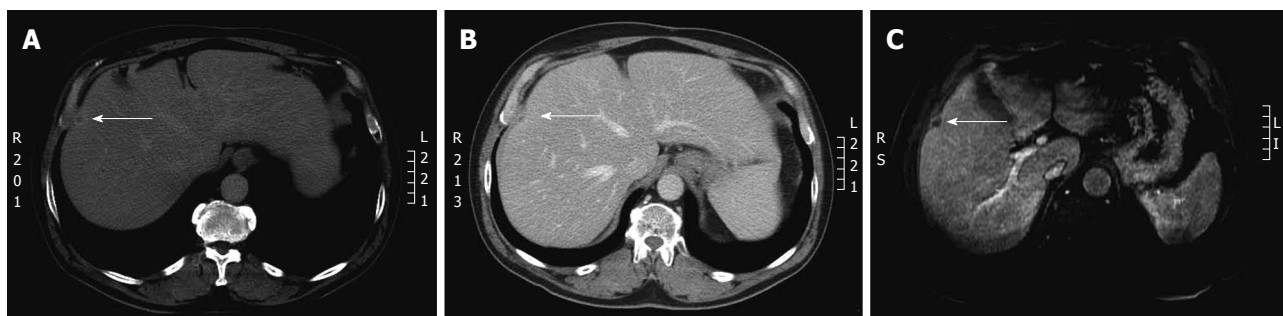
Regarding the definite diagnosis of whether or not these lesions are benign or malignant, consideration



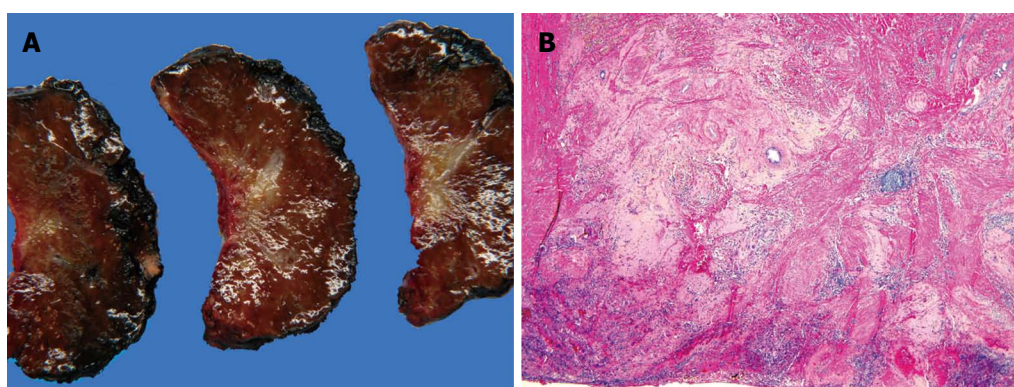
**Figure 2** Example of a small lesion which proved to be malignant after follow-up. A: Positron emission tomography (PET)/computed tomography negative (PET-cold) focal liver lesion (white arrow) in a patient who received chemotherapy in the past; B: Corresponding T1w Gradient Echo magnetic resonance imaging (MRI) image before injection of contrast agent shows a rather hypo-intense focal liver lesion (white arrow); C: Corresponding T1w Gradient Echo MRI image in the venous phase after injection of contrast agent shows a hypo-intense focal liver lesion with ring-enhancement compatible with an (active) malignant focal liver lesion (colorectal cancer liver metastasis) (white arrow).

should include the T stage and the nodal status of the primary tumour and the distribution patterns of lesions as the probability of malignancy of small lung or liver lesions depends on the stage of the primary tumour (T stage) and the nodal status (N stage)<sup>[17,18]</sup>.

Further evaluation with complementary imaging modalities is needed in patients with small CT-indeterminate lesions if a change of M staging would alter the treatment. In the thorax, additional PET/CT may be helpful in the differentiation of a single < 1 cm pulmonary lesion, but it may not be efficient when more than one small lesion is found on MDCT examination. In the liver, US may be the primary modality of choice in cases of advanced liver metastases, whereas contrast-enhanced US<sup>[22]</sup> MRI<sup>[23,24]</sup> and/or US-CT fusion imaging tech-



**Figure 3** Restaging the liver after neoadjuvant therapy in a patient with multiple liver metastases from rectal cancer. A: On computed tomography (CT) before intravenous contrast, all the metastases disappeared except for a small lesion suspicious of residual disease (white arrow); B: Corresponding CT after intravenous contrast showing the challenging lesion (white arrow); C: Also on magnetic resonance imaging the lesion (white arrow) remains suspicious for malignancy providing indication for a liver resection.



**Figure 4** Pathological examination of the resected liver specimen corresponding to the lesion indicated by the white arrow in figure 3. A: Gross specimen: on cut the macroscopic examination shows a star-like whitish lesion; B: At histology (hematoxylin and eosin, x 100) only fibrosis and inflammation are seen indicating a complete response to chemotherapy.

niques<sup>[25]</sup> may complement the initial CT information in candidates for liver resection.

Follow-up CT imaging may be a reasonable approach in patients in whom a change of M staging would alter the clinical management, as malignant lesions would be expected to grow but benign lesions less so.

During restaging after neoadjuvant chemotherapy, besides the complete clinical remission (disappearance on CT, ycM0) achieved in a small number of patients with metastases (mainly in CRC lung or liver metastases), small lesions, suspected as being residual tumour may be encountered (Figure 3): the difficulties in interpreting these lesions are made harder by the response to chemotherapy which may impact on the sensitivity of preoperative imaging studies in identifying all sites of disease<sup>[26]</sup>.

Either chemotherapy itself, or the fatty infiltration it commonly causes, can affect the ability of CT to effectively restage patients, resulting in both false negative and false positive results; thus, in patients with known hepatic steatosis, or in patients receiving neo-adjuvant chemotherapy, liver MRI is often recommended for staging or restaging of hepatic disease<sup>[14]</sup>.

However, only pathologic examination can determine the actual nature of these lesions (Figure 4).

## CONCLUSION

### **Advantages and disadvantages of the revised TNM classification of metastatic status**

Disease management for CRC has evolved in recent years into a multidisciplinary setting and is essentially based on tumour stage.

Cancer staging represents the operational basis for choosing the most appropriate therapy and for evaluating the efficacy of different therapeutic methods; it is an essential component of patient care, cancer research, and control activities, even in light of the impressive progress that has been attained in the fields of clinical strategies and molecular medicine.

The TNM system is subjected to continuous updating through an ongoing expert review of existing data.

Proposals for changes are made in different situations, including when the classification is poorly accepted, poorly used, or criticized in the literature<sup>[27]</sup>: here comes the decision for MX category elimination<sup>[6]</sup>.

As the MX category results in ambiguity (lack of information or uncertainty in assigning a given category) both in applying and in interpreting it for stage assigning, it seems reasonable to abandon it.

This is also in accord with the general rule of the

TNM system which states that, “if there is doubt concerning the correct category to which a particular patient should be allotted (T, N, or M), then the lower (*i.e.*, less advanced) category should be used”<sup>[8]</sup>.

However, the increased detection of small indeterminate lesions due to improved imaging technologies, particularly with MDCT imaging<sup>[20]</sup>, suggests that a separate category is needed.

This is to avoid the application of M0 category to all indeterminate/suspicious lesions, with consequent reduced diagnostic accuracy and staging errors.

### Proposal

Cancer patients with small indeterminate lesions (*e.g.*, in the lung and/or liver) are at risk of developing metastases and therefore need continued follow-up with imaging. The TNM staging system uses an extensive number of prefixes and suffixes, as additional descriptors, their presence indicating cases needing separate analysis<sup>[4]</sup> and ongoing investigation. We propose to add a suffix (*e.g.*, iPUL, iHEP) to cM0 to indicate the presence of “small indeterminate lesions” considered to be benign but to be surveyed on further follow-up imaging to confirm that they are benign by their unchanged size and radiologic characteristics.

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## From fatty liver to fibrosis: A tale of “second hit”

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### Abstract

Although much is known about how fat accumulates in the liver, much remains unknown about how this causes sustained hepatocellular injury. The consequences of injury are recognized as nonalcoholic steatohepatitis (NASH) and progressive fibrosis. The accumulation of fat within the hepatocytes sensitizes the liver to injury from a variety of causes and the regenerative capacity of a fatty liver is impaired. An additional stressor is sometimes referred to as a “second hit” in a paradigm that identifies the accumulation of fat as the “first hit”. Possible candidates for the second hit include increased oxidative stress, lipid peroxidation and release of toxic products such as malondialdehyde and 4-hydroxynonenal, decreased antioxidants, adipocytokines, transforming growth factor (TGF)- $\beta$ , Fas ligand, mitochondrial dysfunction, fatty acid oxidation by CYPs (CYP 2E1, 4A10 and 4A14), and peroxisomes, excess iron, small intestinal bacterial overgrowth, and the generation of gut-derived toxins such as lipopolysaccharide and ethanol. Oxidative stress is one of the most popular proposed mechanisms of hepatocellular injury. Previous studies have specifically observed in-

creased plasma and tissue levels of oxidative stress markers and lipid peroxidation products, with reduced hepatic and plasma levels of antioxidants. There is also some indirect evidence of the benefit of antioxidants such as vitamin E, S-adenosylmethionine, betaine, phlebotomy to remove iron, and N-acetylcysteine in NASH. However, a causal relationship or a pathogenic link between NASH and oxidative stress has not been established so far. A number of sources of increased reactive oxygen species production have been established in NASH that include proinflammatory cytokines such as tumor necrosis factor (TNF)- $\alpha$ , iron overload, overburdened and dysfunctional mitochondria, CYPs, and peroxisomes. Briefly, the pathogenesis of NASH is multifactorial and excess intracellular fatty acids, oxidant stress, ATP depletion, and mitochondrial dysfunction are important causes of hepatocellular injury in the steatotic liver.

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**Key words:** Fatty liver; Oxidative stress; Fibrosis; Nonalcoholic fatty liver diseases; Nonalcoholic steatohepatitis

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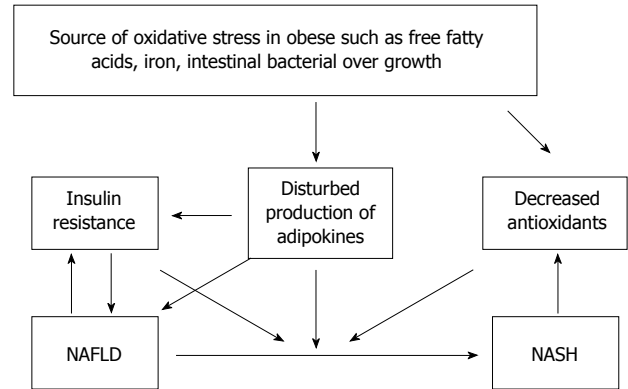
### INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most prevalent forms of chronic liver disease in the United States<sup>[1]</sup>. Contributing factors to this may include the increasingly sedentary lifestyle of the population and increased consumption of a high-fat (HF) diet and high fructose corn syrup (HFCS)<sup>[2]</sup>. In the setting of excessive

central adiposity, insulin resistance is the major underlying cause of fat accumulation in the liver<sup>[3-7]</sup>. Nonalcoholic steatohepatitis (NASH), as a subgroup of NAFLD, is characterized by chronic and progressive liver pathology and may lead to advanced fibrosis, cirrhosis, end-stage liver disease, hepatocellular carcinoma and liver-related death<sup>[8-10]</sup>. One of the important and unresolved problems in NASH is the pathogenesis of hepatocyte injury. One hypothesis for the pathogenesis of NAFLD is the “two-hit” hypothesis<sup>[11]</sup>. According to this paradigm, the primary abnormality (“first hit”) is most likely insulin resistance, which leads to the accumulation of triglycerides within the hepatocytes. Then, a “second hit” induces hepatocyte injury and inflammation (NASH). The mechanisms that cause hepatocyte injury in fatty liver have not been fully elucidated to date. Oxidative stress is one of the most popular proposed mechanisms of hepatocellular injury (Figure 1). It was reported that obesity correlated with systemic oxidative stress in humans and mice<sup>[12-14]</sup>. Obese adults with metabolic syndrome (MS) have higher plasma concentration of oxidative stress biomarkers than obese adults without MS. Increased reactive oxygen species (ROS) production has been selectively shown in adipose tissue of obese mice<sup>[15,16]</sup>. Previous studies also specifically observed increased plasma and tissue levels of oxidative stress markers and lipid peroxidation products with reduced hepatic and plasma levels of antioxidants in patients with NASH<sup>[2-6,12-16]</sup>.

## MOLECULAR SIGNAL OF DEVELOPMENT OF FATTY LIVER IN OBESITY

Obesity is associated with low-grade chronic inflammation in humans, and this chronic inflammation is a link between obesity and insulin resistance<sup>[17-19]</sup>. Indeed, obesity is strongly associated with chronic macrophage accumulation within increased adipose tissue in obese humans. Xu *et al.*<sup>[16]</sup> showed that inflamed macrophages are active within white adipose tissue and this activation occurs after increased adiposity and before insulin resistance. Macrophages secrete cytokines which promote insulin resistance in adipose tissue and eventually increase adipose tissue lipolysis, which causes insulin resistance in both muscle and the liver, besides significant amount of inducible NO synthase and interleukin-6<sup>[15]</sup>. Moreover, a positive correlation between adipocyte size and the content of accumulated macrophages in adipose has been reported. Increased fatty acids or accumulated macrophages might be the reason for this increased ROS production within adipose tissue. These data indicate localized inflammation and systemic consequences such as insulin resistance and increased circulating free fatty acids. Additional evidence that this chronic inflammation causes insulin resistance comes from restoring insulin sensitivity by various anti-inflammatory agents, such as high-dose salicylates *via* IKK- $\beta$  inhibition or anti-tumor necrosis factor (TNF)- $\alpha$

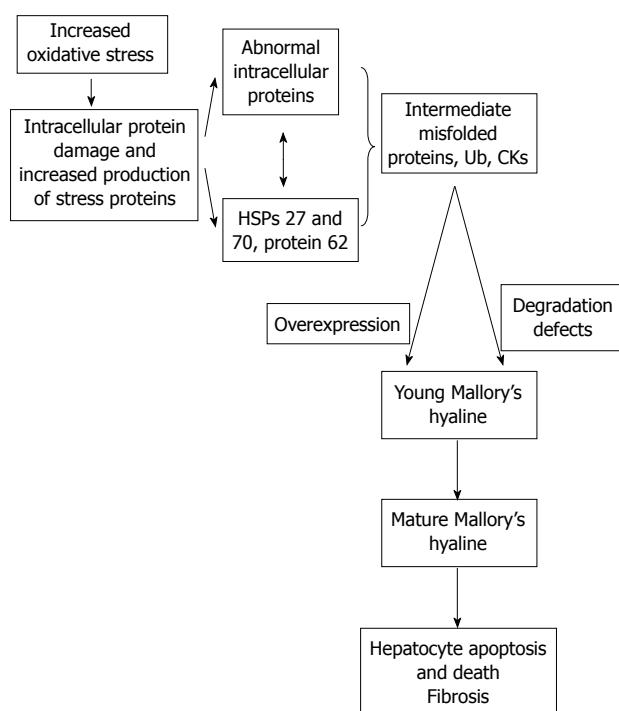


**Figure 1** Oxidative stress is one of the most popular proposed mechanisms of hepatocellular injury and possible source of the oxidant stress as follows: increased free fatty acids supply, iron, intestinal bacterial over growth. NASH: Nonalcoholic steatohepatitis; NAFLD: Nonalcoholic fatty liver disease.

antibody infusion<sup>[20]</sup>.

## OXIDATIVE STRESS AND LIPID PEROXIDATION FOR DEVELOPMENT OF HEPATIC FIBROSIS

A logical and attractive hypothesis is that oxidative stress in TG-loaded hepatocytes is the cause of sustained injury with consequent NASH, fibrosis and cirrhosis<sup>[11]</sup> (Figure 2). The imbalance between the increased ROS and decreased antioxidants leads to lipid peroxidation of polyunsaturated fatty acids, cellular membranes, mitochondrial membranes, and DNA<sup>[21]</sup>. Lipid peroxidation products have longer half-lives and the capability to reach extracellular targets. Lipid peroxidation produces cytotoxic aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal. ROS and these aldehydes further contribute to oxidative stress, decreased ATP production, and increased proinflammatory cytokine release. These events promote hepatocyte injury, necroinflammation and hepatocytes apoptosis. Despite the attractiveness of this hypothesis, most clinical studies only provide correlations between the presence of NASH and elevated indices of oxidant stress, without establishing a causal relationship<sup>[22-27]</sup>. The lipid peroxidation product 4-hydroxynonenal is found more in perivenular zone (zone 3), correlating with the histological lesions of NASH that are predominantly in zone 3<sup>[21]</sup>. Lipid peroxidation is greater in patients with NASH than in patients with simple steatosis. The same study has also shown that increased 4-hydroxynonenal strongly correlates with both the grade of necroinflammation and the stage of NASH, but not with the grade of steatosis, while increased evidence of oxidant damage to DNA as measured by 8-hydroxydeoxyguanosine only correlates with the grade of necroinflammation in patients with NASH.



**Figure 2** Logical and attractive hypothesis is that oxidative stress in TG-loaded hepatocytes is the cause of sustained injury with consequent NASH, fibrosis and cirrhosis. HSP: Heat shock proteins.

## UNDERSTANDING THE MOLECULAR MECHANISMS OF NASH

### Oxidative stress

Oxidative stress might play a role in the pathogenesis of hepatocyte dysfunction and inflammation in NAFLD. Oxidative stress can result from either excess ROS production and/or deficient antioxidant capacity<sup>[12-15,21]</sup>. The enzyme NAD(P)H oxidase catalyzes the transfer of a single electron to molecular oxygen to produce superoxide. Upregulation of NAD(P)H oxidase can raise ROS production, and thereby contribute to the pathogenesis of oxidative stress in HF and high-sucrose-fed mice. Formation of ROS is enhanced by different mechanisms, including xanthine oxidase activation, NADH auto-oxidation, and superoxide dismutase (SOD) inactivation. Experimental evidence supports the concept of diverse ROS, such as superoxide anion, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite.

Assessment of: (1) NAD(P)H oxidase (a major source of ROS) in liver; (2) downregulation of the main antioxidant enzymes, SOD, glutathione peroxidase (GPX), catalase, and heme oxygenase (HO) in liver tissue; (3) level of lipid peroxidation products, MDA + 4-hydroxyalkenals in the liver; (4) plasma concentration of 8-isoprostanes, lipoperoxides in plasma samples by measuring MDA *via* HPLC; and (5) immunoblotting to quantify NADPH oxidase, Mn SOD, Cu Zn SOD, GPX, catalase, and HO-2 levels in liver will help to understand the underlying mechanisms of oxidative stress in the

pathogenesis of NASH<sup>[12,13,22-28]</sup>. A number of sources of increased ROS production have been established in NASH that include proinflammatory cytokines, such as TNF- $\alpha$ , iron overload, overburdened and dysfunctional mitochondria, CYPs, and peroxisomes.

### Sources of oxidants

**Mitochondria and mitochondrial  $\beta$ -oxidation:** The hepatocyte is a cell rich in mitochondria. Each hepatocyte contains approximately 800 mitochondria<sup>[28]</sup>. The hepatocyte mitochondria are the main site of  $\beta$ -oxidation of free fatty acids. The electrons removed from free fatty acids during  $\beta$ -oxidation are shuttled through the mitochondrial respiratory chain (MRC), eventually leading to ATP synthesis and generation of CO<sub>2</sub> and water. Inherent in this process is the dissociation of partially reduced molecular oxygen in the form of superoxide, hydrogen peroxide and the hydroxyl radical, species collectively termed ROS. About 1%-5% of oxygen consumed during cellular respiration is not fully reduced to water during this process under physiological conditions, and the production of these ROS is further increased in dysfunctional mitochondria. Thus, mitochondria have been proposed to play a central role in the pathogenesis of NASH<sup>[29]</sup>. Mitochondria also increase their oxidation capacity for the increased fatty acid flux as observed in obesity and insulin resistant states in humans. However, this increase has its limits and excess free fatty acids are metabolized at other sites in hepatocytes such as peroxisomes ( $\omega$ -oxidation) and the smooth endoplasmic reticulum ( $\omega$ -oxidation). Acyl-CoA oxidase catalyzes the initial reaction of fatty acid oxidation in peroxisomes; a process that generates hydrogen peroxide and thus may contribute to oxidant stress.

Mitochondrial  $\beta$ -oxidation of short-, medium- and long-chain fatty acids involves multiple steps that include entry of long-chain fatty acids into the mitochondria; a process dependent on carnitine shuttle enzymes carnitine palmitoyltransferase (CPT)-I (an outer membrane enzyme) and CPT-II, and the  $\beta$ -oxidation of fatty acids to form progressively shorter acyl-CoA moieties, acetyl-CoA<sup>[29]</sup>. These oxidation processes are associated with the reduction of oxidized NAD<sup>+</sup> and FAD to NADH and FADH<sub>2</sub>. Reoxidation of NADH and FADH<sub>2</sub> to NAD<sup>+</sup> and FAD produces electrons that transfer to the MRC<sup>[29,30]</sup>. Partially reduced oxygen molecules, termed ROS, are constitutively generated during this process when the electrons of NADH and FADH<sub>2</sub> directly react with oxygen and may contribute to oxidant stress if endogenous protective mechanisms are overwhelmed<sup>[30]</sup>. Consistent with the increased flux of nonesterified fatty acid to the liver in obese patients with NAFLD, mitochondrial  $\beta$ -oxidation of fatty acids in the liver is also increased and as such may contribute to increased generation of ROS and oxidant stress.

Excessive fatty acids might use alternative pathways other than mitochondrial  $\beta$ -oxidation to be metabolized and cause mitochondrial injury. These include

peroxisomal and cytochrome P450 (microsomal CYP) oxidation systems regulated by mainly fatty acids and insulin<sup>[31]</sup>. These alternative fatty acid oxidation systems produce more ROS and thus their utilization may be a source of oxidant stress.

**Peroxisomal fatty acid  $\beta$ -oxidation:** Peroxisomal oxidation of fatty acids is the normal route of metabolism of very long chain fatty acids and dicarboxylic acids<sup>[31]</sup>. Peroxisomal oxidation is a four-step pathway in which electrons from FADH<sub>2</sub> and NADH are transferred directly to oxygen. Although this increases the production of hydrogen peroxide, peroxisomes are uniquely endowed with the enzyme catalase that eliminates this reactive oxygen molecule. Fatty acids not oxidized by mitochondria are mainly oxidized by CYP2E1; a process that further increases ROS production within the hepatocytes<sup>[32]</sup>. Increased endogenous substrate burden such as increased levels of free fatty acids and ketone bodies induce CYP2E1 expression in humans. In normal conditions, CYP2E1 oxidation produces oxygen radicals, but the balance between these ROS and the abundance of endogenous antioxidants determines the extent of resulting oxidant stress. Increased hepatic CYP2E1 expression has been demonstrated by immunostaining of paraffin-embedded liver biopsy sections in patients with NASH. In contrast, hepatic content of CYP3A was decreased in all liver sections from patients with NASH. These studies also showed that weight loss decreased hepatic CYP2E1 activity. In parallel, Leclercq *et al.*<sup>[33]</sup> previously had reported that dietary sugar restriction decreased CYP2E1 activity in humans. These novel studies pointed out that insulin rather than ketone bodies, with or without glucose contribution, regulate the expression and activity of hepatic CYP2E1. These metabolic abnormalities increase hepatic CYP2E1 activity and subsequent pro-oxidant production in patients with NAFLD.

**Cytochrome P450 fatty acid  $\omega$  (omega)-oxidation:** Fatty acids can undergo oxidation by the CYP enzymes of the smooth endoplasmic reticulum which is a relatively minor pathway. CYP2E1 and CYP4A isoforms are involved in fatty acid oxidation in conditions with substrate overload such as increased ketone bodies in type 2 diabetes mellitus. CYP4A upregulation particularly occurs in conditions with decreased CYP2E1 activity. The expression of both CYP2E1 and CYP4A mRNA and their protein levels are increased in both obese and diabetic humans<sup>[32,33]</sup>. Their hepatic activity and expression are also reported to be increased in patients with NASH due to the increased substrates, mainly fatty acids and ketone bodies, irrespective of the underlying clinical condition of diabetes or obesity. Nonetheless, the capacity of this enzyme system is very low to handle fatty acids. Oxidation reactions by the CYP enzymes can be major producers of ROS because of a low degree of coupling between substrate binding and their weak affin-

ity to molecular oxygen, leading to the release of species such as superoxide anion radical, hydroxyl radicals, and hydrogen peroxide.

**Mitochondrial dysfunction and ATP depletion:** Mitochondria are the organelles primarily responsible for fatty acid  $\beta$ -oxidation and oxidative phosphorylation; the process responsible for the production of ATP<sup>[30]</sup>. Several observations including decreased mitochondrial enzyme activities and increased fat concentration of skeletal muscle cells in obese or diabetic patients have suggested mitochondrial dysfunction in these disorders. Such abnormalities may increase ROS production and promote both oxidative stress and lipid peroxidation within hepatocytes. Mitochondrial dysfunction is frequently due to a combination of genetic abnormalities, physical inactivity, aging, lipotoxicity (free fatty acids), lipid peroxidation (mitochondrial DNA alterations), and TNF- $\alpha$ <sup>[29,30,34]</sup>. Hepatic mitochondrial abnormalities have been identified in NAFLD, suggesting that mitochondria may be the source or target of injury and that ineffective mitochondrial function resulting in cellular ATP depletion may be important pathophysiological processes in NAFLD and NASH. The presence of megamitochondria, or mitochondrial swelling, is a microscopically detectable structural abnormality of hepatocyte mitochondria found in a variety of liver diseases including NAFLD<sup>[28]</sup>. Crystalline inclusions within the mitochondrial matrix have been documented by electron microscopy in patients with NASH. Hepatic mitochondrial DNA levels and the protein products of the mitochondrial genes are also decreased in patients with NASH. Impaired hepatic MRC function increases ROS production, and if ROS production exceeds antioxidant capabilities, oxidative stress and injury, lipid peroxidation of macromolecules and cellular membranes, mitochondrial DNA damage, direct damage of several mitochondrial enzymes, and further MRC dysfunction with more pro-oxidant production are observed. Mixed macro- and microvesicular steatosis due to  $\beta$ -oxidation defects in the mitochondria was the predominant type of steatosis in this study, and CYP 2E1 expression was upregulated, and levels of the antioxidant glutathione were decreased. Carnitine and CPT-I and CPT-II are required to transfer long-chain free fatty acids into the mitochondria for  $\beta$ -oxidation. Some investigators have reported the role of carnitine deficiency in NAFLD development, while others have observed normal hepatic content of total and free carnitine in patients with NASH.

**Iron, oxidant stress and NASH:** Iron can play a central role in promoting oxidant stress and this is proposed to be the mechanism of progressive liver disease. A large-population based study reported a correlation between elevated serum alanine aminotransferase levels and increased serum transferrin and iron concentrations<sup>[35,36]</sup>. After initial measurements, investigators in-



duced iron depletion to a level of near-iron deficiency by phlebotomy. Interestingly, they observed improvements in both insulin sensitivity and serum alanine aminotransferase activity in some of the patients, indicating that iron may play a role not only in oxidant stress but also in the initial predisposing factor of insulin resistance.

**Free fatty acid toxicity:** In addition to insulin resistance and hyperinsulinemia, obesity and type 2 diabetes mellitus are strongly associated with increased concentrations of free fatty acids in the circulation<sup>[24,26]</sup>. Fatty acids are involved in many important cellular events such as synthesis of cellular membranes, energy storage, and intracellular signaling pathways. However, chronically elevated free fatty acids have the capability to disturb diverse metabolic pathways and induce insulin resistance in many organ systems<sup>[37-39]</sup>. In addition to their metabolic effects, fatty acids could induce cellular apoptosis, also called lipotoxicity, in two ways: direct toxicity and an indirect effect. One proposed mechanism of fatty acid toxicity in hepatocytes is that fatty acids induce translocation of Bax (which is a mitochondrial protein and a member of the Bcl-2 family) to lysosomes and cause lysosomal destabilization, which promotes the release of cathepsin B (a specific lysosomal enzyme), from lysosomes to the cytosol. Subsequently, a cathepsin-B-dependent process induces nuclear factor (NF)- $\kappa$ B activation and TNF- $\alpha$  overexpression in the liver<sup>[26]</sup>. TNF- $\alpha$  might further increase lysosomal destabilization and cathepsin-B-dependent hepatocyte apoptosis. Then, cytochrome c release from dysfunctional mitochondria may occur. Mitochondrial dysfunction causes energy depletion, which activates proteolytic caspases and induces DNA fragmentation and chromatin condensation. NF- $\kappa$ B is a transcriptional factor and has both apoptotic and antiapoptotic effects. In healthy hepatocytes, activation of NF- $\kappa$ B by TNF- $\alpha$  induces Bcl-2 synthesis, which prevents the release of cytochrome c from the mitochondria and subsequent apoptosis. Moreover, while cathepsin B has been demonstrated in hepatocyte lysosomes of healthy control individuals, the majority of hepatocytes in patients with NAFLD show diffuse distribution of cathepsin B in the cytosol, with a positive correlation with the stage of NASH<sup>[40-44]</sup>.

## HEPATIC FIBROGENESIS IN NASH

Hepatic steatosis is the most frequent and initially observed morphological feature of these processes<sup>[45-53]</sup>. Steatosis, inflammation, glycogen nuclei, lipogranulomas, ballooning of hepatocytes, Mallory bodies, and fibrosis are the major features of NAFLD.

Genetic and environmental factors may affect the development of liver fibrosis in NAFLD<sup>[54-62]</sup>. Age, severity of obesity, presence of diabetes, and hyperglycemia are the major nongenetic factors. Elevated plasma glucose, free fatty acids and adipocytokines activate both Kupffer

cells and hepatic stellate cells (HSCs) and stimulate fibrogenesis<sup>[63-65]</sup>. Hepatic connective tissue growth factor (CTGF) mRNA was overexpressed in all NASH patients, while hepatic CTGF mRNA and its protein were upregulated in *fa/fa* rats (obese and diabetic) compared with their lean littermates. The same study also demonstrated upregulation of both CTGF mRNA and its protein in HSCs after exposure to high concentrations of either glucose or insulin. After activation, HSCs proliferate and express  $\alpha$ -smooth muscle actin. Activated HSCs express myogenic markers such as c-myc and myocyte enhancer factor-2, exhibit proinflammatory and profibrogenic properties, migrate and secrete extracellular matrix components (ECM) such as collagen, and regulate the degradation of ECM. Activation of HSCs is the crucial step in liver fibrogenesis. A study of NAFLD patients (16 patients with steatosis alone and 60 with NASH) demonstrated that activation of HSCs was positive in almost all cases, and markedly in two thirds of patients, and it was correlated with the degree and location of hepatic fibrosis. HSC activation and upregulation of profibrogenic genes were observed in rats on an HF diet. Lipid-peroxidation-associated inflammation and HSC activation with increased TGF- $\beta$ 1 mRNA expression in methionine-choline-deficient steatohepatitis models have also been reported.

Oxidative stress may also participate in the activation of HSCs and the development of fibrosis in NAFLD. The intracellular NADPH oxidase pathway produces ROS, and the disruption of NADPH oxidase protects mice from developing severe liver injury. Lipid peroxidation products enhance the production of both TGF- $\beta$  and collagen.

Currently, proposed mechanisms for the transformation from NASH to NASH-associated hepatocellular carcinoma are severe and cumulative oxidative stress to the hepatocytes, production of damaged DNA, defective or inhibited DNA repair systems, chronic continued hepatocyte injury and inflammatory infiltration, impaired antioxidant systems, and increased cell cycle of hepatocytes<sup>[65-67]</sup>. Animal and human studies have also indicated that a connection between age, sex and the disease might be possible<sup>[66-70]</sup>.

In conclusion, a liver with excess fat may be more vulnerable to stressors than a normal liver. The factors that play key roles in the development of NASH from NAFLD remain uncertain.

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## Fructose as a key player in the development of fatty liver disease

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### Abstract

We aimed to investigate whether increased consumption of fructose is linked to the increased prevalence of fatty liver. The prevalence of nonalcoholic steatohepatitis (NASH) is 3% and 20% in nonobese and obese subjects, respectively. Obesity is a low-grade chronic inflammatory condition and obesity-related cytokines such as interleukin-6, adiponectin, leptin, and tumor necrosis factor- $\alpha$  may play important roles in the development of nonalcoholic fatty liver disease (NAFLD). Additionally, the prevalence of NASH associated with both cirrhosis and hepatocellular carcinoma was reported to be high among patients with type 2 diabetes with or without obesity. Our research group previously showed that consumption of fructose is associated with adverse alterations of plasma lipid profiles and metabolic changes in mice, the American Lifestyle-Induced Obesity Syndrome model, which included consumption of a high-fructose corn syrup in amounts relevant to that consumed by some Americans. The observation

reinforces the concerns about the role of fructose in the obesity epidemic. Increased availability of fructose (e.g., high-fructose corn syrup) increases not only abnormal glucose flux but also fructose metabolism in the hepatocyte. Thus, the anatomic position of the liver places it in a strategic buffering position for absorbed carbohydrates and amino acids. Fructose was previously accepted as a beneficial dietary component because it does not stimulate insulin secretion. However, since insulin signaling plays an important role in central mechanisms of NAFLD, this property of fructose may be undesirable. Fructose has a selective hepatic metabolism, and provokes a hepatic stress response involving activation of c-Jun N-terminal kinases and subsequent reduced hepatic insulin signaling. As high fat diet alone produces obesity, insulin resistance, and some degree of fatty liver with minimal inflammation and no fibrosis, the fast food diet which includes fructose and fats produces a gene expression signature of increased hepatic fibrosis, inflammation, endoplasmic reticulum stress and lipopapoptosis. Hepatic *de novo* lipogenesis (fatty acid and triglyceride synthesis) is increased in patients with NAFLD. Stable-isotope studies showed that increased *de novo* lipogenesis (DNL) in patients with NAFLD contributed to fat accumulation in the liver and the development of NAFLD. Specifically, DNL was responsible for 26% of accumulated hepatic triglycerides and 15%-23% of secreted very low-density lipoprotein triglycerides in patients with NAFLD compared to an estimated less than 5% DNL in healthy subjects and 10% DNL in obese people with hyperinsulinemia. In conclusion, understanding the underlying causes of NAFLD forms the basis for rational preventive and treatment strategies of this major form of chronic liver disease.

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**Key words:** Nonalcoholic; Fatty liver; Diabetes; Insulin resistance; Cytokines; Obesity; Fructose



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## INTRODUCTION

Excessive accumulation of triglycerides in hepatocytes in the absence of significant alcohol consumption occurs in about 20%-30% of adults<sup>[1-5]</sup>. Excessive fat in the liver, called nonalcoholic fatty liver disease (NAFLD), predisposes to the development of nonalcoholic steatohepatitis (NASH). NASH constitutes the subset of NAFLD that is most worrisome because it is a significant risk factor for developing cirrhosis and its complications, including hepatocellular carcinoma (HCC)<sup>[6-9]</sup>. Because the accumulation of excess fat in the liver is a prerequisite for the development of NASH, understanding the underlying causes of NAFLD forms the basis for rational preventive and treatment strategies of this major form of chronic liver disease.

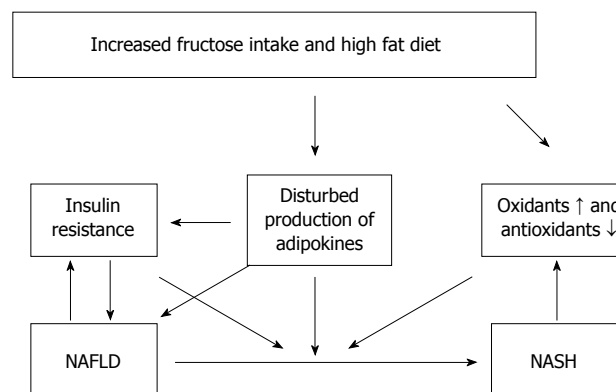
Obesity is a low-grade chronic inflammatory condition and obesity-related cytokines such as interleukin-6 (IL-6), adiponectin, leptin, and tumor necrosis factor (TNF)  $\alpha$  may play important roles in the development of NAFLD. The prevalence of NASH is 3% and 20% in nonobese and obese subjects, respectively. Additionally, the prevalence of NASH associated with both cirrhosis and HCC was reported to be high among patients with type-2 diabetes with or without obesity.

## OBESITY EPIDEMIC

A balance exists between energy demand and intake in the human body. Obesity is one of the major abnormalities of this well preserved equilibrium. Obesity, and its consequences such as insulin resistance and the metabolic syndrome, is a growing threat to the health of people in developed nations<sup>[10]</sup>. A diet based on high cholesterol, high saturated fat, and high fructose (cafeteria or fast food type) recapitulates features of the metabolic syndrome and NASH with progressive fibrosis (Figure 1).

## "FAST FOOD" OR "CAFETERIA" TYPE DIET COMPOSED OF HIGH SATURATED FATS, CHOLESTEROL, AND FRUCTOSE

The basis of the composition of "fast food" or "cafeteria" style food is high saturated fats, cholesterol, and fructose<sup>[11]</sup>. As the high fat diet produces obesity, insulin resistance, and some hepatic steatosis with minimal inflammation and no fibrosis, the fast food diet produces a gene expression signature of increased hepatic fibrosis, inflammation, endoplasmic reticulum stress and lipopapoptosis (Figure 2). Our research group previously



**Figure 1** Diet based on high cholesterol, high saturated fat, and high fructose (cafeteria or fast food type) recapitulates features of the metabolic syndrome and nonalcoholic fatty liver disease and nonalcoholic steatohepatitis with progressive fibrosis in human and mice. NAFLD: Nonalcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

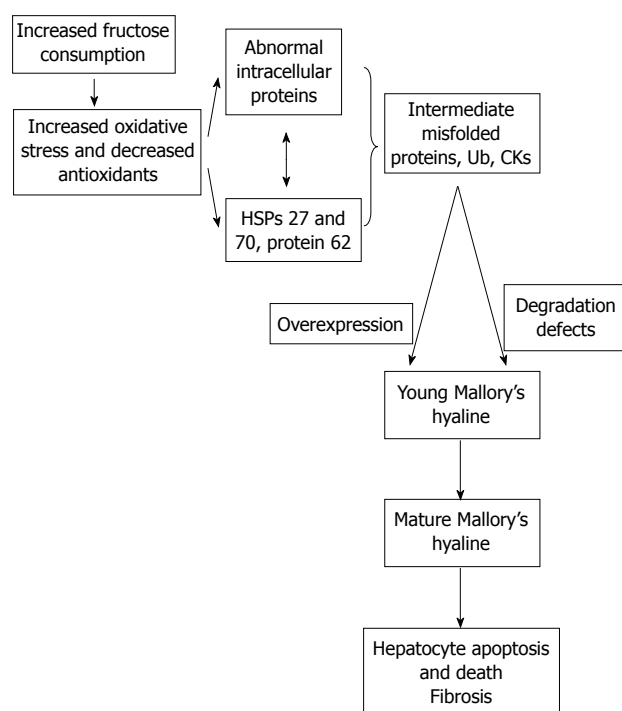
showed that consumption of fructose is associated with adverse alterations of plasma lipid profiles and metabolic changes in mice, the American Lifestyle-Induced Obesity Syndrome (ALIOS) model, which included consumption of a high-fructose corn syrup (HFCS) in amounts relevant to that consumed by some Americans<sup>[11]</sup>. The observation that the ALIOS mice indeed consumed a greater quantity of food beyond the additional calories consumed from the HFCS when fed HFCS compared with control water supports this observation and reinforces the concerns about the role of fructose in the obesity epidemic<sup>[12-15]</sup>. In adolescents, higher fructose consumption is associated with multiple markers of cardiometabolic risk, but it appears that these relationships are mediated by visceral obesity.

The most commonly used HFCS in soft drinks and other carbohydrate-sweetened beverages is a blend composed of 55% fructose, 41% glucose, and 4% complex polysaccharides. Fructose has increasingly been used as a sweetener since the introduction of high-fructose corn syrups in the 1960s<sup>[10-13,16]</sup> and is now an abundant source of dietary carbohydrate in the United States. The annual per capita consumption of extrinsic or added fructose was approximately 0.2 kg in 1970 to approximately 28 kg in 1997. This increased consumption has been linked to the increased prevalence of obesity, type 2 diabetes and fatty liver in the United States.

The liver is exquisitely sensitive to changes in nutrient delivery and is uniquely suited to metabolize ingested simple sugars, such as fructose and glucose<sup>[13,14]</sup>. Stress-activated protein kinases, principally the c-Jun N-terminal kinases (JNK), are activated by cell stress-inducing stimuli. Increased fructose supply provokes a hepatic stress response involving activation of JNK and subsequent reduced hepatic insulin signaling.

## UNIQUE METABOLISM OF FRUCTOSE

Fructose, glucose, and galactose are the 3 major dietary



**Figure 2** As the high fat diet produces obesity, insulin resistance, and some hepatic steatosis with minimal inflammation with no fibrosis, the fast food diet produces a gene expression signature of increased hepatic fibrosis, inflammation, and endoplasmic reticulum stress and lipooapoptosis. HSP: Heat shock proteins.

monosaccharides. Sucrose (glucose-fructose), lactose (glucose-galactose), and maltose (glucose-glucose) are the major disaccharides. Dietary fructose occurs in 2 forms: mono- or disaccharide. The rate of fructose absorption appears to be between that of mannose and glucose<sup>[12-15]</sup>. Fructose is absorbed by carrier-mediated facilitated diffusion, an energy-dependent process. The fructose carrier is a member of the glucose transport family and is referred to as glucose transporter 5. Sucrose is cleaved to glucose and fructose by sucrase, an enzyme located in the brush border of small intestine enterocytes.

Fructose was previously accepted as a beneficial dietary component because it does not stimulate insulin secretion. However, since insulin signaling plays an important role in the central mechanisms of NAFLD, this property of fructose may be undesirable<sup>[13-15]</sup>. Additionally, fructose may prevent suppression of ghrelin secretion, resulting in impaired satiety mechanisms<sup>[14]</sup>. In large quantities, fructose can also stress the liver by depleting hepatic energy supplies. Normal subjects and patients with NASH exhibited a similar depletion of hepatic ATP levels after an injection of fructose, but recovery of ATP levels after depletion was slower in NASH patients compared with healthy controls. A mixture of fructose and glucose might induce metabolic abnormalities that differ from sucrose, a disaccharide cleaved to fructose and glucose in the small intestine.

Phosphorylation of glucose by glucokinase is a rate-de-

termining step in hepatic glucose metabolism. In contrast to glucose, phosphorylation of fructose in the liver occurs *via* the enzyme fructokinase. In addition, the metabolism of fructose 1-phosphate in the liver occurs independently of phosphofructokinase, a second rate-determining step in glucose metabolism<sup>[13-15]</sup>. As a result, the liver is the primary site of fructose extraction and metabolism, with extraction approaching 50% to 70% of fructose delivery. Therefore, increased availability of fructose (*e.g.*, high-fructose corn syrup) will increase not only abnormal glucose flux but also fructose metabolism in the hepatocyte. Thus, the anatomic position of the liver places it in a strategic buffering position for absorbed carbohydrates and amino acids.

Fructose extraction and metabolism by the liver are exceptionally high compared to glucose due both to the extensive amount of fructokinase that phosphorylates fructose to fructose 1-phosphate in the liver and to the subsequent metabolism of fructose 1-phosphate at the triose phosphate level, which bypasses flux control at phosphofructokinase<sup>[13-16]</sup>. Previous studies comparing the metabolism of fructose and glucose in postabsorptive humans over short intervals have shown that fructose is used faster than glucose and that more is converted to liver glycogen. Fructose oxidation represented a significant portion of fructose metabolism, accounting for 56% to 59% of the ingested fructose and approximately 33% of the infused fructose. It is likely that extrahepatic lactate oxidation subsequent to hepatic fructolysis contributed significantly to the estimated rate of fructose oxidation. Thus, increments in fructose after infusion produced immediate changes in hepatic and extrahepatic substrate metabolism, but did not induce changes in overall glucose production. An immediate fructose infusion in humans induced both hepatic and extrahepatic insulin resistance. These data are consistent with the notion that high concentrations of fructose elicit adaptations in the liver that include metabolic intermediates, gene expression, and insulin action.

## SYSTEMIC AND HEPATIC INSULIN RESISTANCE IN NAFLD

While insulin receptor defects cause severe insulin resistance, most patients with insulin resistance have impaired post-receptor intracellular insulin signaling. Insulin binds  $\alpha$ -subunits of its receptor, which is a cell surface receptor on the major insulin sensitive cells such as skeletal muscle, adipocytes, and hepatocytes, leading to autophosphorylation of the cytoplasmic domains ( $\beta$ -subunits) of the receptor<sup>[2,5,17]</sup>. The insulin receptor has intrinsic tyrosine kinase activity activated by insulin binding and the autophosphorylated receptor activates its substrates that include insulin receptor substrate (IRS)-1, IRS-2, Src homology collagen, and adaptor protein with a pleckstrin homology and Src homology 2 domain by tyrosine phosphorylation. These phosphory-

lated docking proteins bind and activate several downstream components of the insulin signaling pathways. Activated IRS-1 associates with phosphatidylinositol 3-kinase, which then activates Akt. These events and insulin-dependent inhibition of hepatic glucose output maintain glucose homeostasis. Insulin also affects glucose homeostasis indirectly by its regulatory effect on lipid metabolism. Any interference in this insulin signaling pathway causes glucotoxicity, insulin resistance and, when islet beta cells are capable of responding, compensatory hyperinsulinemia.

Hepatic expression of insulin receptor protein in humans and the levels of both IRS-1 and IRS-2 in animals were decreased in chronic hyperinsulinemic states<sup>[11]</sup>. IRS-1 was more closely linked to glucose homeostasis with the regulation of glucokinase expression while IRS-2 was more closely linked to lipogenesis with the regulation of lipogenic enzymes sterol regulatory element-binding protein-1c (SREBP-1c) and fatty acid synthase<sup>[18,19]</sup>. Additional physiological roles of insulin include regulating the metabolism of macronutrients and stimulating cellular growth. Insulin activates synthesis and inhibits catabolism of lipids while shutting off the synthesis of glucose in the liver.

Adipose tissue is one of the major insulin sensitive organs in the human body and the process of differentiation of preadipocytes to adipocytes is induced by insulin<sup>[17,18]</sup>. Within the adipose tissue, insulin stimulates triglyceride synthesis and inhibits lipolysis by upregulating lipoprotein lipase activity which is the most sensitive pathway in insulin action, facilitating free fatty acid uptake and glucose transport, inhibiting hormone sensitive lipase, and increasing gene expression of lipogenic enzymes.

## PROINFLAMMATORY SIGNALING IN INSULIN RESISTANCE

Protein kinase C theta (PKC $\theta$ ) and inhibitor  $\kappa$ B kinase  $\beta$  (IKK- $\beta$ ) are two proinflammatory kinases involved in insulin downstream signaling<sup>[17,18]</sup>. They are activated by lipid metabolites such as high plasma free fatty acid concentrations and there is a positive relationship between the activation of PKC $\theta$  and the concentration of intermediate fatty acid products. PKC $\theta$  activates both IKK- $\beta$  and JNK, leading to increased Ser 307 phosphorylation of IRS-1 and insulin resistance. Activation or overexpression of IKK- $\beta$  diminishes insulin signaling and causes insulin resistance whereas inhibition of IKK- $\beta$  improves insulin sensitivity. Inhibition of IKK- $\beta$  activity prevented insulin resistance due to TNF- $\alpha$  in cultured cells. IKK- $\beta$  phosphorylates the inhibitor of nuclear factor kappa B (NF- $\kappa$ B), leading to the activation of NF- $\kappa$ B by the translocation of NF- $\kappa$ B to the nucleus. NF- $\kappa$ B is an inducible transcription factor and promotes specific gene expression in the nucleus. For example, NF- $\kappa$ B regulates the production of multiple inflama-

tory mediators, such as TNF- $\alpha$  and IL-6. TNF- $\alpha$  and reactive oxygen species could also activate NF- $\kappa$ B<sup>[19-22]</sup>. In contrast, antioxidants inhibit this activation. NF- $\kappa$ B has both apoptotic and anti-apoptotic effects. The finding that NF- $\kappa$ B deficient mice were protected from high-fat diet-induced insulin resistance suggests that NF- $\kappa$ B directly participates in processes that impair insulin signaling. High-dose salicylates also inhibit NF- $\kappa$ B and subsequently improve insulin sensitivity. These subsequently promote hepatic and systemic insulin resistance. The study group also showed that these results were reversed by curcumin which inhibits NF- $\kappa$ B activity. Curcumin also has the ability to induce antioxidant enzymes and scavenge ROS.

Suppressors of cytokine signaling (SOCS) and inducible nitric oxide synthase are two inflammatory mediators recently recognized to play a role in insulin signaling<sup>[23-25]</sup>. Induction of SOCS proteins (SOCS 1-7 and cytokine-inducible src homology 2 domain-containing protein) by proinflammatory cytokines might contribute to the cytokine-mediated insulin resistance in obese subjects<sup>[26-30]</sup>. In fact, the isoforms of SOCS are the members of a negative feedback loop of cytokine signaling, regulated by both phosphorylation and transcription events. SOCS-1, and particularly SOCS-3, are involved in the inhibition of insulin signaling either by interfering with IRS-1 and IRS-2 tyrosine phosphorylation or by the degradation of their substrates. SOCS-3 might also regulate central leptin action and play a role in the leptin resistance of obese human subjects. SOCS might be a link between leptin and insulin resistance because insulin levels are increased in leptin resistant conditions due to the diminished insulin suppression effect of leptin because of insufficient leptin levels. Moreover, SOCS proteins might involve insulin/insulin like growth factor-1 signaling. SOCS-1 knockout mice showed low glucose concentrations and increased insulin sensitivity. SREBP-1c is one of the key mediators of lipid synthesis from glucose and other precursors (*de novo* lipogenesis) in the liver. Indeed, SOCS proteins markedly induce *de novo* fatty acid synthesis in the liver by both the up-regulation of SREBP-1c and persistent insulin resistance with hyperinsulinemia which stimulates SREBP-1c-mediated gene expression. Liver is the insulin clearance organ. Thus, decreased insulin clearance in patients with NAFLD further elevates insulin levels in the circulation and *de novo* lipogenesis in the liver. SOCS-1 and SOCS-3 may exert these effects by inhibiting signal transduction and activator of transcription proteins (STAT), particularly STAT-3, *via* binding Janus tyrosine Kinase (JAK) tyrosine kinase because this binding diminishes the phosphorylation ability of JAK kinase to STAT-3. STAT-3 inhibits the activation of SREBP-1c. Specific STAT-3 knockout mice showed markedly increased expression of SREBP-1c and subsequently increased fat content in the liver. Conversely, inhibition of SOCS proteins, particularly SOCS-3, improved both insulin sensitivity and the activation of

SREBP-1c which eventually reduced liver steatosis and hypertriglyceridemia in db/db mice.

Nitric oxide synthase-2 (NOS2) or inducible nitric oxide synthase (iNOS) production are also induced by proinflammatory cytokines<sup>[31]</sup>. A high-fat diet in rats causes up-regulation of iNOS mRNA expression and increases iNOS protein activity. Increased production of NOS2 might reduce insulin action in both muscle and pancreas and decreased iNOS activity protects muscles from the high-fat diet induced insulin resistance. It was also shown that leptin deficient ob/ob mice without iNOS were more insulin sensitive than ob wild-type mice. Thus, the production of nitric oxide may be one link between inflammation and insulin resistance.

## SOURCES OF LIVER FAT

Accumulation of triglycerides as fat droplets within the cytoplasm of hepatocytes is a prerequisite for subsequent events of NASH. Accumulation of excess triglyceride in hepatocytes is generally the result of increased delivery of non-esterified fatty acids (NEFAs), increased synthesis of NEFAs, impaired intracellular catabolism of NEFAs, impaired secretion as triglyceride, or a combination of these abnormalities<sup>[32]</sup>. Recent techniques, such as isotope methodologies, multiple-stable-isotope approach and gas chromatography/mass spectrometry, provided valuable information regarding the fate of fatty acids during both fasting and fed states<sup>[33]</sup> such as the relative contribution of three fatty acid sources to the accumulated fat in NAFLD: adipose tissue, *de novo* lipogenesis, and dietary fat. Additionally, these studies reported that the plasma NEFA pool is the main contributor of both hepatic triglycerides in the fasting state and very low-density lipoproteins (VLDL)-triglycerides in both fasting and fed states.

## DYSREGULATED PERIPHERAL LIPOLYSIS

A study showed that adipose tissue makes a major contribution to the plasma NEFA pool, contributing 81.7% in the fasted state and 61.7% in the fed state<sup>[33]</sup>. Additionally, the contribution of dietary lipids to the plasma NEFA pool was found to be only 26.2% and 10.4% in fed and fasted states, respectively, in the same study. Finally, the contribution of newly made fatty acids (originating from the adipose tissue and liver) to the plasma NEFA pool was 7.0% and 9.4% for the fasted and fed states, respectively.

The liver takes up free fatty acids from the circulating NEFA pool and the rate of uptake depends only on the plasma free fatty acid concentrations. Hepatic NEFA uptake continues despite increased hepatic content of fatty acids and triglycerides<sup>[34]</sup>. The concentration of free fatty acids is increased in the portal circulation rapidly when lipolysis occurs in visceral adipose tissue. These products directly flux to the liver *via* the splanchnic circulation and contribute to hepatic triglyceride synthesis, NAFLD, and

hepatic insulin resistance.

## HEPATIC *DE NOVO* LIPOGENESIS

Hepatic *de novo* lipogenesis (fatty acid and triglyceride synthesis) is increased in patients with NAFLD<sup>[35-39]</sup>. Stable-isotope studies showed that increased *de novo* lipogenesis (DNL) in patients with NAFLD contributed to fat accumulation in the liver and the development of NAFLD<sup>[33]</sup>. Specifically, DNL was responsible for 26% of accumulated hepatic triglycerides and 15%-23% of secreted VLDL triglycerides in patients with NAFLD compared to an estimated less than 5% DNL in healthy subjects and 10% DNL in obese people with hyperinsulinemia. Interestingly, Donnelly and colleagues demonstrated the similarity between VLDL-triglycerides and hepatic-triglycerides regarding contributions of fatty acid sources (62% *vs* 59% for NEFA contribution, respectively; 23% *vs* 26% for DNL, respectively; and 15% *vs* 15% for dietary fatty acids, respectively) in NAFLD patients. Substrates used for the synthesis of newly made fatty acids by DNL are primarily glucose, fructose, and amino acids; oleic acid (18:1, a  $\omega$ -6 monounsaturated fatty acid, which is relatively resistant to peroxidation) is the major end product of *de novo* fatty acid synthesis<sup>[40-42]</sup>. Moreover, simple sugars have the ability to stimulate lipogenesis<sup>[33]</sup>. Ingested carbohydrates are a major stimulus for hepatic delayed neuronal loss and are thus more likely to directly contribute to NAFLD than dietary fat intake<sup>[43-46]</sup>.

In conclusion, fructose has increasingly been used as a sweetener since the introduction of high-fructose corn syrups in the 1960s and is now an abundant source of dietary carbohydrate in the United States<sup>[47-50]</sup>. The most commonly used HFCS in soft drinks and other carbohydrate-sweetened beverages is a blend composed of 55% fructose, 41% glucose, and 4% complex polysaccharides<sup>[51-55]</sup>. This increased consumption has been linked to the increased prevalence of obesity and type 2 diabetes and fatty liver in the United States by increased fructose supply, which provokes a hepatic stress response involving activation of JNK and subsequent reduced hepatic insulin signaling<sup>[56-59]</sup>. Understanding the underlying causes of NAFLD forms the basis for rational preventive and treatment strategies of this major form of chronic liver disease.

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## Histone deacetylase inhibitors and pancreatic cancer: Are there any promising clinical trials?

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### Abstract

Pancreatic cancer, although not very frequent, has an exceptionally high mortality rate, making it one of the most common causes of cancer mortality in developed countries. Pancreatic cancer is difficult to diagnose, allowing few patients to have the necessary treatment at a relatively early stage. Despite a marginal benefit in survival, the overall response of pancreatic cancer to current systemic therapy continues to be poor, and new therapies are desperately needed. Histone deacetylase (HDAC) enzymes play an important role in the development and progression of cancer and HDAC inhibitors (HDACIs) have been shown to induce differentiation and cell cycle arrest, activate the extrinsic or intrinsic pathways of apoptosis, and inhibit invasion, migration and angiogenesis in different cancer cell lines. As a result of promising preclinical data, various HDACIs are being tested as either monotherapeutic agents or in combination regimens for both solid and hematological malignancies. Vorinostat was the first HDACI approved by the Food and Drug Administration for patients with cutaneous T-cell lymphoma. The use of HDACIs in clinical trials, in pretreated and relapsed patients suffering

from advanced pancreatic cancer is discussed. Unfortunately, clinical data for HDACIs in patients with pancreatic cancer are inadequate, because only a few studies have included patients suffering from this type of neoplasm and the number of pancreatic cancer patients that entered HDACIs phase II/III trials, among others with advanced solid tumors, is very limited. More studies recruiting patients with pancreatic cancer remain to determine the efficiency of these therapies.

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**Key words:** Pancreatic cancer; Histone deacetylases; Histone deacetylase inhibitors; Clinical trials

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### INTRODUCTION

Worldwide, over 200 000 people die annually of pancreatic cancer. The incidence of pancreatic cancer varies greatly across regions, with the highest incidence and mortality rates found in developed countries, which suggests roles for lifestyle and environmental factors. Deaths from pancreatic cancer rank fourth among cancer-related deaths in the United States. Risk factors for pancreatic cancer include, among others, high-fat diet, smoking, chronic pancreatitis, primary sclerosing cholangitis, hereditary pancreatitis, family history of pancreatic cancer, and diabetes mellitus. Age seems to be a significant risk factor, with incidence increasing with age.

Due to few early illness indicators and lack of screening tests, pancreatic cancer is difficult to diagnose and often at the time of presentation, the cancer has already become advanced. The overall 5-year survival rate among

patients with pancreatic cancer is < 5%. Approximately 20% of patients present with localized, potentially curable tumors. The majority (95%) of cases of pancreatic cancer are adenocarcinomas, resembling the pancreatic ductal cell. Metastasis of this cancer can be either local, most often involving the spleen, adrenal glands and transverse colon, or distant<sup>[1,2]</sup>.

## TREATMENT OF ADVANCED PANCREATIC CANCER

### Conventional chemotherapies

For over a decade, gemcitabine-based therapy has been considered a first-line treatment for locally advanced and metastatic pancreatic cancer. Preference for gemcitabine over 5-fluorouracil (5-FU) was established in the mid-1990s, when a phase III trial comparing gemcitabine monotherapy to 5-FU demonstrated clinical benefit in gemcitabine-treated patients with advanced pancreatic cancer<sup>[3]</sup>. The value of radiotherapy in the management of locally advanced pancreatic cancer remains unclear<sup>[4]</sup>.

Numerous attempts have been made to improve the efficacy of standard gemcitabine monotherapy in patients with advanced pancreatic cancer, but little success has been achieved. Several phase III trials were undertaken with gemcitabine in combination with a range of chemotherapy agents. However, the combinations of gemcitabine with 5-FU<sup>[5]</sup>, as well as irinotecan, oxaliplatin, pemetrexed, exatecan and cisplatin<sup>[6-10]</sup>, all failed to show superiority over gemcitabine monotherapy.

Capecitabine is an orally administered fluoropyrimidine that is metabolized in both liver and tumor cells into 5-FU, resulting in high intratumoral 5-FU concentrations. Phase III trial results on capecitabine and gemcitabine combination appeared to be contradictory. In a recent phase III trial, the combination significantly improved objective response rate and progression-free survival (PFS), but did not show superiority in overall survival (OS) in patients with advanced pancreatic cancer<sup>[11]</sup>.

In addition, folfirinox (5-FU/leucovorin, irinotecan, and oxaliplatin) treatment of metastatic pancreatic cancer patients in a randomized phase II trial of folfirinox versus gemcitabine, indicated that response rate was more than 30%. Furthermore, the median OS was 11.1 mo in the folfirinox group as compared with 6.8 mo in the gemcitabine group, indicating that folfirinox is an option for the treatment of patients with metastatic pancreatic cancer<sup>[12]</sup>.

### Targeted therapies

Targeted therapies have also been investigated for advanced pancreatic cancer. The matrix metalloproteinase inhibitors (MMPi) marimastat and talomastat (BAY 12-9566) inhibit enzymes that play a key role in extracellular matrix (ECM) degradation, and angiogenesis. In phase III trials, neither marimastat monotherapy nor marimastat with gemcitabine, improved OS compared with gemcitabine monotherapy<sup>[13]</sup>.

The farnesyl transferase enzyme Kras regulator tipifarnib in combination with gemcitabine did not improve OS compared with gemcitabine monotherapy in a phase III trial<sup>[14]</sup>.

Erlotinib is a small-molecule tyrosine kinase inhibitor (TKI) of the human epidermal growth factor receptor (EGFR). A multicenter, randomized, double-blind, placebo-controlled phase III clinical trial of erlotinib in combination with gemcitabine, in patients with locally advanced or metastatic pancreatic adenocarcinoma met its primary endpoint, with the combination regimen being the first gemcitabine combination to demonstrate a statistically significant survival advantage over gemcitabine monotherapy and the regimen was consequently approved for metastatic disease<sup>[15]</sup>.

Cetuximab, an anti-EGFR monoclonal antibody, blocks the extracellular EGFR domain, preventing ligand-dependent or independent activation and downstream signaling. An open-label, randomized phase III trial, unfortunately, recently failed to demonstrate a clinically significant advantage of the addition of cetuximab to gemcitabine for response and OS<sup>[16]</sup>.

Bevacizumab is a recombinant, humanized IgG1 monoclonal antibody that selectively binds to vascular endothelial growth factor (VEGF), inhibiting its interaction with VEGF receptor-1 and -2, on the surface of endothelial cells. Despite recently reported negative results, a number of phase II and III studies are underway in advanced pancreatic cancer that include bevacizumab and cetuximab in combination with other agents<sup>[17]</sup>.

Finally, a wide range of molecular-targeted agents that interact with crucial pathways for cell survival in pancreatic cancer are currently being explored. These include agents that target polyADP-ribose polymerase, histone deacetylase (HDAC), Src/Abl kinases, and mammalian target of rapamycin<sup>[18]</sup>.

Given the positive data observed in phase III trials, gemcitabine, erlotinib and capecitabine are likely to form the base for future treatment strategies for advanced pancreatic cancer.

### Histone acetyltransferases and HDACs

The organization of chromatin is crucial for the regulation of gene expression. Accumulating evidence suggests that the acetylation and deacetylation of histones play significant roles in transcriptional regulation of eukaryotic cells. Histone acetylation and deacetylation are catalysed by specific enzyme families, Histone acetyltransferases (HATs) and HDACs, respectively<sup>[19,20]</sup>. There are at least four groups of proteins with intrinsic HAT activity, while 18 HDACs belonging to four distinct classes have been described in mammalian cells. Eleven of the HDACs are zinc dependent, classified on the basis of homology to yeast HDACs: Class I includes HDACs 1, 2, 3 and 8; Class II A includes HDACs 4, 5, 7 and 9; Class II B, HDACs 6 and 10; and Class IV, HDAC11. Class III HDACs, sirtuins 1-7, have an absolute requirement for NAD<sup>+</sup>, are not zinc dependent, and generally not inhib-

ited by compounds that inhibit zinc-dependent deacetylases<sup>[21]</sup>. Apart from deacetylating histones, HDACs have also been reported to interact with non-histone proteins, involved in numerous important cell pathways including control of gene expression, regulation of cell proliferation, differentiation, migration and death. The balance between acetylation and deacetylation is an important factor in regulating gene expression and disruption of HAT or HDAC activity is possibly associated with cancer development<sup>[22,23]</sup>.

### HDAC inhibitors

HDAC inhibitors (HDACIs) are divided into different classes based on their chemical properties, including hydroxamic acids, such as suberoylanilide hydroxamic acid (SAHA), trichostatin A (TSA), LBH589 (panobinostat) and PXD101 (belinostat); short chain fatty acids, such as sodium butyrate (NaBu), 4-phenylbutyrate (4-PB) and valproic acid; cyclic tetrapeptides, such as trapoxin, apicidin and depsipeptide-also known as FK228 or romidepsin; benzamides, such as MS-275, CI-994 and MGCD0103, and a variety of other chemical compounds and synthetic inhibitors. HDACIs have three common structural characteristics: a Zn-binding moiety, an opposite capping group, and a straight chain alkyl, vinyl or aryl linker connecting the two<sup>[24,25]</sup>.

HDAC inhibition is able to promote a variety of different anticancer mechanisms including apoptosis, cell differentiation and cell cycle inhibition. HDACIs are a new class of antineoplastic agents currently being evaluated in clinical trials. While these agents have been studied extensively in the laboratory, only recently has their mechanism of action begun to be elucidated. Several HDACIs are now in various stages of development, including clinical trials as monotherapy and in combination with other anticancer drugs and radiation<sup>[26,27]</sup>.

In the present review, the available so far data regarding the different classes of HDACIs used in clinical trials including patients with pancreatic cancer are presented in Table 1.

### Hydroxamic acids

SAHA (N-hydroxy-N'-phenyl-octanediamide, vorinostat) is a synthetic hydroxamic acid, which is structurally related to the natural product, TSA (7-[4-(dimethylamino)phenyl]-N-hydroxyl-4,6-dimethyl-7-oxo-(2E,4E,6R)-2,4-heptadienamide), produced by selected strains of *Streptomyces platensis*, *Streptomyces hygroscopicus* Y-50 or *Streptomyces sioyaensis*. Hydroxamic acids have a high affinity to bio-metals, including Fe<sup>3+</sup>, Ni<sup>2+</sup> and Zn<sup>2+</sup>. The synthesis of SAHA and its potency to induce differentiation of murine erythroleukemia (MEL) cells was first reported in 1996. SAHA and TSA comprise a hydroxamic-acid-based metal-binding domain that coordinates the catalytic Zn<sup>2+</sup> in the HDAC active site, a 5 (TSA) or 6 (SAHA)-membered carbon-based linker that mimics the C $\alpha$  functional group of lysine, and a hydrophobic motif that interacts with the periphery of the HDAC binding pocket<sup>[28]</sup>.

**SAHA:** SAHA (vorinostat) is now undergoing several clinical trials. One current phase I trial is studying the side effects and best dose of SAHA given together with flavopiridol in treating patients with advanced solid tumors. Another trial is studying SAHA in patients with metastatic or unresectable solid tumors or lymphoma and liver dysfunction. Combination with doxorubicin is also under survey for solid tumors, as well as that with bortezomib, vinorelbine, gemcitabine and other agents like paclitaxel and carboplatin, fluorouracil/leucovorin and oxaliplatin. A phase I / II trial is studying the highest tolerable dose of SAHA that can be given in combination with radiotherapy to patients with locally advanced pancreatic cancer, as well the efficacy of combined therapy. Another phase I clinical trial is now examining the safety, pharmacokinetics, pharmacodynamics and efficacy of *in vivo* proteasome inhibitor NPI-0052 in combination with oral SAHA in patients with non-small cell lung cancer, pancreatic cancer, melanoma or lymphoma. A phase I / II study of SAHA in combination with radiotherapy and infusional 5-FU in patients with locally advanced adenocarcinoma of the pancreas is under way. Finally, SAHA in combination with capecitabine plus radiotherapy is also being evaluated in patients with non-metastatic pancreatic cancer. The above studies are recruiting for participants and possible patients suffering from pancreatic cancer remain to determine the efficiency of these therapies<sup>[29]</sup>.

### Cyclic peptides

**FK228 (FR901228, depsipeptide, romidepsin):** Depsipeptide (1S,4S,7Z,10S,16E,21R)-7-ethylidene-4,21-bis (1-methylethyl)-2-oxa-12,13-dithia-5,8-,20,23-tetraazabicyclo[8.7.6]tricos-16-ene-3,6,9,19,22-pentone, is a bicyclic peptide isolated from *Chromobacterium violaceum* and has demonstrated potent *in vitro* cytotoxic activity against human tumor cell lines and *in vivo* efficacy against human tumor xenografts. Upon entering cells, FK228 is reduced to an active compound, capable of preferentially interacting with the zinc in the active site of the HDAC class I enzymes, however, it is still generally classified as a broad-spectrum inhibitor as it does inhibit class II enzymes. It was approved by the United States Food and Drug Administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL)<sup>[30]</sup>.

Among other current clinical trials for patients with advanced solid tumors, FK228 is being studied in combination with gemcitabine in patients with pancreatic cancer. This phase I / II dose escalation trial is designed to determine the maximum tolerated dose for the combination, as well as evaluate toxicities and objective disease responses. Furthermore, another phase II trial is studying the effectiveness of FK228 in patients who have locally advanced or metastatic neuroendocrine tumors; among them pancreatic islet tumors<sup>[29]</sup>.

### Short-chain fatty acids

**Valproic acid:** Valproic acid (VPA) is now an established antiepileptic drug, by affecting the function of the neu-



**Table 1 Clinical studies on histone deacetylase inhibitors for treatment of patients with pancreatic cancer**

HDACI	Patients total number	Patients with PC number	Time schedule	Dosage schedule (HDACI)	MTD (HDACI)	Responses (for patients with pancreatic cancer treated with HDACI)			Main toxicities	Ref.
						PR	SD	PD		
VPA + epirubicin phase I	48	1	q21 d	15-160 mg/kg per day <i>iv/po</i> d1, d2	140 mg/kg per day	1	-	-	Neutropenia, thrombocytopenia, hypocalcaemia, fatigue, QTc prolongation	[33]
4-PB phase I	21	1	q28 d	60-360 mg/kg <i>iv</i> 2 × daily d1-d5, d8-d12	300 mg/kg per day	-	-	1	Fatigue, headache, nausea, vomiting, confusion	[36]
MS-275 + 13-cis retinoic acid phase I	Not defined	1	q28 d	4-5 mg/m <sup>2</sup> <i>po</i> , once weekly	5 mg/m <sup>2</sup>	-	1	-	Hyponatremia, neutropenia, anemia, fatigue	[39]
MS-275 phase I	27	1	a: q14 d b: q28 d c: q28 d	2-6 mg/m <sup>2</sup> <i>po</i> a: one dose 1 <sup>st</sup> wk b: 2 × weekly for 3 wk c: 1 × weekly for 3 wk	a: 6 mg/m <sup>2</sup> b: not defined c: 4 mg/m <sup>2</sup>	-	-	1	Hypophosphatemia, hyponatremia, nausea, asthenia, fatigue, anorexia <sup>1</sup>	[40]
CI-994 phase II	17	17	Continues	8 mg/m <sup>2</sup> per day <i>po</i>	-	-	2	N/D	Thrombocytopenia, fatigue, anorexia, nausea, vomiting, bruising, hematuria	[42]
CI-994 phase I	53	1	a: acute q21 d b: chronic q10 wk	a: 10-15 mg/m <sup>2</sup> per day <i>po</i> d1-d14 b: 5-12.5 mg/m <sup>2</sup> per day <i>po</i> d1-d56	a: 15 mg/m <sup>2</sup> b: 8 mg/m <sup>2</sup>	-	-	1	Thrombocytopenia, neutropenia, nausea, vomiting, diarrhea, fatigue, hoarseness, paresthesias, alopecia	[43]
CI-994 + capecitabine phase I	54	4 sch. c	a: q21 d b: q42 d c: q21 d	a: 4-10 mg/m <sup>2</sup> per day <i>po</i> d1-d14 b: 6 mg/m <sup>2</sup> per day <i>po</i> d1-d35 c: 4-8 mg/m <sup>2</sup> per day <i>po</i> d1-d14	a: 10 mg/m <sup>2</sup> b: (abandoned) c: 8 mg/m <sup>2</sup>	-	-	4	Thrombocytopenia, anemia, anorexia, diarrhea, nausea, vomiting, fatigue	[44]
CI-994 + carboplatin paclitaxel phase I	30	2	q21 d	4-6 mg/m <sup>2</sup> per day <i>po</i> d1-d14 or d1-d7	6 mg/m <sup>2</sup> (d1-d7)	-	-	2	Thrombocytopenia, neutropenia, fatigue, alopecia, nausea	[45]
CI-994 + gemcitabine <i>vs</i> gemcitabine alone phase II	174	174 (86 + 88)	q28 d	6 mg/m <sup>2</sup> per day <i>po</i> d1-d21	-	8/68	-	60/68	Thrombocytopenia, neutropenia, anemia, nausea, vomiting, anorexia, diarrhea	[46]
MGCD-0103 phase I	38	2	q21 d	12.5-56 mg/m <sup>2</sup> per day <i>po</i> d1, 3, 5, 8, 10, 12	56 mg/m <sup>2</sup> RPTD: 45 mg/m <sup>2</sup>	-	-	2	Fatigue, anorexia, nausea, vomiting, diarrhea, abdominal pain, dehydration	[48]
LAQ824 phase I	39	3	q21 d	6-100 mg/m <sup>2</sup> per day <i>iv</i> d1-d3	72 mg/m <sup>2</sup> RPTD: < 72 mg/m <sup>2</sup>	-	-	3	Thrombocytopenia, fatigue, anorexia, nausea, vomiting, diarrhea, nonspecific ST-segment, hyperbilirubinemia, transaminitis <sup>2</sup>	[50]

<sup>1</sup>The patient with pancreatic cancer experienced grade 3 hypophosphatemia as DLT and was finally excluded from study after cycle 2 due to PD; <sup>2</sup>At 100 mg/m<sup>2</sup> one patient with advanced pancreatic cancer, developed grade 4 hyperbilirubinemia associated with febrile neutropenia on day 3. The patient developed thrombocytopenia on day 8 of treatment and anemia, necessitating platelet and blood transfusions. This was followed by an episode of atrial fibrillation on day 13 and then acute renal failure. The patient finally died on d18. PC: Pancreatic cancer; PD: Progressive disease; PR: Partial response; RPTD: Recommended dose for phase II; SD: Stable disease.

rotransmitter GABA. The finding that VPA was an effective inhibitor of HDACs arose from the observations that VPA was able to relieve transcriptional repression of a peroxisomal proliferation and activation of a glucocorticoid receptor (GR)-PPAR $\epsilon$  hybrid receptor, suggesting that it acts on a common mechanism in gene regulation, such as histone deacetylation, rather than on individual transcription factors or receptors. Consistent with this finding, it was shown that VPA causes hyperacetylation of the N-terminal tails of histones H3 and H4 *in vitro* and *in vivo*, and was found to inhibit HDAC enzymatic activity at a concentration of 0.5 mmol/L<sup>[31]</sup>. VPA has shown potent antitumor effects in a variety of *in vitro* and *in vivo* systems, by modulating multiple pathways including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation and senescence. Most preclinical and clinical data on the anticancer effects of VPA have been generated for malignant hematological diseases<sup>[32]</sup>.

A phase I clinical trial investigated the safety, toxicity and maximum-tolerated dose of VPA and the topoisomerase II inhibitor epirubicin in solid tumors. Forty-eight patients with different malignancies were enrolled; one with pancreatic cancer. The patient suffering from pancreatic cancer experienced a partial response at 100 mg/kg VPA and 100 mg/m<sup>2</sup> on day 3 of the cycle, while no dose-limiting toxicities occurred. All patients with a partial response had at least a twofold increase in histone acetylation<sup>[33]</sup>.

Phase I clinical trials are currently testing VPA in combination with other agents such as erlotinib, 5-FU, cyclophosphamide, bevacizumab, and azacytidine, as well as epirubicin to determine safety, tolerability and effectiveness in treating patients with advanced solid tumors. In addition, another phase II trial is studying VPA combined with the hypomethylating factor hydralazine. A phase I clinical trial is undertaking recruitment to determine maximum tolerated doses of VPA in combination with sunitinib, sorafenib, dasatinib, erlotinib, lapatinib, or lenalidomide for the treatment of patients with advanced solid tumors, as well as to estimate the safety and treatment response<sup>[29]</sup>.

**4-PB:** 4-PB is a short-chain fatty acid known to inhibit reversibly class I and II HDACs. It is considered as an HDAC inhibitor of the first generation, as the HDAC inhibitory effect is not specific. Working concentrations are rather high, in the millimolar range, and the effects are pleiotropic. 4-PB is known to exert multiple effects in the cell, including the modulation of protein isoprenylation, which importantly regulates the ras proto-oncoprotein, and activation of the nuclear steroid PPAR<sup>[34]</sup>. 4-PB exerts a potent antitumor effect *in vitro* and has been shown to cause growth inhibition and differentiation in various human cancer cell lines<sup>[35]</sup>.

A phase I dose escalating trial to evaluate twice daily *iv* 4-PB infusion has been undertaken. 4-PB was administered for five consecutive days for a total of 20 doses over two consecutive weeks from 60 to 360 mg/kg per

day. Twenty-one patients with different malignancies, including one with pancreatic carcinoma, participated in the trial. Dose limiting toxicities were fatigue and headache, while no significant myelosuppression was seen. Three patients with brain malignancies remained stable for an average of 6 mo<sup>[36]</sup>.

A current phase I clinical trial is investigating oral phenylbutyrate three times daily in patients with refractory solid tumors. Combination with azacytidine is also being studied to determine effectiveness and maximum dose in patients with advanced or metastatic solid tumors<sup>[29]</sup>.

**NaBu:** NaBu has multiple effects on cultured mammalian cells that include inhibition of proliferation, induction of differentiation and induction or repression of gene expression. Sodium butyrate inhibits most HDACs except class III HDAC and class II HDAC6 and-10. Promoters of butyrate-responsive genes have butyrate response elements, and the action of butyrate is often mediated through Sp1/Sp3 binding sites<sup>[37]</sup>.

In a recruiting phase I clinical trial, the butyrate pro-drug tributyrin is being studied in patients with various advanced solid tumors<sup>[29]</sup>.

### Benzamides

**MS-275:** This synthetic benzamide derivative (3-pyridylmethyl-N-{4-[(2-aminophenyl)carbamoyl]benzyl} carbamate) has been shown to inhibit HDACs, and has antitumor activity in many preclinical models. The first clinical trial with this agent in 2005 included patients with advanced solid tumors or lymphoma. At high concentrations of MS-275, there is a marked induction of reactive oxygen species, mitochondrial damage, caspase activation and apoptosis. Treatment of sensitive tumor cell lines with MS-275 induces gelsolin, a maturation marker, and produces a change in the cell cycle distribution with a decrease in S phase and an accumulation of cells in G<sub>1</sub>. The *in vivo* therapeutic efficacy of MS-275 has been shown in a variety of human tumor xenograft models<sup>[38]</sup>.

A phase I study determined the maximum tolerated dose, the dose limiting toxicity and the pharmacokinetic or pharmacodynamic profile of MS-275 in combination with 13-cis-retinoic acid. Patients with advanced solid tumors were treated with MS-275 orally, once weekly, and 13-cis-retinoic acid orally, 1 mg/kg twice daily, for 3 wk every 4 wk. One patient suffering from pancreatic cancer remained on treatment for 6 mo and a patient with renal cell carcinoma showed a partial response in the lungs. Side effects included hyponatremia, neutropenia, anemia and fatigue<sup>[39]</sup>.

Another phase I study evaluated the toxicity and pharmacokinetic profiles of MS-275 in patients with refractory solid tumors and lymphomas, including one patient suffering from metastatic pancreatic cancer, on three different schedules. The patient with metastatic pancreatic cancer developed grade 3 hypophosphatemia, thus meeting criteria for dose limiting toxicity, and was

finally removed from study due to disease progression after cycle 2. Objective responses were only observed in patients receiving the every-other-week dosing schedule, but the numbers of patients enrolled were too small to determine whether this dosing regimen was truly more efficacious<sup>[40]</sup>.

**CI-994:** CI-994 or N-acetyldinaline [4-(acetylamino)-N-(2-amino-phenyl) benzamide] is a novel oral compound with a wide spectrum of antitumor activity in preclinical models. The mechanism of action may involve inhibition of histone deacetylation and cell cycle arrest. CI-994 is currently undergoing clinical trials. Although several changes in cellular metabolism induced by the drug have been characterized, the primary molecular mechanism of its antitumor activity remains unknown<sup>[41]</sup>.

A phase II trial of CI-994 in patients with advanced pancreatic cancer evaluated the antitumor activity and safety of CI-994. CI-994 was administered orally at 8 mg/m<sup>2</sup> per day. Seventeen patients were enrolled, including 15 with metastatic disease. Among patients evaluable for response, stable disease for 8 wk occurred in two patients (12%). Overall, median time to progressive disease was 6 wk and median survival was 10 wk, with one patient alive at 41 wk. Grade 3 thrombocytopenia occurred in eight patients but grade 4 in none. Most common non-hematological toxicities were generally grade 1 or 2 and included fatigue, anorexia, nausea, vomiting and bruising. According to this study, CI-994 was well tolerated but resulted in no objective responses in patients with advanced pancreatic cancer<sup>[42]</sup>.

A phase I study in patients with solid tumors was carried out to determine the maximum tolerated daily oral dose for CI-994 administered on a chronic basis. Fifty-three patients, most of them suffering from colorectal, lung and renal malignancies, including one with pancreatic cancer, received CI-994 daily for treatment durations ranging from 2 to 10 wk. Antitumor effects were documented in four patients, including a durable partial response in one pretreated non-small cell lung cancer patient and stable disease in three patients with non-small cell lung, colon and renal cancers<sup>[43]</sup>.

Another study investigated the toxicity profile, maximum tolerated dose and pharmacokinetics of CI-994 in combination with capecitabine. Fifty-four patients were treated according to three different dosing schemes in which the capecitabine dose was fixed and the CI-994 dose was escalated. In schedule C, 22 patients, including four with pancreatic cancer, were treated with capecitabine 2000 mg/m<sup>2</sup> per day and CI-994 for 2 of 3 wk. One partial response was achieved at the 4 mg/m<sup>2</sup> dose level of schedule A in a patient with colorectal cancer. Disease stabilization was seen in adenocarcinoma of unknown primary origin, appendiceal cancer, breast cancer, colorectal cancer and mesothelioma<sup>[44]</sup>.

A phase I study of oral CI-994 in combination with carboplatin and paclitaxel in patients with advanced solid tumors was carried out. A total of 30 patients were en-

tered into five treatment cohorts, including two suffering from pancreatic cancer. Five patients achieved a partial response (non-small cell lung, colon, unknown primary origin) and two patients achieved a complete response (esophageal and bladder cancer). It was observed that patients whose histone H3 acetylation in peripheral blood lymphocytes was at least 1.5-fold greater after treatment had an objective clinical response or stable disease<sup>[45]</sup>.

A randomized, double-blind, placebo-controlled, multicenter study compared whether CI-994 plus gemcitabine improved OS, duration of response, time to treatment failure, and quality of life compared to gemcitabine alone. Patients had diagnosis of advanced or metastatic adenocarcinoma of the exocrine pancreas and were not considered surgical candidates. A total of 174 patients received CI-994 6 mg/m<sup>2</sup> per d orally on days 1-21 plus gemcitabine 1000 mg/m<sup>2</sup> on days 1, 8 and 15 or placebo plus gemcitabine 1000 mg/m<sup>2</sup> on days 1, 8 and 15 of each 28-d cycle. There was no observed difference in survival time between the two cases. The estimated median survival was 194 and 214 d and objective response rates based on investigator assessments were 12% and 14%, respectively. In addition, pain responses did not differ significantly. Treatment with CI-994 was associated with more cases of grade 3/4 thrombocytopenia, anemia and leukopenia than with placebo, while non-hematological toxicities such as nausea, vomiting, anorexia and diarrhea were identical with both treatments. Consequently, in this study, CI-994 in combination with gemcitabine did not appear to offer any benefit compared to gemcitabine as a single agent for the treatment of pancreatic cancer<sup>[46]</sup>.

A randomized phase II trial comparing the effectiveness of gemcitabine with or without CI-994 in patients with advanced pancreatic cancer is still ongoing<sup>[29]</sup>.

**MGCD0103:** MGCD0103 is an isotype-specific aminophenylbenzamide that inhibits HDAC classes I and IV, with almost no class II effect. MGCD0103 is well tolerated and exhibits favorable pharmacokinetic and pharmacodynamic profiles, demonstrating target inhibition and clinical responses. It induces cell death and autophagy, synergizes with proteasomal inhibitors and affects non-histone targets, such as microtubules<sup>[47]</sup>.

In a phase I study MGCD0103 was given three times weekly orally to patients with advanced solid tumors to determine safety, tolerability and pharmacokinetics. Thirty-eight patients were enrolled and completed a total of 99 cycles of MGCD0103, including two with pancreatic cancer. No objective tumor responses were observed. Five patients with previously progressive colorectal, renal cell and lung cancers had stable disease for four or more cycles. Furthermore, MGCD0103 exerted dose-dependent HDAC inhibitory activity and was able to induce histone acetylation in peripheral leukocytes<sup>[48]</sup>.

Oral MGCD0103 three times weekly is currently being studied in combination with gemcitabine, in patients with advanced solid tumors. In this phase I / II study, patients with locally advanced or metastatic pancreatic

cancer can participate. Maximum tolerated dose of MGCD0103 and objective response of patients remain to be determined<sup>[29,49]</sup>.

### Other HDACIs

A phase I study of LAQ824 has determined the safety, maximum tolerated dose, and pharmacokinetic-pharmacodynamic profile in patients with advanced solid tumors. Thirty-nine patients were recruited and were eligible for assessment of toxicity, including three with pancreatic cancer. At 100 mg/m<sup>2</sup>, one patient who had advanced pancreatic cancer, developed grade 4 hyperbilirubinemia associated with febrile neutropenia on day 3. The patient finally died after an episode of atrial fibrillation and acute renal failure 18 d after the first infusion. All patients treated with LAQ824 at 12 mg/m<sup>2</sup> or above showed increase in histone acetylation. No objective responses were documented. One patient with hepatocellular carcinoma and two with fibrosarcoma and papillary carcinoma of the thyroid showed disease stabilization<sup>[50]</sup>.

A phase I study is investigating safety, pharmacodynamic, antitumor activity, and pharmacokinetics of PXD101 (belinostat) alone and in combination with 5-FU in patients with advanced solid tumors or lymphoma<sup>[51]</sup>. Another recruiting phase I trial is studying the safety, tolerability and pharmacokinetics of orally administered PXD101 in combination with carboplatin and/or paclitaxel in patients with advanced solid tumors or lymphoma<sup>[52]</sup>.

A recruiting phase I study is evaluating the pharmacokinetics and safety of oral LBH589 (panobinostat) in patients with advanced solid tumors and varying degrees of renal function. Another phase II trial will determine tumor response, toxicity and tolerability of LBH589 in patients with gastrointestinal neuroendocrine tumors. A phase I A, dose-escalating study of *in vivo* LBH589 in adult patients with advanced solid tumors is ongoing. Another phase I study will evaluate the safety and tolerability of the combination of LBH589 and paclitaxel/carboplatin in patients with metastatic or locally advanced solid tumors. Finally, a phase I dose escalation trial of LBH589 and gemcitabine in patients with solid malignancies has been temporarily suspended<sup>[53,54]</sup>.

Other HDACIs, such as CHR-3996, CRA-024781, SB939 and R306465, are under phase I trials for the treatment of patients with advanced solid tumors<sup>[29]</sup>.

### CONCLUSION

The experimental results of HDACIs led to their use in clinical trials, especially in pretreated and multiply relapsed patients at an advanced cancer stage. Additionally, HDACIs constitute a promising treatment for cancer due to their low toxicity. The first HDACIs tested in clinical trials have shown encouraging antitumor effects, at doses well tolerated by patients. Vorinostat (SAHA) was the first HDACI to be approved by the FDA for clinical use in patients with hematological malignancy (CTCL).

HDACIs alone and in combination with a variety of cytotoxic or other targeted anticancer agents are now being tested. To date, at least 10 different HDACIs, including SAHA, VPA, NaBu, MS-275, CI-994, FK228, PXD101, LAQ824 and others, are in phase II or III clinical trials for the treatment of hematological and solid tumors.

Despite efforts in recent decades, conventional treatments such as surgery, radiation and chemotherapy, have slightly affected the course of pancreatic cancer. The development of effective systemic treatments, capable of reversing the biology of this aggressive disease, remains a critical requirement. Unfortunately, clinical data for HDACIs on patients with pancreatic cancer are inadequate (Table 1). So far, only a few studies have included patients suffering from this type of neoplasm. Additionally, the number of pancreatic cancer patients that entered HDACIs phase II / III trials, among others with advanced solid tumors, is very limited. Although HDACIs are recognized as some of the most promising agents, more studies recruiting candidates with pancreatic cancer remain to determine the efficacy of these therapies.

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## DNA and histone methylation in gastric carcinogenesis

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### INTRODUCTION

Gastric cancer (GC) is the fourth most frequent cancer and is the second leading cause of cancer-related death worldwide<sup>[1]</sup>. Histologically, gastric tumors are divided into intestinal and diffuse types according to the Lauren classification<sup>[2]</sup>. The intestinal type of GC mostly progresses through the successive steps of normal gastric mucosa, leading to acute and chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and finally a gastric tumor<sup>[3]</sup>. In contrast, the sequence of events in the development of diffuse type GC is poorly understood, although a subset of diffuse type GC appears to develop independently of atrophic gastritis or intestinal metaplasia<sup>[4,5]</sup>. Differences in the clinicopathological characteristics between these two histological types indicate that development occurs through distinct molecular pathways<sup>[6-10]</sup>. Each histological type is a consequence of a progressive accumulation of different genetic and epigenetic alterations.

Epigenetics refers to a number of modifications in the chromatin structure that affect gene expression without altering the primary DNA sequence, and these changes lead to transcriptional activation or silencing of the gene. Interestingly, epigenetic modifications of DNA can also increase mutagenesis and influence the interactions between DNA and carcinogens and ultraviolet light<sup>[11]</sup>. Epigenetic modifications play a central role in gastric car-

### Abstract

Epigenetic alterations contribute significantly to the development and progression of gastric cancer, one of the leading causes of cancer death worldwide. Epigenetics refers to the number of modifications of the chromatin structure that affect gene expression without altering the primary sequence of DNA, and these changes lead to transcriptional activation or silencing of the gene. Over the years, the study of epigenetic processes has increased, and novel therapeutic approaches that target DNA methylation and histone modifications have emerged. A greater understanding of epigenetics and the therapeutic potential of manipulating these processes is necessary for gastric cancer treatment. Here, we review recent research on the effects of aberrant DNA and histone methylation on the onset and progression of gastric tumors and the development of compounds that target enzymes that regulate the epigenome.

cinogenesis<sup>[12]</sup>. Recent reports indicate that infection with *Helicobacter pylori* (*H. pylori*) or Epstein-Barr virus (EBV), pathogens with a substantial role in development of GC, are associated with elevated levels of aberrant DNA methylation in GC<sup>[13-16]</sup>. The study of epigenetic processes has increased in recent years, and novel therapeutic approaches that target DNA methylation and histone modifications have emerged. A greater understanding of epigenetics and the therapeutic potential of intervention into these processes is necessary to help GC treatment.

In this review, after a brief introduction to the methylation machinery, we focus on the roles that aberrant DNA and histone methylation play in the onset and progression of gastric tumors, and the development of compounds that target enzymes that regulate the epigenome.

## METHYLATION MACHINERY

DNA methylation refers to the addition or subtraction of a methyl moiety at the 5 position of the cytosine ring within CpG dinucleotides that are usually located in CpG-rich regions or CpG islands and around the gene promoter. DNA methylation in gene promoter regions represses transcription of their downstream genes associated with the suppression of gene expression<sup>[17]</sup>. However, methylation in gene bodies does not block transcription and is sometimes associated with active transcription<sup>[18]</sup>. Methylation status is controlled by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B)<sup>[19]</sup>. DNMT1 maintains the existing methylation patterns following DNA replication, whereas DNMT3A and DNMT3B target unmethylated CpGs to initiate methylation and are highly expressed during embryogenesis and minimally expressed in adult tissues<sup>[20]</sup>. Another DNA methyltransferase family member, DNMT3L, interacts with DNMT3A and DNMT3B to facilitate methylation of retrotransposons<sup>[21]</sup>. Many studies have shown that overexpression of DNA methyltransferases is closely related to tumorigenesis, although the role of DNMT3L in cancer is still unclear (Table 1). In addition, *H. pylori* infection may increase DNA methyltransferase activity through upregulation of the epidermal growth factor and its receptor or via the release of inflammatory mediators, such as nitric oxide<sup>[22]</sup>. In particular, *DNMT1* overexpression has been associated with EBV infection in GC<sup>[23-25]</sup>.

DNA methylation has also been implicated in the regulation of higher order chromatin structure, the maintenance of genome integrity, and stable patterns of gene expression. These biological effects of DNA methylation are, at least in part, mediated by proteins that preferentially bind to methylated DNA<sup>[26]</sup>. Methylated DNA is specifically recognized by a set of proteins called methyl-CpG-binding proteins (MBPs), which belong to three different structural families: methyl-CpG binding domain proteins (MBDs), Kaiso domain proteins, and SET and RING finger-associated domain (SRA) domain proteins<sup>[27,28]</sup>. MBD family proteins (MeCP2, MBD1, MBD2, MBD3 and MBD4) bind methylated CpG (5mCpG) through a conserved protein motif called the methyl-

CpG binding domain<sup>[29,30]</sup>. Over the last decade, proteins that utilize different structures to recognize and bind DNA or its components have been identified. In 2001, Prokhortchouk *et al.*<sup>[31]</sup> identified Kaiso proteins, which bind methylated DNA through a zinc finger motif. Other MBPs including UHRF1 and UHRF2 were identified, and these proteins use the SRA to bind 5mCpG<sup>[32,33]</sup>.

In cancer, the roles of MBPs are related to their functions as transcriptional repressors or chromatin remodelers (Table 1)<sup>[34-36]</sup>. However, a few studies have reported MBPs in GC (Table 1). Mutations in *MBD4* have been found in gastric tumors in association with microsatellite instability<sup>[37,38]</sup>. *MBD4* encodes a protein that interacts with the mismatch repair protein hMLH1. Therefore, it has been postulated that mutations in *MBD4* may result in mismatch repair deficiency<sup>[39]</sup>.

The processes of DNA methylation and histone modification often involve dynamic interactions that either reinforce or inhibit epigenetic changes. Thus, histone modification can also alter chromatin remodeling, and this is a possible mechanism for decreased gene expression<sup>[39-41]</sup>.

The nature of the interaction between DNA and histones, which are composed of pairs of the four core proteins H2A, H2B, H3, and H4, alters the accessibility of DNA transcription sites to RNA polymerase II and other transcription factors. The interaction between histones and DNA is thought to be under epigenetic control, because specific amino acid residues on specific histone core proteins are subjected to post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation, proline isomerization, and ADP ribosylation<sup>[42,43]</sup>. Histone acetylation and methylation are the only modifications that have been clinically associated with pathological epigenetic disruption in cancer cells<sup>[44]</sup>. In this review, we focus on histone methylation modifications.

Histones can be mono-, di-, or trimethylated at lysine and arginine residues by histone methyltransferases (HMTs) or demethylated by histone demethylases (HDTs). Depending on the residue and the level of methylation, the chromatin may be transcriptionally active or inactive. In general, trimethylation at H3K4 and H3K36 or monomethylation at H3K27, H3K9, H4K20, H3K79, and H2BK5 is associated with transcriptional activation. In contrast, trimethylation at H3K27, H3K9, and H4K20 or monomethylation at H3K27, H3K9, H4K20, H3K79, and H2BK5 is associated with transcriptional repression<sup>[44]</sup>.

A growing number of studies have analyzed the HMTs and HDMs in tumor cells, whereas few genes involved in histone methylation activity have been described for GC (Table 1). EZH2, an HMT that plays a role in trimethylation of H3K27 and leads to silencing of important genes in carcinogenesis, is overexpressed in several types of cancer, including GC<sup>[45,46]</sup>. Cai *et al.*<sup>[47]</sup> reported that EZH2 plays an important role in the multi-step process of intestinal-type GC. In addition, Fujii *et al.*<sup>[48]</sup> demonstrated that silencing of *EZH2* by siRNA resulted in a lower H3K27me3 protein level in GC cells.



**Table 1** Methylation machinery in gastric cancer

Gene	Function	Alteration in cancer	Ref.
<i>DNMT1</i>	Maintenance of methylation Repression of transcription	Upregulation Mutation	Kanai <i>et al</i> <sup>[93]</sup> Fang <i>et al</i> <sup>[94]</sup> Ding <i>et al</i> <sup>[95]</sup> Yang <i>et al</i> <sup>[96]</sup> Mutze <i>et al</i> <sup>[97]</sup>
<i>DNMT3A</i>	<i>De novo</i> methylation during embryogenesis Imprint establishment Repression	Upregulation Mutation	Ding <i>et al</i> <sup>[95]</sup> Fan <i>et al</i> <sup>[98]</sup> Yang <i>et al</i> <sup>[96]</sup>
<i>DNMT3B</i>	<i>De novo</i> methylation during embryogenesis Repeat methylation Repression	Upregulation Mutation	Ding <i>et al</i> <sup>[95]</sup> Su <i>et al</i> <sup>[99]</sup> Hu <i>et al</i> <sup>[100]</sup> Yang <i>et al</i> <sup>[96]</sup>
<i>MeCP2</i>	Transcription repression	Upregulation Mutation	Wada <i>et al</i> <sup>[101]</sup>
<i>MBD1</i>	Transcription repression	Upregulation Mutation	-
<i>MBD2</i>	Transcription repression DNA demethylase	Downregulation Mutation	Kanai <i>et al</i> <sup>[102]</sup>
<i>MBD3</i>	Transcription repression, but requires MBD2 to recruit it to methylated DNA	Upregulation Mutation	-
<i>MBD4</i>	Transcription repression DNA repair Glycosylase domain, repair of deaminated 5-methyl C	Downregulation Mutation	Pinto <i>et al</i> <sup>[38]</sup> D'Errico <i>et al</i> <sup>[37]</sup>
<i>Kaiso</i>	Transcription repression	Upregulation	Ogden <i>et al</i> <sup>[103]</sup>
<i>G9a</i>	Histone methyltransferase	Gene Repression	Lee <i>et al</i> <sup>[104]</sup>
<i>RIZ1</i>	Histone methyltransferase	Underexpression	Oshimo <i>et al</i> <sup>[105]</sup>
<i>PRDM2</i>	Histone methyltransferase	Mutation	Pan <i>et al</i> <sup>[106]</sup>
<i>SUZ12</i>	Histone methyltransferase	Upregulation	Yoo <i>et al</i> <sup>[107]</sup>
<i>BM11</i>	Histone methyltransferase	Upregulation	Liu <i>et al</i> <sup>[108]</sup> Xiao <i>et al</i> <sup>[109]</sup> Lu <i>et al</i> <sup>[110]</sup> Zhang <i>et al</i> <sup>[111]</sup> Li <i>et al</i> <sup>[112]</sup>
<i>EVH1</i>	Histone methyltransferase	Chromosomal rearrangement	Takahata <i>et al</i> <sup>[113]</sup>
<i>EZH2</i>	Histone methyltransferase	Amplification Upregulation Mutation	Mattioli <i>et al</i> <sup>[114]</sup> Varambally <i>et al</i> <sup>[115]</sup> Fujii <i>et al</i> <sup>[48]</sup> Cai <i>et al</i> <sup>[47]</sup> Choi <i>et al</i> <sup>[46]</sup> Zhou <i>et al</i> <sup>[116]</sup>
<i>NSD2/MMSET</i>	Histone methyltransferase	Upregulation	Hudlebusch <i>et al</i> <sup>[117]</sup>
<i>SUV39H1 -2</i>	Histone methyltransferase	Translocation	
<i>LSD1/BHC110</i>	Histone demethylase	Polymorphism	Li <i>et al</i> <sup>[84]</sup>
<i>JARID1A-D</i>	Histone demethylase	Downregulation Upregulation	Magerl <i>et al</i> <sup>[118]</sup> Zeng <i>et al</i> <sup>[51]</sup>
<i>JMJD2A</i>	Histone demethylase	Inactivation	
<i>JHDM3A</i>	Histone demethylase	Mutation	Li <i>et al</i> <sup>[119]</sup>
<i>JMJD1A-C</i>	Histone demethylase	Upregulation Downregulation	Katoh <i>et al</i> <sup>[120]</sup>

*DNMT*: DNA methyltransferase; *EVH1*: Domain containing 1; *EZH2*: Enhancer of zest homolog2; *JARID*: Jumonji, AT-rich interactive-domain; *JHDM*: JmJc domain-containing histone demethylase 1; *JMJD*: Jumonji domain containing 2; *LSD1*: Lysine specific demethylase; *MBD*: Methyl-CpG-binding domain; *NSD2*: Nuclear receptor-binding SET-domain protein 2; *PRMT*: Protein arginine methyltransferase 1; *RIZ1*: Retinoblastoma protein-interacting zinc finger 1; *SUV39H*: Suppressor of variation 3-9 homolog.

Among the HDTs, RBP2 is a newly identified member of the JARID family of proteins, and RBP2 specifically targets tri- and dimethylated H3K4 for demethylation in cancer<sup>[49,50]</sup>. Zeng *et al*<sup>[51]</sup> reported that *RBP2* is overexpressed in GC and suggested that HDT inhibition by targeting RBP2 may be an anticancer strategy.

## DNA METHYLATION

DNA methylation contributes to cancer mainly through

DNA hypo- or hypermethylation. DNA hypomethylation, which refers to the loss of DNA methylation, affects chromosomal stability and increases aneuploidy<sup>[52]</sup>. DNA hypermethylation, which refers to the gain of methylation at a locus originally unmethylated, usually results in stable transcriptional silencing, which functions in regulating gene expression<sup>[53,54]</sup>.

Global DNA hypomethylation is usually considered one of the hallmarks of cancer cells, because aberrant hypermethylation-vulnerable genes are overlapped by

**Table 2** Aberrant DNA methylation in gastric cancer

Gene	Role	Aberrant methylation	Ref.
<i>ABCB1</i>	Multidrug resistance	Hyper	Poplawski <i>et al</i> <sup>[121]</sup> , Tahara <i>et al</i> <sup>[122]</sup> , Lee <i>et al</i> <sup>[123]</sup>
<i>ADAM23</i>	Tissue cell invasion and metastasis	Hyper	Takada <i>et al</i> <sup>[124]</sup> , Watanabe <i>et al</i> <sup>[125]</sup> , Kim <i>et al</i> <sup>[126]</sup>
<i>ALDH2</i>	Oxidative pathway of alcohol metabolism	Hypo	Balassiano <i>et al</i> <sup>[127]</sup>
<i>APC</i>	Tissue cell invasion and metastasis	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Ksiao <i>et al</i> <sup>[63]</sup> , Shin <i>et al</i> <sup>[69]</sup> , Geddert <i>et al</i> <sup>[129]</sup>
<i>ARPC1B (p41ARC)</i>	Signal transduction		
<i>BNIP3</i>	Cell morphology	Hyper	Maekita <i>et al</i> <sup>[130]</sup> , Shin <i>et al</i> <sup>[69]</sup>
<i>BRCA1</i>	Apoptosis	Hyper	Murai <i>et al</i> <sup>[131]</sup> , Hiraki <i>et al</i> <sup>[132]</sup> , Sugita <i>et al</i> <sup>[133]</sup>
<i>BRCA1</i>	DNA repair	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Ryan <i>et al</i> <sup>[134]</sup>
<i>CAV1</i>	Tissue cell invasion and metastasis	Hyper	Yamashida <i>et al</i> <sup>[135]</sup>
<i>CDH1</i>	Tissue invasion and metastasis	Hyper	Leal <i>et al</i> <sup>[136]</sup> , Bernal <i>et al</i> <sup>[136]</sup> , Borges <i>et al</i> <sup>[61]</sup> , Tahara <i>et al</i> <sup>[122]</sup> , Al-Moundhri <i>et al</i> <sup>[137]</sup> , Balassiano <i>et al</i> <sup>[127]</sup>
<i>CHFR</i>	Cell cycle regulation	Hyper	Oki <i>et al</i> <sup>[138]</sup> , Hiraki <i>et al</i> <sup>[139]</sup> , Hu <i>et al</i> <sup>[140]</sup>
<i>DAPK</i>	Apoptosis	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Zou <i>et al</i> <sup>[74]</sup> , Hu <i>et al</i> <sup>[140]</sup> , Tahara <i>et al</i> <sup>[122]</sup> , Sugita <i>et al</i> <sup>[133]</sup>
<i>FHIT</i>	Apoptosis	Hyper	Leal <i>et al</i> <sup>[136]</sup> , Bernal <i>et al</i> <sup>[128]</sup>
<i>FLNC</i>	Cell morphology	Hyper	Kim <i>et al</i> <sup>[126]</sup> , Shi <i>et al</i> <sup>[141]</sup>
<i>GATA4/5</i>	Transcriptional factor	Hyper	Akiyama <i>et al</i> <sup>[142]</sup> , Wen <i>et al</i> <sup>[143]</sup>
<i>HAND1</i>	Cell differentiation	Hyper	Maekita <i>et al</i> <sup>[130]</sup> , Shin <i>et al</i> <sup>[69]</sup> , Shi <i>et al</i> <sup>[141]</sup>
<i>HRAS</i>	Signal transduction	Hypo	Fang <i>et al</i> <sup>[144]</sup> , Luo <i>et al</i> <sup>[145]</sup>
<i>IGFBP3</i>	Cell cycle regulation	Hyper	Gigek <i>et al</i> <sup>[146]</sup> , Ryan <i>et al</i> <sup>[134]</sup> , Chen <i>et al</i> <sup>[147]</sup>
<i>LOX</i>	Tissue cell invasion and adhesion	Hyper	Maekita <i>et al</i> <sup>[130]</sup> , Shin <i>et al</i> <sup>[69]</sup> , Tamura <i>et al</i> <sup>[148]</sup>
<i>MGMT</i>	DNA repair	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Hibi <i>et al</i> <sup>[149]</sup> , Ksiao <i>et al</i> <sup>[63]</sup> , Zou <i>et al</i> <sup>[74]</sup> , Schneider <i>et al</i> <sup>[14]</sup> , Hiraki <i>et al</i> <sup>[139]</sup> , Balassiano <i>et al</i> <sup>[127]</sup> , Shi <i>et al</i> <sup>[141]</sup>
<i>MLF1</i>	Cell differentiation	Hyper	Watanabe <i>et al</i> <sup>[125]</sup> , Shi <i>et al</i> <sup>[141]</sup> , Yamashita <i>et al</i> <sup>[135]</sup>
<i>MLH1</i>	DNA repair	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Poplawski <i>et al</i> <sup>[121]</sup> , Hiraki <i>et al</i> <sup>[139]</sup> , Kim <i>et al</i> <sup>[150]</sup> , Shin <i>et al</i> <sup>[58]</sup>
<i>MOS</i>	Cell cycle regulation	Hypo	Shin <i>et al</i> <sup>[58]</sup>
<i>MTHFR</i>	DNA synthesis	Hypo	Balassiano <i>et al</i> <sup>[127]</sup>
	DNA repair		
	DNA methylation		
<i>MYC</i>	Cell cycle regulation	Hypo	Fang <i>et al</i> <sup>[144]</sup> , Luo <i>et al</i> <sup>[145]</sup>
<i>P14ARF</i>	Cell cycle regulation	Hyper	Balassiano <i>et al</i> <sup>[127]</sup> , Geddert <i>et al</i> <sup>[129]</sup>
	Apoptosis		
	Cell differentiation		
<i>P16</i>	Cell cycle regulation	Hyper	Ksiao <i>et al</i> <sup>[63]</sup> , Dong <i>et al</i> <sup>[151]</sup> , Zou <i>et al</i> <sup>[74]</sup> , Shin <i>et al</i> <sup>[69]</sup> , Hu <i>et al</i> <sup>[140]</sup> , Ryan <i>et al</i> <sup>[134]</sup> , Geddert <i>et al</i> <sup>[129]</sup> , Balassiano <i>et al</i> <sup>[124]</sup> , Al-Moundhri <i>et al</i> <sup>[137]</sup> , Shin <i>et al</i> <sup>[58]</sup>
<i>PRDM5</i>	Cell differentiation	Hyper	Watanabe <i>et al</i> <sup>[125]</sup> , Shu <i>et al</i> <sup>[152]</sup>
<i>RAR-beta 2</i>	DNA binding	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Ksiao <i>et al</i> <sup>[63]</sup>
	Activation transcription		
<i>RASSF1A/RASSF2</i>	DNA repair	Hyper	Zou <i>et al</i> <sup>[74]</sup> , Guo <i>et al</i> <sup>[153]</sup> , Shin <i>et al</i> <sup>[58]</sup>
	Cell cycle regulation		
<i>RORA</i>	Cell differentiation	Hyper	Watanabe <i>et al</i> <sup>[125]</sup> , Yamashida <i>et al</i> <sup>[131]</sup>
<i>RPRM</i>	Cell cycle regulation	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Schneider <i>et al</i> <sup>[14]</sup>
<i>RUNX3</i>	Signal transduction	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Sakakura <i>et al</i> <sup>[154]</sup> , Lee <i>et al</i> <sup>[104]</sup> , Zou <i>et al</i> <sup>[74]</sup> , Hiraki <i>et al</i> <sup>[139]</sup> , Tamura <i>et al</i> <sup>[148]</sup> , Hu <i>et al</i> <sup>[140]</sup> , Fan <i>et al</i> <sup>[155]</sup> , Al-Moundhri <i>et al</i> <sup>[137]</sup>
<i>SHP1</i>	Signal transduction	Hyper	Bernal <i>et al</i> <sup>[128]</sup> , Ksiao <i>et al</i> <sup>[63]</sup>
<i>TERT</i>	Cell senescence	Hyper	Kang <i>et al</i> <sup>[67]</sup> , Wang <i>et al</i> <sup>[75]</sup> , Gigek <i>et al</i> <sup>[177]</sup>
<i>TFF1</i>	Repair gene	Hyper	Carvalho <i>et al</i> <sup>[156]</sup> , Ryan <i>et al</i> <sup>[134]</sup>
<i>THBD</i>	Inflammation response	Hyper	Maekita <i>et al</i> <sup>[130]</sup> , Shin <i>et al</i> <sup>[69]</sup>
<i>TWIST1</i>	Cell differentiation	Hyper	Kang <i>et al</i> <sup>[67]</sup> , Schneider <i>et al</i> <sup>[14]</sup>

*ABCB1*: ATP-binding cassette, sub-family B (MDR/TAP), member 1; *ADAM23*: ADAM metalloproteinase domain 23; *ALDH2*: Aldehyde dehydrogenase 2 family (mitochondrial); *APC*: Adenomatous polyposis coli; *ARPC1B (p41ARC)*: Actin related protein 2/3 complex, subunit 1B, 41kDa; *BNIP3*: Adenovirus E1B 19kDa interacting protein 3; *BRCA1*: Breast cancer 1 gene; *CAV1*: Cavinolin 1; *CDH1*: Cadherin 1; *CHFR*: Checkpoint with forkhead and ring finger domains; *DAPK*: Dapkin death associated protein kinase; *FHIT*: Fragile histidine triad gene; *FLNC*: Filamin C, gamma; *GATA4/5*: GATA binding protein 4/5; *GSTP1*: Glutathione S-transferase pi 1; *HAND1*: Heart and neural crest derivatives expressed 1; *HRAS*: v-Ha-ras Harvey rat sarcoma viral oncogene homolog; *IGFBP3*: Insulin-like growth factor; binding protein 3; *LOX*: Lysyl oxidase; *MGMT*: O-6-methylguanine-DNA methyltransferase; *MLF1*: Myeloid leukemia factor 1; *MLH1*: MutL homolog 1; *MOS*: Moloney murine sarcoma viral oncogene homolog; *MTHFR*: Methylene tetrahydrofolate reductase (NADPH); *MYC*: v-myc myelocytomatosis viral oncogene homolog (avian); *P14ARF*: Cyclin-dependent kinase inhibitor 2A; *P16*: Cyclin-dependent kinase inhibitor 2A; *PRDM5*: PR domain containing 5; *RAR-beta 2*: Retinoic acid receptor  $\beta$  2 gene; *RASSF1A/RASSF2*: Ras association (RalGDS/AF-6) domain family member 1/member 2; *RORA*: RAR-related orphan receptor A; *RPRM*: TP53 dependent G<sub>2</sub> arrest mediator candidate; *RUNX3*: Runt-related transcription factor 3; *SHP1*: Hematopoietic cell-specific protein-tyrosine phosphatase; *TERT*: Telomerase reverse transcriptase; *TFF1*: Trefoil factor 1; *TFPI2*: Tissue factor pathway inhibitor 2; *THBD*: Thrombomodulin; *TWIST1*: Twist homolog 1.

genes targeted by hypomethylation<sup>[55,56]</sup>. Compare *et al*<sup>[57]</sup> suggested that global DNA hypomethylation may be implicated in GC associated with *H. pylori* infection at an early stage. At the individual gene level, DNA hypomethylation is often associated with activation of proto-oncogenes.

In GC, few studies have shown promoter hypomethylation associated with the activation of proto-oncogenes (Table 2). In particular, Shin *et al*<sup>[58]</sup> reported that the hypomethylation of the *MOS* promoter in GC was associated with tumor invasion, lymph node metastasis, and the diffuse type. A number of genes involved in cell cycle regulation, tumor cell invasion, DNA repair, chromatin remodeling, cell signaling, transcription, and apoptosis are known to be silenced by hypermethylation in GC (Table 2).

Multiple reports have been published regarding gene hypermethylation in both intestinal and diffuse types of GC. Interestingly, the methylation profile differs between the intestinal and diffuse types of GC<sup>[54]</sup>.

The epithelial cadherin gene *CDH1*, which is a well-studied gene involved in cancer, is downregulated in gastric tumors and is hypermethylated more frequently in the diffuse type than in the intestinal type of GC. Loss of *CDH1* during tumor progression has led to the notion that this is a tumor suppressor gene<sup>[59,60]</sup>. In addition, mapping of the *CDH1* promoter has revealed a positive association between hypermethylation and older age, as well as a significant correlation between DNA hypermethylation and the A allele of the -160 C→A polymorphism. The A allele has been described to increase the risk of developing GC in association with the methylation status<sup>[61]</sup>. Unlike the *CDH1* gene, the *P16* gene is hypermethylated mainly in the intestinal type of GC<sup>[54,62,63]</sup>. This epigenetic mark was recently associated with tumor location and *H. pylori* infection in GC<sup>[64]</sup>.

Other studies have also described a number of genes that are silenced by hypermethylation in association with *H. pylori* or EBV infection: *APC*, *SHP1*, *p14*, and *CDH1*<sup>[63,65-67]</sup>. According to Chan *et al*<sup>[68]</sup>, the eradication of *H. pylori* infection significantly reduces the methylation index of the *CDH1* promoter. In contrast, it has been shown that a portion of the aberrant DNA methylation induced by *H. pylori* infection may persist even after the infection has disappeared<sup>[69,70]</sup>. Shin *et al*<sup>[58]</sup> observed that the methylation levels in *MOS* remained significantly increased in patients with previous *H. pylori* infection compared with *H. pylori*-negative subjects.

Moreover, hypermethylation of several gene promoters has also been observed in the premalignant stages of GC, suggesting that aberrant methylation occurs early during gastric carcinogenesis<sup>[59,71-74]</sup>. For example, the methylation levels of the catalytic subunit of the telomerase gene (*bTERT*) promoter are increased during gastric carcinogenesis. Wang *et al*<sup>[75]</sup> reported that the *bTERT* promoter was more methylated in GC than in precancerous lesions and non-neoplastic gastric tissues. Therefore, it has been suggested that the degree of methylation of the *bTERT* promoter may be useful in the early diagnosis

of GC and/or may have an impact on the anti-telomerase strategy for cancer therapy. Other studies, however, showed that methylation of the *bTERT* promoter and resultant gene expression were opposite to the general model of regulation by DNA methylation, which is usually dependent on the CpG islands studied<sup>[76,77]</sup>.

Recently, aberrant hypermethylation of the newly associated metastatic suppressor gene *RECK* was found to be associated with GC development and may also be useful for early diagnosis and treatment<sup>[78]</sup>. These abovementioned findings lead to the possibilities that epigenetic alterations may also occur at different stages of gastric tumorigenesis.

## HISTONE METHYLATION

Histone modifications leading to gene expression alterations have been described in several cancer types, but the methylation status of chromatin is still unclear for GC. Using the ChIP-on-chip technique, Zhang *et al*<sup>[79]</sup> identified candidate genes with significant differences in H3K27me3 in GC samples compared to adjacent non-neoplastic gastric tissues. These genes included oncogenes, tumor suppressor genes, cell cycle regulators, and genes involved in cell adhesion. Moreover, these investigators demonstrated that higher levels of H3K27me3 produce gene expression changes in *MMP15*, *UNC5B*, and *SHH*.

In 2011, Kwon *et al*<sup>[80]</sup> showed that *LAMB3* and *LAMC2* were overexpressed in GC samples in comparison with non-neoplastic adjacent tissue samples. Furthermore, these researchers demonstrated that overexpression of these genes was a result of the enrichment of H3K4me3 in the gene promoter. Using immunohistochemistry, Park *et al*<sup>[81]</sup> showed that higher levels of H3K9me3, which is a repressive mark, was associated with higher T stage, lymphovascular invasion, and recurrence in gastric tumors. They also observed that the level of H3K9me3 was correlated with patient survival, because stronger methylation corresponded to a worse prognosis and intermediate methylation to an intermediate prognosis.

Taken together with results from previous studies, these results have suggested that histone methylation results in a worse prognosis by inactivating certain tumor suppressor genes<sup>[82,83]</sup>. Moreover, Li *et al*<sup>[84]</sup> used GC cell lines to demonstrate that the PRC1 member CBX7 initiated trimethylation of H3K9 at the *P16* locus through recruitment and/or activation of the HMT SUV39H2 to the target locus. This finding links two repressive epigenetic landmarks, H3K9me3 formation and PRC1 binding within the silenced domains in euchromatin, and builds up a full pathway for epigenetic inactivation of *P16* by histone modifications.

Recently, Angrisano *et al*<sup>[85]</sup> reported that *H. pylori* infection is followed by activation of *iNOS* gene expression, chromatin changes at the *iNOS* promoter (including decreased H3K9 methylation and increased H3K4 methylation), and selective release of MBD2 from the *iNOS*

promoter in a GC cell line.

## METHYLATION INHIBITOR DRUGS

The silencing of cancer-related genes by DNA methylation and chromatin modification are reversible and may represent a viable epigenetic therapeutic target. In the last decade, drugs that modify chromatin or DNA methylation status have been used alone or in combination in order to affect therapeutic outcomes<sup>[86]</sup>. Specially, cytosine analogs (5-azacytidine and 5-aza-2'-deoxycytidine) are powerful mechanism-based inhibitors of DNA cytosine methylation. These cytosine analogs are incorporated into the DNA of replicating cells after the drugs have been metabolized to the appropriate dNTP. After incorporation into the DNA, the analogs interact with DNA methyltransferases to form covalent intermediates, and this interaction inhibits DNA methylation in subsequent rounds of DNA synthesis<sup>[87]</sup>. Both drugs have been approved by the US Food and Drug Administration for use in hematological malignancy treatment<sup>[88]</sup>.

In GC, surgery remains the primary curative treatment for gastric tumors. Currently, adjuvant and neo-adjuvant therapies are accepted<sup>[89]</sup>; however, so-called epigenetic therapy has not yet been used in treatment of GC patients.

In the past few years, epigenetic screening techniques using treatment with a demethylating agent have been developed to identify genes with epigenetic aberrations in GC cell lines. Zheng *et al.*<sup>[90]</sup> treated a GC cell line with 5-aza-2'-deoxycytidine and performed DNA methylation array analysis of these cells with six normal mucosal samples from healthy patients. These results revealed 82 hypermethylated gene promoters. These authors investigated 15 candidate genes by methylation-specific PCR and confirmed five highly methylated promoters: *BX141696*, *WT1*, *CYP26B1*, *KCN4A*, and *FAM84A*. All of these, except *FAM84A*, also showed DNA hypermethylation in serum of GC patients, suggesting that serum DNA offers a readily accessible bioresource for methylation analysis.

A similar study conducted by Jee *et al.*<sup>[91]</sup> described 11 selected genes and validated the genes in three GC cell lines and in non-neoplastic gastric tissue by bisulfate sequencing. Differential DNA hypermethylation was observed in *GPX1*, *IGFBP6*, *IRF7*, *GPX3*, *TFPI2*, and *DMRT1* promoter regions in GC cells but not in non-neoplastic tissues. Moreover, a poor survival rate was observed in those individuals with higher methylation status at the *TFPI2* gene. *TFPI2* is a serine protease inhibitor, which negatively regulates the enzymatic activities of trypsin, plasmin, and a tissue factor complex. Therefore, it has been proposed that this gene inactivation may be implicated in human carcinogenesis and metastasis<sup>[92]</sup>.

## CONCLUSION

In summary, aberrant DNA methylation and histone modification play a crucial role in gastric carcinogenesis.

Thus, the recognition of the methylation machinery, genes with aberrant methylation status, and histone methylation levels in gastric carcinogenesis exemplified in this review allow us to contemplate the possibility of dealing with the aforementioned oncological issue in a new way that may have a significant impact on the therapy and management of GC.

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## Management of small hepatocellular carcinoma in cirrhosis: Focus on portal hypertension

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### INTRODUCTION

The incidence of hepatocellular carcinoma (HCC) is rising worldwide being currently the fifth most common cancer and third cause of cancer-related mortality<sup>[1]</sup>. HCC accounts for more than 90% of primary liver cancers, and it usually arises in the setting of long-term underlying liver disease. In fact, HCC is now the first cause of death among cirrhotic patients<sup>[2]</sup>. According to the Barcelona Clinic Liver Cancer (BCLC) algorithm, HCC can be classified into 5 stages when considering variables related to tumor burden, liver function and health status. Additionally, this algorithm links tumor stage with a specific treatment strategy<sup>[3]</sup>. Despite recent advancements in HCC management such as the introduction of the molecular targeted agent sorafenib for advanced stages<sup>[4]</sup>, the mainstream potential curative therapies in HCC are still resection, transplantation and percutaneous ablation.

HCC surveillance programs for cirrhotic patients have enabled the identification of small nodules with higher frequency<sup>[5]</sup>, and nowadays account for 10%-15% of patients diagnosed in the West and almost 30% in Japan<sup>[6]</sup>. "Small HCC" is a term frequently used to define tumors less than 2 cm in diameter. This cut-off is based

### Abstract

The incidence of hepatocellular carcinoma (HCC) is rising worldwide being currently the fifth most common cancer and third cause of cancer-related mortality. Early detection of HCC through surveillance programs have enabled the identification of small nodules with higher frequency, and nowadays account for 10%-15% of patients diagnosed in the West and almost 30% in Japan. Patients with small HCC can be candidates for potential curative treatments: liver transplantation, surgical resection and percutaneous ablation, depending on the presence of portal hypertension and co-morbidities. This review will analyze recent advancements in the clinical management of these individuals, focusing on issues related to the role of portal hypertension, the debate between resection and ablative therapies and the future impact of molecular technologies.

on the outstanding outcomes of patients with these tumors treated with surgical resection when compared to those with larger ones<sup>[7]</sup>. However, small HCC can be two different entities in pathology: vaguely and distinctly nodular<sup>[7]</sup>. Vaguely nodular tumors are well-differentiated without local invasiveness and around 12 mm in size; whereas distinctly nodular are frequently larger (16 mm) and often show local invasiveness features such as micro-vascular invasion<sup>[8]</sup>. European Association for the Study of the Liver (EASL) and American Association for the Study of Liver Diseases (AASLD) guideless for HCC management enables an accurate diagnosis of tumors larger than 1 cm in cirrhotic livers when a dynamic imaging technique [computed tomographic (CT) or magnetic resonance (MR)] show the so-called “hallmark” features of HCC: uptake in the arterial phase with early wash-out in the portal phase<sup>[9]</sup>. For nodules between 1-2 cm, this criterion is still maintained particularly in referral centers with a dense experience in HCC management. In case these imaging features are absent, diagnosis still requires a biopsy. According to the BCLC algorithm, patients with small HCC can be candidates for potential curative treatments, depending on portal hypertension and co-morbidities. This short review will analyze recent advancements in the clinical management of these individuals, focusing on issues related to the role of portal hypertension, the debate between resection and ablative therapies and the future impact of molecular technologies.

## TREATMENT STRATEGY OF SMALL HCC AND IMPACT OF PORTAL HYPERTENSION ON POSTOPERATIVE OUTCOME

The EASL<sup>[9]</sup> and AASLD<sup>[10]</sup> guidelines recommend three alternatives for the treatment of patients with small HCC, depending on the presence of portal hypertension and co-morbidities: liver transplantation, surgical resection and percutaneous ablation. Overall, these therapies achieve a 5-year survival rate between 60%-75% in early HCC (BCLC 0/A), even though in small HCC survival rates can even be higher. About 80% of HCC develop in patients with underlying chronic liver disease, where liver failure and portal hypertension may be present. Since different treatment options are available for patients with small HCC, allocation to a given option should rely on evidence-based criteria, considering expected outcomes rather than merely treatment feasibility<sup>[11]</sup>.

As for surgical resection, peri-operative mortality has decreased to 2%-3% with current surgical techniques. On the other hand a pioneer study by our group in Barcelona<sup>[12]</sup> showed that in a series of 29 Child A cirrhotic patients and HCC less than 5 cm undergoing liver resection surgery, hepatic venous pressure gradient (HVPG)  $\geq 10$  mmHg was independently associated with unresolved decompensation 3 mo following surgery. In a larger series published later, post-operative survival of patients with Child A cirrhosis without clinically significant portal hy-

pertension (CSPH) was 70% at 5 years, while it dropped to 25%-50% in patients with CSPH<sup>[3]</sup>. In addition, even small increases in bilirubin, above the 1 mg/dL cutoff, were also independently associated with increased mortality. The impact of PH-related variables on the risk of post-operative liver failure and mortality has been confirmed in many Western and in Eastern series<sup>[3,12-24]</sup> (Table 1). In these studies 3-year survival was in mean 59% (range: 45%-71%) in patients with clinical signs of portal hypertension, while it increased to 72% (62%-81%) in patients without any clinical sign of the syndrome. A similar difference is observed analyzing post-operative liver decompensation and 5-year survival, and similar results were obtained in a Japanese cohort of resected HCC<sup>[25]</sup>. Overall these data show that even if the presence of CSPH is not an absolute contraindication for performing liver resection for HCC, it clearly increases the risk of post-operative complications and death, suggesting that other available options such as liver transplantation (see below) should be considered in this subgroup of patients. Hence, the best candidates for surgical resection are patients with solitary tumors, preserved liver function and absence of CSPH. Consequently, the European and American Associations for the study of the Liver (EASL<sup>[9]</sup> and AASLD<sup>[10]</sup>) guidelines for HCC management recommend surgical resection as the first-line option in patients with small HCC without CSPH or liver dysfunction.

A main drawback of liver resection is the risk to develop a tumor recurrence, that accounts for 70% at 5 years<sup>[26]</sup> either because of true metastasis or *de novo* HCC.

By definition, patients with small HCC are within Milan criteria for liver transplantation<sup>[27]</sup>, and besides treating the tumor liver transplantation provides a solution for the underlying liver disease. However, the scarcity of donors limits its feasibility and increases time in the waiting list. Even though there are no robust data, the risk of dropout from the waiting list seems lower in patients listed with small HCC. Hence, liver transplantation is the best option for patients in BCLC stage 0/A, CSPH and absence of other medical conditions that contraindicate this procedure.

### Local ablation

Local ablation is considered as a competitive alternative to resection or transplantation in patients with small HCC<sup>[28]</sup> not suitable for surgery. Both radiofrequency ablation (RFA) and percutaneous ethanol injection (PEI) are standard of care techniques for tumor ablation, being used worldwide. They are both able to induce necrosis through different mechanisms. PEI presents a higher post-treatment rate of recurrence that can reach 40% at two years in larger lesions<sup>[29,30]</sup>. RFA has shown its ability to better control the disease with a lower local recurrence rate (2%-18% at 2 years) when compared with PEI (11%-45%) in randomized control trials (RCT)<sup>[31-35]</sup>. However, differences in survival were not described. Therefore, current guidelines recommend the use of PEI in cases where RFA is not feasible for technical reasons<sup>[9]</sup>. Five-year

**Table 1** Relevant reported studies describing the impact of hepatic venous pressure gradient or portal-hypertension related variables in patients with potentially resectable or resected hepatocellular carcinoma

Ref.	Patients included (n)	Portal hypertension-related variables studied	Outcome
Llovet <i>et al</i> <sup>[3]</sup>	43	HVPG	CSPH independently associated with 5-yr post-operative mortality
Bruix <i>et al</i> <sup>[12]</sup>	29	HVPG	CSPH independently associated with PLF at 3-mo
Berzigotti <i>et al</i> <sup>[13]</sup>	63	Spleen size; platelet count; platelet count/spleen diameter; liver stiffness; LSPS PH risk score	Best single predictor of CSPH: liver stiffness; combination with spleen size and platelet count improved the results (AUROC LSPS 0.852; PH risk score 0.884)
Boleslawski <i>et al</i> <sup>[14]</sup>	43	HVPG Platelet count; spleen size; esophageal varices = indirect signs of PH	CSPH independently associated with increased PLF and 90-d mortality. Indirect signs of PH showed no discriminative ability
Capussotti <i>et al</i> <sup>[15]</sup>	217	Platelet count; spleen size; esophageal varices	PH associated with lower 3-yr and 5-yr survival
Cescon <i>et al</i> <sup>[16]</sup>	90	Liver stiffness; platelet count; spleen size; esophageal varices	LS (but not other signs) independently associated with the risk of PLF
Chen <i>et al</i> <sup>[17]</sup>	190	Intraoperative measurement of PVP	PVP independently associated with PLF on multivariate analysis
Cucchetti <i>et al</i> <sup>[18]</sup>	241	Platelet count; spleen size; esophageal varices	PH associated with lower 3-yr and 5-yr survival, but not after adjusting for MELD, albumin and extent of resection no
Figueras <i>et al</i> <sup>[19]</sup>	39	HVPG	CSPH associated with increased risk of morbidity
Giulante <i>et al</i> <sup>[20]</sup>	588	Platelet count; spleen size; esophageal varices	PH independently associated with increased mortality
Imamura <i>et al</i> <sup>[21]</sup>	532	Varices, hypersplenism or hepatofugal portal flow	PH associated with a higher risk of post-operative ascites
Ishizawa <i>et al</i> <sup>[22]</sup>	203	Platelet count	Platelet count < 100 × 10 <sup>3</sup> /mL independently associated with PLF
Kim <i>et al</i> <sup>[23]</sup>	72	Liver stiffness	LS predicted PLF with good accuracy; LS better than ICG15
Llop <i>et al</i> <sup>[24]</sup>	79	Liver stiffness	CSPH predicted with good accuracy
Ishizawa <i>et al</i> <sup>[25]</sup>	434	Platelet count; spleen size; esophageal varices	PH associated with lower 3-yr and 5-yr survival

HVPG: Hepatic venous pressure gradient; PH: Portal hypertension; LS: Liver stiffness; PVP: Portal vein pressure; PLF: Post-operative liver failure; CSPH: Clinically significant PH (HVPG ≥ 10 mmHg); AUROC: Area under receiver operating characteristic curve; MELD: Model for end-stage liver disease.

survival with RFA reaches 70%<sup>[36,37]</sup>, with the best results in patients with good liver function (Child-Pugh A) and small tumors<sup>[38]</sup>. Although resection remains the first-line option for patients without CSPH, well-preserved liver function and solitary tumors less than 2 cm, there is an increasing debate as to whether ablative therapies (particularly RFA) could be a competitive option in these patients. Theoretically, in these solitary small tumors RFA could be as effective as surgery in terms of oncological results, and avoid all the possible complications related to the surgical procedure. However, available evidence is still limited as the few RCT available evaluating face-to-face both therapeutic options show controversial results (Table 2). In addition, there are several methodological issues that preclude reaching any robust conclusion from these trials (*e.g.*, treatment allocation, patient selection criteria, trial implementation, short follow-up, *etc.*). In fact, none of them was specifically design to only include patients with HCC less than 2 cm. Replacement of resection by RFA as first line therapy in patients with early HCC cannot be recommended at this point. Further evidence will be needed to appropriately address this issue.

Tumor recurrence is the main drawback in patients treated with resection or ablation, since the risk factor for HCC development (*i.e.*, cirrhosis) is still present<sup>[39]</sup>. Early recurrence usually occurs within the 2 years after surgery and it is due to true metastatic spread. However, late recurrence appears later on, usually beyond 2 years, and it is consider as *de novo* tumor developed as a consequence of the carcinogenic effect of underlying cirrhosis (field ef-

fect)<sup>[21]</sup>. Besides clinical differences<sup>[21]</sup>, these two patterns of recurrence also differ in their molecular profile<sup>[40]</sup>. A large Japanese study found that non-anatomical resection, high AFP serum levels and presence of microscopic vascular invasion were risk factors for early recurrence, whereas grade of hepatitis activity, multiplicity and gross tumor classification impacted mostly late recurrence<sup>[21]</sup>.

## PORTAL HYPERTENSION ASSESSMENT IN PATIENTS WITH CIRRHOSIS AND SMALL HCC

The above-mentioned data underscore the need of an accurate discrimination of the presence of portal hypertension in patients with small HCC. Portal hypertension (PH) is a clinical syndrome hemodynamically defined as an increase in the pressure gradient across the liver (between portal pressure and inferior vena cava pressure) above the normal value of 5 mmHg<sup>[41]</sup>. In patients with cirrhosis, this gradient can be estimated by its clinical equivalent-the HVPG-which is assessed at hepatic vein catheterization and avoids the need to directly puncture the portal vein<sup>[41]</sup>. An elevated HVPG between 6 to 9 mmHg defines subclinical portal hypertension, whereas an HVPG ≥ 10 mmHg defines clinically CSPH, since all the potential complications of the syndrome (*e.g.*, varices, ascites, *etc.*) can appear above this threshold<sup>[41]</sup>. The gold standard for the diagnosis and assessment of PH is the measurement of the HVPG, which is obtained as



**Table 2** Randomized controlled trials comparing surgical resection and percutaneous ablation in patients with early hepatocellular carcinoma

Ref.	Treatment allocation	Sample size (n)	Serum bilirubin	Tumor median size	Nodules	Median follow-up	Recurrence rate	1/3/5 yr survival
Chen <i>et al</i> <sup>[17]</sup>	Multidisciplinary team of doctors	90 (resection)	> 2 mg/dL (33%)	< 3 cm (52%)	Single (100%)	NR	NR	94%/68%/NR
Huang <i>et al</i> <sup>[66]</sup>	Consecutive enrolment	71 (ablation)	> 2 mg/dL (26%)	< 3 cm (46%)	Single (100%)	NR	NR	93%/73%/NR
		38 (ablation)	NR	≤ 2 cm (55%)/2-3 cm (45%)	Single (79%)	37.7 ± 14.5	47%	100%/95%/92%
		38 (resection)	NR	≤ 2 cm (63%)/2-3 cm (37%)	Single (89%)	38.4 ± 16.4	39%	97%/89%/87%
Huang <i>et al</i> <sup>[67]</sup>	Consecutive enrolment	115 (ablation)	15.3 ± 4.6 μmol/L	≤ 3 cm (49%)	Single (73%)	NR	63%	86.9%/69.6%/54.78%
		115 (resection)	16.4 ± 5.3 μmol/L	≤ 3 cm (39%)	Single (84%)	NR	41%	98.26%/92.17%/75.6%
Feng <i>et al</i> <sup>[68]</sup>	Consecutive enrolment	84 (ablation)	17.2 μmol/L	≤ 2 cm (37%)/> 2 cm and < 4 cm (63%)	Single (57%)	NR	42%	96%/87.6%/NR
		84 (resection)	15.1 μmol/L	≤ 2 cm (30%)/> 2 cm and < 4 cm (46%)	Single (62%)	NR	32%	93.1%/83.1%/NR

NR: Not reported.

the difference between “wedged” (occluded) and “free” hepatic venous pressures. This method is safe, objective, reproducible, and accurate; and it provides prognostic information independent of liver function. It is currently the best marker to predict clinical events in patients with liver diseases within research protocols<sup>[42]</sup>.

Since development of gastroesophageal varices is a direct consequence of CSPH, the presence of esophageal varices on endoscopy is a 100% specific sign of CSPH. However, approximately 50% of well compensated patients without any evident sign of portal hypertension (*e.g.*, without esophageal varices) already show CSPH on hemodynamic assessment<sup>[43]</sup>. In the specific setting of well-compensated patients without varices, CSPH independently increases the risk of developing esophageal varices, clinical decompensation and HCC during follow-up<sup>[43-45]</sup>, resulting in a higher mortality. Hence, CSPH should be always assessed in patients with cirrhosis for prognostic stratification<sup>[41]</sup>.

HVPG measurement has some limitations such as cost, invasiveness (even if minor) and the need for a specific training for its performance and interpretation. Since it is not available in all centers, noninvasive surrogate methods to diagnose portal hypertension are needed, and have been widely evaluated. Non-invasive markers include laboratory tests, ultrasonography and liver stiffness. Regarding routine laboratory tests, the objective components of Child-Pugh score (albumin, bilirubin, INR) correlate with HVPG<sup>[46-48]</sup> and with the prevalence and grade of esophageal varices in cirrhotic patients. Interestingly this correlation is also observed in patients with compensated cirrhosis<sup>[49]</sup> suggesting that there is a close correlation between liver structural damage and the onset of portal hypertension and hepatocellular dysfunction. Low platelet count, either alone or in combination with spleen size<sup>[50-52]</sup> is associated with CSPH and esophageal varices, and is the single most commonly reported non-invasive sign of portal hypertension. Nonetheless, there is not any established cut-off for platelet count able to accurately diagnose or exclude CSPH in patients with cirrhosis<sup>[13]</sup>.

Ultrasound (US) is the first-line imaging technique used in patients with suspected cirrhosis and portal hypertension, since it is cheap, repeatable, and allows direct visualization of the anatomical changes induced by PH<sup>[53,54]</sup>. US signs of PH in cirrhosis are multiple<sup>[55,56]</sup>; overall, the sensitivity of the technique is moderate, but its specificity is above 90%. Hence, the presence of one or more US signs of PH allows to diagnose CSPH, but the absence of signs do not exclude CSPH, suggesting that it should be investigated by more sensitive techniques (*e.g.*, HVPG measurement). Splenomegaly, defined as an increase in spleen diameter above 12 cm, is among the most commonly reported signs. It is sensitive but poorly specific, while porto-collateral vessels have a low sensitivity but 100% specificity for the diagnosis of CSPH. This last sign can be therefore considered a reliable surrogate of PH if detected either on US or on CT/MR imaging.

Liver stiffness (LS) measured by transient elastography (Fibroscan<sup>®</sup>) is a well-accepted objective non-invasive method to reliably estimate liver fibrosis<sup>[57]</sup>. It has been shown that LS and HVPG show a good correlation in patients with compensated cirrhosis<sup>[13,58]</sup>. LS cut-off for the detection of CSPH varies across studies, but it is widely accepted that values above 21 kPa have a high specificity for CSPH. This has been recently confirmed by a longitudinal study using clinical endpoints that showed that HVPG ≥ 10 mmHg and LS > 21 kPa equally predict clinical decompensation in patients with compensated cirrhosis at baseline<sup>[59]</sup>, making LS the most accurate method so far for predicting CSPH. Preliminary data suggests that spleen stiffness has a better correlation with HVPG than LS, but data are still limited<sup>[60]</sup>.

The combination of different non-invasive methods offers potential benefits by integrating complementary information. In a recent study by our group where HVPG was used as the gold standard for diagnosing CSPH, the combination of LS, platelet count and spleen size either as LSPS<sup>[23]</sup> or as a newly calculated PH risk score improved the accuracy of LS alone for detecting CSPH, and allowed a correct diagnosis in 86% of cases<sup>[13]</sup>.

The EASL<sup>[9]</sup> and AASLD<sup>[10]</sup> practice guidelines for HCC management recommend evaluation of portal hypertension prior treatment decision in HCC patients with a single tumour. For this purpose, HVPG measurement is the best available option and should be considered the standard-of-care. However, it cannot be routinely performed in all hospitals. Platelet count below  $100 \times 10^3/\text{mL}$  is often used to identify CSPH, especially when associated with splenomegaly<sup>[13]</sup>. In this regard, it should be underscored that both platelet count and spleen size are inaccurate for diagnosing CSPH in this specific setting<sup>[13]</sup>. Specifically, while in patients with both signs CSPH is highly probable, the absence of thrombocytopenia and/or splenomegaly cannot exclude CSPH. This has been recently confirmed in a prospective study in patients undergoing surgery for HCC including HVPG measurement<sup>[14]</sup> and reinforces the notion that more objective and accurate methods are still needed to accurately diagnose or exclude CSPH in patients with potentially resectable HCC.

Given that liver stiffness by transient elastography is currently considered the single most reliable non-invasive surrogate marker of PH, researchers from Eastern and Western countries evaluated its accuracy to predict CSPH and PH-related complication after surgery for HCC. A study including 72 patients found that LS had a good accuracy (area under receiver operating characteristic curve 0.824) in discriminating which patients would decompensate after surgery, being superior than the test usually used in Eastern countries (indocyanine green clearance at 15 min)<sup>[61]</sup>. Our group tested LS accuracy in the diagnosis of CSPH versus the gold-standard measurement of HVPG in a series of 90 patients with Child A cirrhosis and potentially resectable HCC<sup>[24]</sup>. In the 79 patients in whom LS was feasible (88% of applicability), the correlation with HVPG was lower than previously published in patients without HCC, suggesting that factors related to tumor location might interfere with LS measurements<sup>[24]</sup>. Nonetheless, results demonstrated that a cut-off of 13.6 kPa was 90% sensitive to detect CSPH, while the 21.1 kPa cutoff was highly specific. In other words, CSPH could be reasonably excluded in patients with  $LS < 13.6$  kPa, and reliably diagnosed in those with  $LS \geq 21.1$  kPa. This simple rule could eventually decrease the need for HVPG measurement by half, being confined to those patients with intermediate LS levels (between 13.6–21.1 kPa) and those with unreliable measurements (*e.g.*, obesity, *etc.*). Furthermore, combination of LS with platelet count and spleen diameter has the potential of reducing the number of patients in this “grey zone”, allowing to correctly diagnose CSPH in 85% of patients, although these findings need validation in independent series.

## MOLECULAR PROGNOSTIC BIOMARKERS IN SMALL HCC

There is a pressing need to incorporate prognostic and predictive biomarkers in HCC management. Genome-wide expression studies have been applied in the HCC

field trying to provide physicians with better tools to characterize early lesions and even to optimize patient selection for personalized therapies.

Different studies indicate that HCC can be broadly classified according to its molecular features in two major subclasses<sup>[62]</sup>. One is characterized by molecular signals of proliferation and cell cycle, usually enriched in TP53 inactivation; whereas the second subclass is characterized by *CTNNB1* mutations and enrichment in WNT target genes *GLUL*, *LGR5*, and *LECT2*. The subclass related to proliferation can be further divided according to the activation of other cascades such as transforming growth factor-beta signaling, insulin-like growth factor<sup>[63]</sup>, Notch<sup>[64]</sup>, *etc.*

Besides molecular classification, a number of studies have reported gene signatures able to predict prognosis in HCC (thoroughly reviewed elsewhere<sup>[62]</sup>), not only generated from the tumor but also from the adjacent non-tumoral cirrhotic tissue. Recently an integrated prognostic model that combines genomic information from both the tumor and the adjacent tissue together with clinical-pathologic data was able to accurately predict outcome in patients with a single nodule early HCC<sup>[65]</sup>. Despite all these data from molecular profiling studies, gene signatures have not been yet incorporated clinical practice guidelines<sup>[62]</sup>.

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## Involvement of parasympathetic pelvic efferent pathway in psychological stress-induced defecation

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### Abstract

**AIM:** To investigate the role of the pelvic nerve pathway in stress-induced acceleration of colorectal transit and defecation in rats.

**METHODS:** Surgical transection of rectal nerves (rectal branches of the pelvic nerve), vagotomy (Vag) or adrenalectomy (Adx) were performed bilaterally in rats. Number of fecal pellet output of these rats was measured during 1-h water avoidance stress (WAS). To evaluate the colonic transit, rats were given phenol red through the catheter indwelled in the proximal colon and subjected to WAS. After WAS session, entire colon and rectum were isolated and distribution of phenol red was measured. Distal colonic and rectal transit was evaluated using glass bead. Rats were inserted the glass bead into the distal colon and evacuation rate of the bead was measured. Neural activation was assessed by immunohistochemical staining of c-Fos and PGP9.5 in colonic whole-mount preparations of longi-

tudinal muscle myenteric plexus (LMMP).

**RESULTS:** In the sham-operated rats (sham op), WAS significantly increased defecation and accelerated colorectal transit with marked elevation of plasma corticosterone level. Compared with sham-operated rats, increase in the excretion of fecal pellets during WAS was significantly reduced by rectal nerve transection (RNT) (sham op:  $6.9 \pm 0.8$  vs RNT:  $4.3 \pm 0.6$ ,  $P < 0.05$ ) or Vag (sham op:  $6.4 \pm 0.8$  vs Vag:  $3.7 \pm 1.1$ ,  $P < 0.05$ ), although corticosterone level remained elevated. Adx-rats significantly increased the defecation despite the lower corticosterone level. Distribution pattern of phenol red showed RNT inhibited distal colonic and rectal transit accelerated by WAS, while Vag inhibited proximal colonic transit. Suppression of distal colonic and rectal transit by RNT was further confirmed by the bead evacuation rate (sham op: 80.0% vs RNT: 53.8%). WAS significantly increased the number of c-Fos-immunoreactive neural cells in the LMMP of the proximal and distal colon, whereas c-Fos expression was decreased by RNT in the distal colon (sham op:  $9.0 \pm 2.0$  vs RNT:  $4.4 \pm 1.0$ ,  $P < 0.05$ ) and decreased by Vag in the proximal colon.

**CONCLUSION:** Pelvic nerve conveys WAS stimuli from the brain to the distal colon, and directly activate the myenteric neurons, followed by the increase of its motility.

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**Key words:** Colonic transit; Fecal pellet output; Pelvic nerve; Psychological stress; Vagus nerve

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## INTRODUCTION

Stress is a key factor in causing abnormal bowel habits, such as constipation and diarrhea, and has been known to exacerbate these symptoms<sup>[1-4]</sup>. Accumulating evidence suggests that various stressors stimulate colonic motor functions in humans and animals<sup>[5-7]</sup>. In patients with irritable bowel syndrome, psychological and physical stressors increase colonic slow-wave, contractile, and motor activities<sup>[8]</sup>. In laboratory animals, acute exposure to restraint, cold restraint, or water avoidance stress (WAS) enhances colonic motor activity (*e.g.*, colonic motility, colonic transit, and defecation)<sup>[9-11]</sup>. The stress response is a complicated process that involves the endocrine and nervous systems. Corticotropin-releasing factor (CRF) level is increased in the hypothalamic paraventricular nuclei (PVN), amygdala, and Barrington's nucleus after acute exposure to stressful stimuli<sup>[12-15]</sup>. CRF is released in the PVN, resulting in the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary into the bloodstream. ACTH then increases the release of glucocorticoid from the cortex of the adrenal gland, which is called the hypothalamic-pituitary-adrenal axis (HPA-axis)<sup>[16]</sup>. Another endocrine system, the sympathetic-adrenal-medullary axis (SAM-axis), is activated by stress and subsequently induces the adrenal medulla to release adrenaline and noradrenaline<sup>[17]</sup>. These two major bodily pathways cause metabolic, cardiovascular, immune, and behavioral responses<sup>[18-20]</sup>. The gastrointestinal tract from the esophagus to the distal colon is innervated by parasympathetic vagal efferent fibers from the dorsal motor nucleus in the medulla oblongata<sup>[21]</sup>. Recently, it was demonstrated that vagotomy (Vag) reduced the restraint stress-induced increase in colonic transit in rats<sup>[22,23]</sup>. This evidence suggests that physical stress causes colonic dysmotility *via* parasympathetic vagal activation. On the other hand, the efferent fiber from Barrington's nucleus projects to the sacral spinal cord and terminates in the region of the preganglionic pelvic nerves<sup>[24,25]</sup>. This pelvic efferent is also classified as the parasympathetic nerve and projects to the distal colon and rectum<sup>[26]</sup>. In rats, activation of the pelvic nerve regulates distal colonic and rectal motility under physiological conditions<sup>[27]</sup>, and electrical nerve stimulation elicits contractions in the rectum<sup>[28]</sup>. Million *et al.*<sup>[29]</sup> demonstrated that WAS activates the sacral parasympathetic nucleus. However, it is still unclear whether activation of the pelvic nerve is involved in altered colonic and rectal motilities under stressed conditions.

In this study, we examined the role of the pelvic nerve pathway in the activation of defecation, colorectal transit, and myenteric neurons under psychological stress by surgically transecting the rectal branches of the pelvic nerve in rats. Additionally, we compared the differences between colorectal functional changes in pelvic nerve transected rats and vagotomized rats.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan)

weighing 300-400 g were maintained under a 12-h/12-h light dark cycle (8:00-20:00) with free access to food and water. All animal experiments were approved by the ethical committee for animal experiments of Yakult Institute. All animals were kept in individual cages in a controlled environment with constant temperature ( $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) for at least 1 wk before experiments.

### WAS

Rats were placed on a rectangular platform (11 cm  $\times$  7 cm  $\times$  8 cm) in the center of a plastic cage (41 cm  $\times$  25 cm  $\times$  20 cm) for a 1-h or 2-h period. The cage was filled with water kept at room temperature to the height of 1 cm from the top of the platform. Control rats were placed on the same platform for 1 or 2 h in a cage without water (sham stress). After loading stress, the number of fecal pellets excreted in the cage was counted. WAS and sham stress tests were carried out between 9:00 and 12:00. The animals were placed in a plastic cage for 1 h the day before the experiments, and the rats that excreted more than 5 fecal pellets were excluded from the stress experiment. Each rat was exposed to WAS only once.

### Measurement of fecal pellet output and plasma corticosterone

The number of fecal pellets found in the tank was counted and blood samples were collected into heparinized tubes from the tail vein immediately after the 1-h WAS or sham stress session. Blood samples were centrifuged and plasma was stored at  $-80^{\circ}\text{C}$  until measurement. The plasma corticosterone concentration was analyzed by the Correlate-EIA corticosterone enzyme immunoassay kit (Enzo Life Sciences, Farmingdale, NY, United States) according to the manufacturer's instructions.

### Surgical procedure

Rectal nerve transection (RNT), subdiaphragmatic Vag, or adrenalectomy (Adx) was performed in rats that were anesthetized by intraperitoneal injection (*ip*) of pentobarbital sodium (50 mg/kg; Somnopentyl, Kyoritsu Seiyaku, Tokyo, Japan). We transected the rectal nerve branches from the pelvic plexus to avoid the impairment of the micturition reflex in the present study. A midline incision was made in the abdomen and the rectal nerves were bilaterally transected at a short distance from the major pelvic ganglion. Anterior and posterior branches of the Vag were resected above the hepatic and celiac branches, and the adrenal glands were removed bilaterally. These procedures were performed using forceps under a microscope. Sham-operated animals underwent the same procedure without the above-described resection and removal. The abdominal wall and skin were sutured after completion of surgery. Rats that did not show elevated body weight during the recovery period were excluded from the experiments. Experiments were performed 7-10 d after the surgery.

### Assessment of colonic transit

Rats were anesthetized by pentobarbital sodium (50 mg/

kg, *ip*). A polyethylene catheter (PE-50, Becton, Dickinson and Company, Franklin Lakes, NJ, United States) was inserted from the cecum, and the tip was positioned in the proximal colon 1 cm distal to the cecocolonic junction. The other side of the catheter was tunneled subcutaneously to the posterior neck. Rats were given phenol red (10 mg/mL in a volume of 0.4 mL, Sigma-Aldrich, St Louis, MO, United States) through the catheter that was positioned in the proximal colon and subjected to WAS or sham stress for 1-h at 7-10 d after surgery. After the stress session, rats were euthanized with an *ip* overdose of pentobarbital sodium. The entire colon and rectum were isolated and divided into 8 equal segments, where the number of the segments from 1 to 8 was consistent with each segment that was isolated from the start of the proximal colon to the rectum. Segments 1-5 were defined as proximal colon while segments 6-8 were defined as distal colon and rectum. The number of fecal pellets in segments 6-8 was counted. Each segment was transferred into tubes and washed 3 times in phosphate buffered saline (PBS), to which followed the addition of 5 mol/L NaOH by one fifth volume. The absorbance was measured at 560 nm to calculate the volume of phenol red. The geometric center was calculated using the following equation.

Geometric center =  $\Sigma$  (fraction of phenol red per segment  $\times$  segment number)

### Assessment of transit in distal colon and rectum

The rats were fasted overnight with water *ad libitum* before the experiment. A single 5-mm glass bead was inserted into the distal colon (3 cm proximal to the anus). The rat was subjected to WAS or sham stress for 2-h immediately after bead insertion and the evacuation rate was monitored over a 2-h period.

### Immunohistochemical analysis

Rats were euthanized with an *ip* overdose of pentobarbital sodium 1 h after the WAS or sham stress session. The proximal colon (2 cm distal to the cecocolonic junction) and distal colon (4 cm proximal to the anus) were isolated and placed in 0.01 mol/L PBS (pH 7.4). The isolated colon was opened along the mesenteric border, stretched and pinned flat on a Silicon-coated petri dish (Shin-Etsu Chemical, Tokyo, Japan), and fixed overnight in 4% paraformaldehyde in 0.1 mol/L phosphate buffer at 4 °C. The following day, tissues were washed (3  $\times$  10 min) in PBS, the mucosa, submucosa, and circular muscle were removed, and whole mount preparations of longitudinal muscle myenteric plexus (LMMP) were prepared. The preparations were washed (3  $\times$  10 min) in PBS and incubated in 5% normal donkey serum (Jackson Immuno Research Laboratories, West Grove, PA, United States) in PBS containing 0.3% Triton X-100 (PBS-T) for 30 min at room temperature. The preparations were incubated with rabbit anti-c-Fos (1:10000; Ab-5; Merck Calbiochem, Darmstadt, Germany) and mouse anti-protein gene product 9.5 (PGP9.5, a marker of enteric neurons, 1:50; ab8189; Abcam, Cambridge, United Kingdom) overnight

at 4 °C. The preparations were washed (3  $\times$  10 min) in PBS-T and incubated with donkey anti-rabbit Alexa 488 (Molecular probes, Eugene, OR, United States) and donkey anti-mouse IgG Cy3-conjugated antibody (Millipore, Billerica, MA, United States). After washing (3  $\times$  10 min) in PBS-T, the preparations were mounted on glass slides using VECTASHIELD (Vector Laboratories, Burlingame, CA, United States) and analyzed by the Zeiss LSM 510 confocal microscope with LSM Image Browser software (Carl Zeiss, Jena, Germany). The number of c-Fos immunoreactive (IR) cells was counted in 10 randomly selected ganglia in each preparation per rat and the means of c-Fos-IR cells per rat were used to calculate the mean of each group.

### Statistical analysis

All results are presented as mean  $\pm$  SE. Comparisons between 2 groups were evaluated using the unpaired Student's *t* test or  $\chi^2$  test. Comparisons between the mean values of multiple groups were performed with one-way analysis of variance followed by Dunnett's multiple comparison test. *P* values of < 0.05 were considered statistically significant.

## RESULTS

### Effect of RNT, Vag and Adx on the WAS-induced increase in defecation

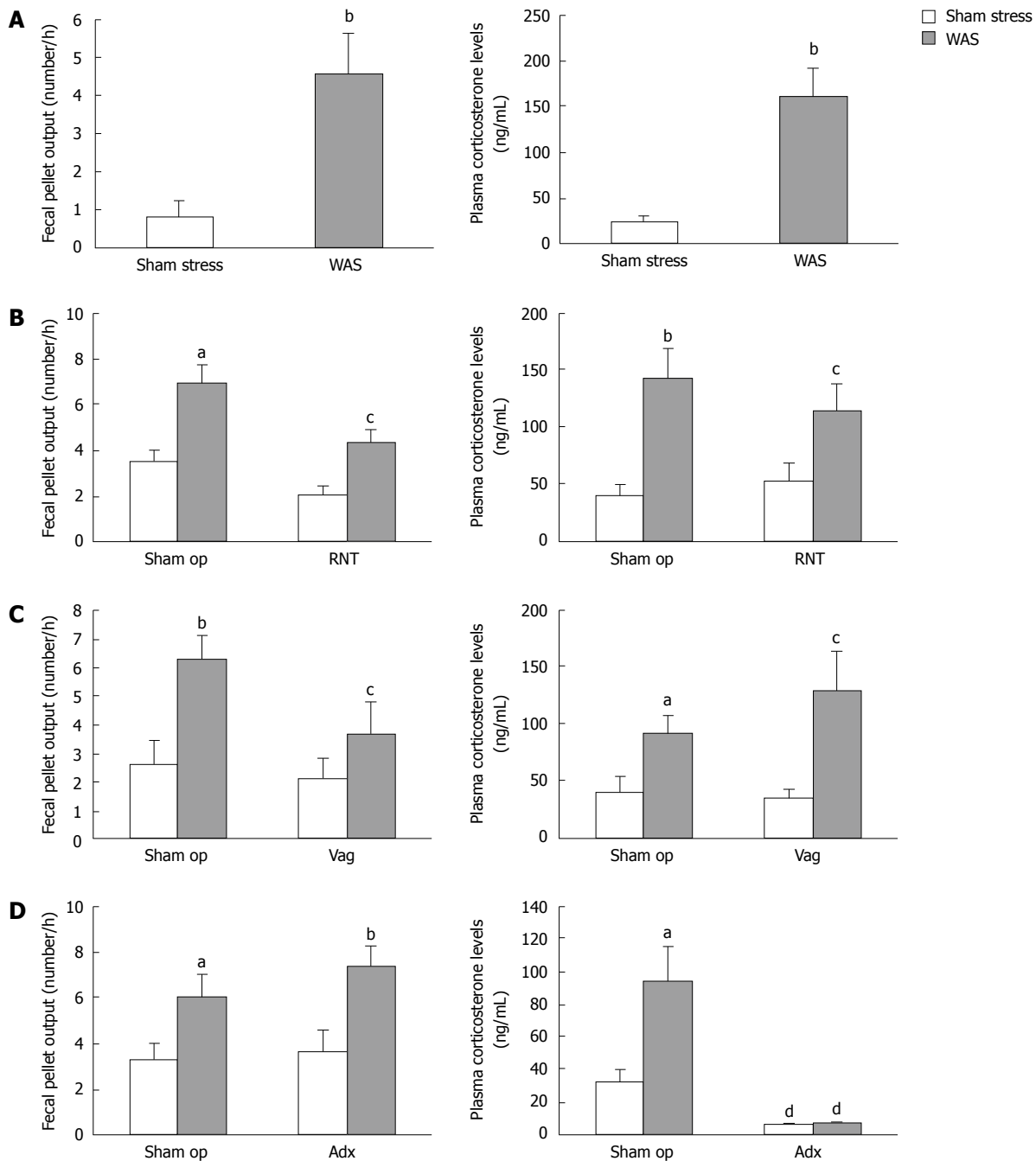
When rats were subjected to acute WAS for 1-h, the number of excreted fecal pellets was significantly increased compared to rats under sham stress ( $4.6 \pm 1.0$  *vs*  $0.8 \pm 0.4$ ,  $P < 0.01$ ). Plasma corticosterone levels markedly rose in rats just after WAS compared with those under sham stress ( $161.6 \pm 29.5$  ng/mL *vs*  $24.2 \pm 5.3$  ng/mL,  $P < 0.01$ ) (Figure 1A).

The sham-operated rats in RNT or Vag (Figure 1B-C) evacuated more fecal pellets and showed higher level of corticosterone in plasma after WAS treatment. The WAS-induced increase in fecal pellet output was significantly suppressed by treating RNT beforehand (sham op-WAS:  $6.9 \pm 0.8$ , RNT-WAS:  $4.3 \pm 0.6$ ,  $P < 0.05$ ), although the elevation of corticosterone was almost equally observed in RNT-rats and sham-operated rats (Figure 1B). Defecation under the condition of sham stress did not differ in RNT-rats and sham-operated rats. Similarly, Vag significantly reduced the WAS-induced increase in fecal pellet output (sham op-WAS:  $6.4 \pm 0.8$ , Vag-WAS:  $3.7 \pm 1.1$ ,  $P < 0.05$ ), but did not suppress the increase in corticosterone levels induced by WAS (Figure 1C).

On the other hand, WAS significantly increased the number of fecal pellets in Adx-rats compared with sham stress ( $7.4 \pm 0.8$  *vs*  $3.6 \pm 0.9$ ). The baseline levels of plasma corticosterone were lower in Adx-rats than in sham-operated rats, and an obvious change in plasma corticosterone level was not observed after WAS in Adx-rats (Figure 1D).

### Effect of RNT and Vag on WAS-induced acceleration of colonic transit

We evaluated whether the pelvic nerve pathway is in-



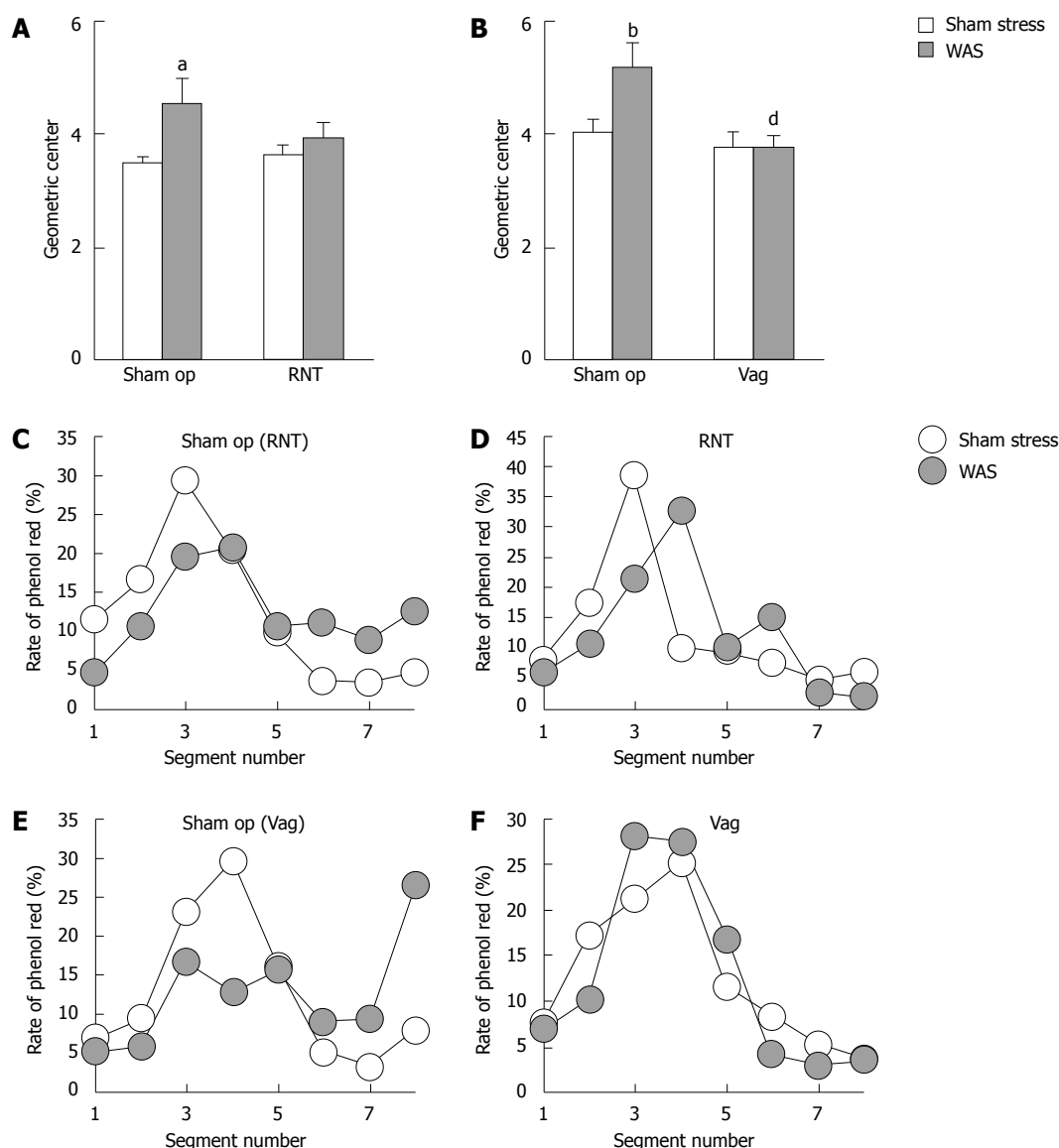
**Figure 1** Involvement of parasympathetic nerves and corticosterone in the accelerated defecation induced by acute water avoidance stress. A: The naive rats showed increases in defecation and corticosterone levels after 1-h of water avoidance stress (WAS) compared with sham stress; B-D: Effects of rectal nerve transection (RNT), vagotomy (Vag) and adrenalectomy (Adx) on WAS-induced increases in defecation and plasma corticosterone in rats. Each value represents the mean  $\pm$  SE ( $n = 6-12$  per group). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the response of sham stress rats; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs sham-operated (sham op) rats.

involved in the colonic transit during the stress session. Phenol red, a non-absorbable marker, was injected into the proximal colon *via* a catheter before stress, and geometric center was measured in the rats under the different stress conditions. In sham-operated rats, WAS significantly increased the geometric center ( $4.6 \pm 0.4$  vs  $3.5 \pm 0.1$ ,  $P < 0.05$ , Figure 2A), and also RNT-rats were found to exhibit the higher geometric center after WAS treatment although the difference was not statistically significant (Figure 2A). In contrast, WAS-induced increase of geometric center was completely suppressed in Vag-rats

(sham op-WAS:  $5.2 \pm 0.4$ , Vag-WAS:  $3.8 \pm 0.2$ ,  $P < 0.001$ , Figure 2B).

In sham-stressed RNT- and Vag-rats, the phenol red was distributed over a wide range from segments 1-8 with peaks at segments 3-4. The phenol red contents decreased in segments 1-4 and increased in segments 6-8 in sham-operated rats after WAS loading compared with sham stress (Figure 2C and E). In RNT-rats, the peak of phenol red at segment 3 during sham stress shifted to segments 4-6 after WAS loading (Figure 2D). However, the distribution pattern of phenol red did not change in





**Figure 2** Involvement of parasympathetic nerves in the bowel movement. A, B: Effects of rectal nerve transection (RNT) (A) and vagotomy (Vag) (B) on the colonic transit presented by geometric center of moved phenol red; C-F: Relative distribution of phenol red through the entire colon and rectum after water avoidance stress (WAS) in sham-operated rats (C, E), RNT-rats (D) and Vag-rats (F). Each value represents the mean of 6-8 rats. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the response of sham stress rats; <sup>c</sup> $P < 0.01$  vs sham-operated (sham op) rats.

Vag-rats even after WAS was treated (Figure 2F).

The number of luminal feces in the distal colon and rectum was higher in RNT-rats and Vag-rats than in sham-operated rats during sham stress (baseline values) [sham op (RNT):  $2.3 \pm 0.5$ , RNT-rats:  $4.1 \pm 0.4$ , sham op (Vag):  $2.1 \pm 0.4$ , Vag-rats:  $3.0 \pm 0.5$ , Table 1]. WAS tended to reduce luminal feces in sham-operated and Vag-rats, with differences of  $-0.6$  and  $-1.8$  between luminal feces in sham stress and in WAS, respectively (Table 1). The number of fecal pellets was increased by WAS in RNT-rats, but was decreased in sham op- and Vag-rats. (B)-(A) indicates the differences of the influx and efflux of fecal pellets from the distal colon and rectum. However, the difference between sham stress and WAS was  $0.1$  feces in RNT-rats, indicating that the luminal feces in segments 6-8 did not decrease under WAS in these rats.

### Effect of RNT and Vag on WAS-induced acceleration of distal colonic and rectal transit

Preganglionic parasympathetic pelvic nerves project from the sacral spinal cord to the distal colon and rectum<sup>[26]</sup>. Thus, to investigate the effect of RNT on WAS-induced distal colonic and rectal transit, a glass bead was inserted until 3 cm depth from the anus into the distal colon, and the evacuation rate of the bead was monitored over a 2-h period.

In sham-operated rats, WAS significantly increased the rate of bead evacuation to 80% compared to 38% in sham stress, but this increase was not observed in RNT-rats subjected to WAS (Figure 3A). On the other hand, Vag did not affect the WAS-induced increase in the bead evacuation rate (Figure 3B).

**Table 1** Number of luminal fecal pellets remaining in the distal colon and rectum after sham stress or water-avoidance stress

	Number of fecal pellets in the distal colon and rectum		
	Sham stress (A)	WAS (B)	(B) - (A)
Sham op (Vag)	2.1 ± 0.4	1.5 ± 0.2	-0.6
Vag	3.0 ± 0.4	1.2 ± 0.5 <sup>a</sup>	-1.8
Sham op (RNT)	2.3 ± 0.5	1.1 ± 0.3	-1.2
RNT	4.1 ± 0.4 <sup>c</sup>	4.3 ± 0.4 <sup>d</sup>	0.1

Each value represents the mean ± SE ( $n = 6-8$  per group). <sup>a</sup> $P < 0.05$  vs sham stress; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs sham operated (sham op) rats. WAS: Water-avoidance stress; Vag: Vagotomy; RNT: Rectal nerve transection.

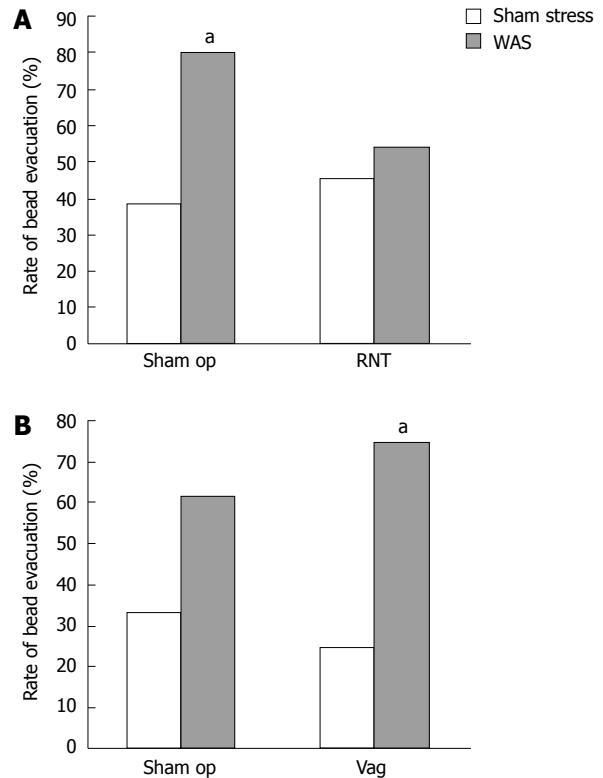
### c-Fos expression in LMMP of RNT- and Vag-rats after WAS treatment

After rats were exposed to WAS for 1 h, the number of c-Fos-IR cells was examined in whole mount preparations of LMMP from the distal colon at 0, 1, 2 and 4 h later. The maximum increase after WAS was found at 0 h, and this number returned to a normal level within 4 h (data not shown). Therefore, we measured c-Fos expression immediately after the WAS session (1-h) in all of the following experiments using denervated rats.

The number of c-Fos-IR cells per ganglia in the LMMP of both the proximal and distal colon (Figures 4 and 5) was increased by WAS compared with sham stress. RNT significantly reduced the WAS-induced increase of c-Fos-IR cells in the LMMP by 52% in the distal colon compared with sham-operated rats ( $4.4 \pm 1.0$  vs  $9.0 \pm 2.0$ ,  $P < 0.05$ , Figure 5B). In contrast, Vag inhibited the increase in c-Fos-IR cells after WAS in the LMMP of proximal colon but not of distal colon ( $1.7 \pm 1.0$  vs  $5.2 \pm 1.3$ , Figure 5C and D).

## DISCUSSION

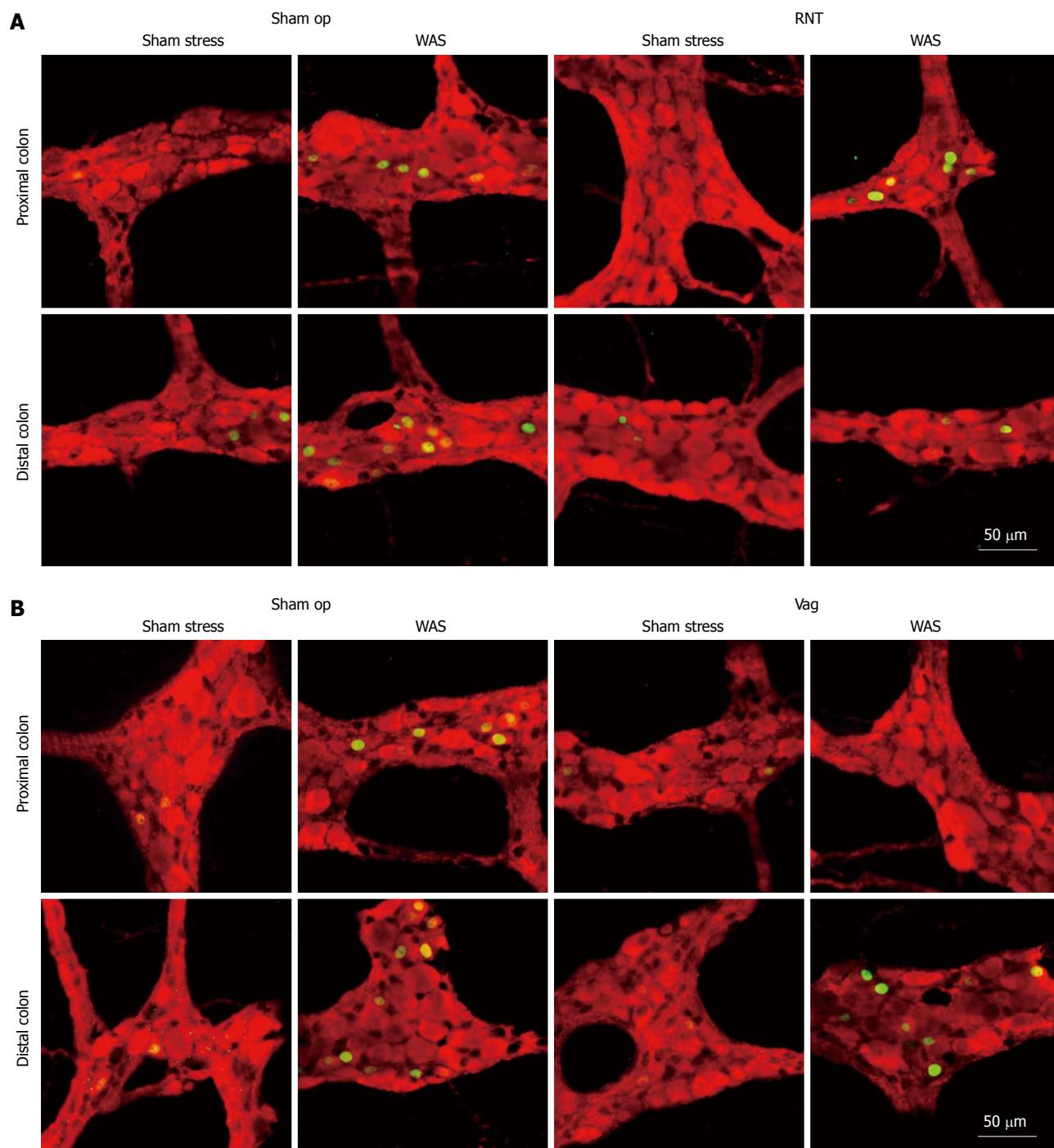
In the present study, we demonstrated the functional importance of the pelvic nerve pathway in the response to psychological stress by showing that transection of the rectal branches of the pelvic nerve (RNT) inhibited the WAS-induced increase in fecal pellet output in rats. This inhibitory effect is supported further by the observation that the RNT inhibited the increased propulsive activity of the distal colon and rectum as shown in the analysis of the colorectal excretion rate of glass beads. Therefore, it is likely that WAS accelerates distal colonic and rectal motilities *via* activation of the pelvic nerve, resulting in increased defecation in the rat. Lenz<sup>[22]</sup> and Nakade *et al.*<sup>[23]</sup> showed that Vag reduced the restraint stress-induced increase in colonic motility in rats, suggesting that physical stress causes colonic dysmotility *via* parasympathetic vagal activation. Vag also suppressed the WAS-induced increase in fecal pellet output in our study, which is consistent with their results. Our study also confirmed that proximal part of the colonic transit was accelerated in RNT-rats, whereas it was inhibited in Vag-rats under stressed conditions. In fact, many hard fecal pellets remained in the distal colon and rectum of RNT-rats in



**Figure 3** Involvement of parasympathetic nerves in the fecal expulsion through the distal colon. A, B: Effects of rectal nerve transection (RNT) (A) and vagotomy (Vag) (B) on the rate of bead evacuation from the distal colon during 2-h water avoidance stress (WAS). The increased rate of bead evacuation in sham-operated rats was inhibited in RNT-rats. Each value represents the mean ± SE ( $n = 11-15$  per group). <sup>a</sup> $P < 0.05$  vs sham stress rats.

spite of WAS loading. In contrast, luminal fecal pellets in the distal part of the colon and rectum were decreased in sham-operated and Vag-rats after WAS. These results suggest that pelvic nerve activation by WAS accelerates motor activities of distal colon and rectum, but not in those of proximal colon. On the other hand, Vagus nerve activation accelerates the proximal colonic transit, but not the distal colonic and rectal transit. Therefore, the difference in projection sites of the gut between the pelvic and vagal efferent nerves may be responsible for the differential actions in the gut<sup>[21,26]</sup>. The pelvic nerve innervates the distal colon and rectum, and regulates rectal motility in the physiological conditions<sup>[27]</sup>. In this study, a larger number of fecal pellets remained in the distal colon and rectum in RNT-rats without stress, compared with sham-operated rats. These observations suggest that the pelvic nerve pathway may be involved in colorectal motility in the non-stressed conditions. In addition, our findings demonstrate that acute psychological stress stimulates colorectal transit *via* the pelvic nerve pathway.

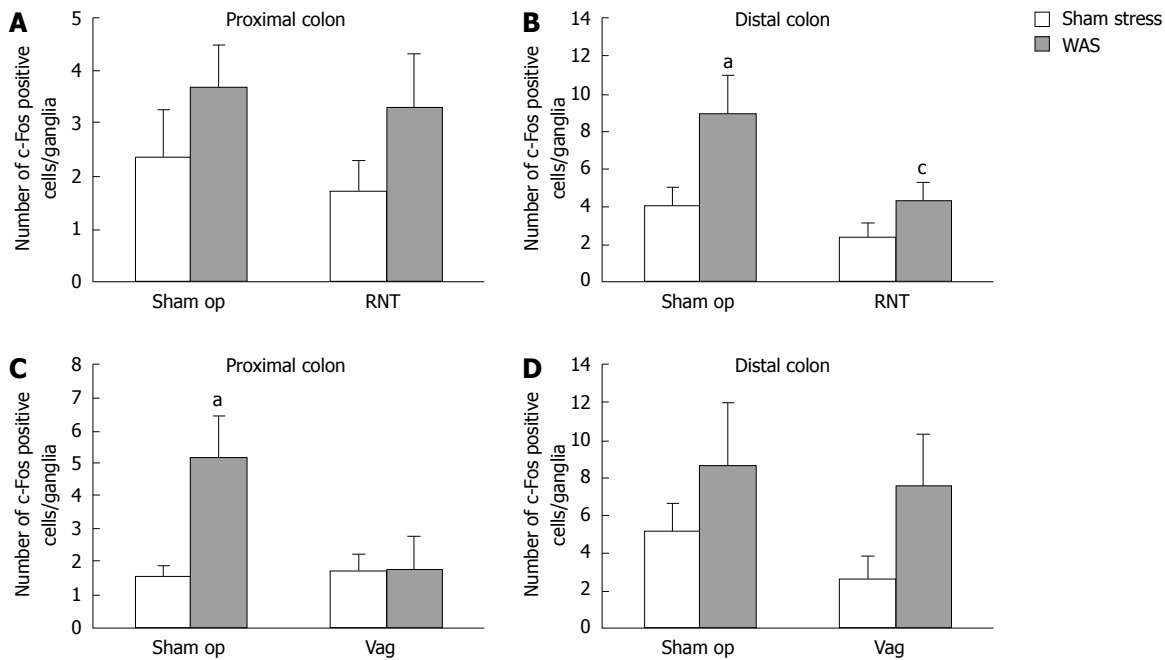
Recently, c-Fos protein expression has been used as a marker of neural cell activation<sup>[30]</sup>. c-Fos expression was significantly increased in PGP9.5-positive myenteric neurons in the proximal and distal colon after WAS. This finding is similar to that of a study by Miampamba *et al.*<sup>[31]</sup>, who reported increased c-Fos expression in the



**Figure 4** Induction of c-Fos expression after exposure to water avoidance stress. Confocal microscope images showing the staining of c-Fos (green) and protein gene product 9.5 (PGP9.5, red) in whole mount preparations of longitudinal muscle myenteric plexus in rectal nerve transection (RNT) (A) and vagotomy (Vag) rats (B). WAS: Water avoidance stress; Sham op: Sham-operated.

myenteric ganglia of the proximal and distal colon after WAS. Enhanced c-Fos expression was correlated with the number of fecal pellets in the rats and was significantly suppressed in the distal, but not proximal, colon by the denervation of pelvic nerve branches after WAS. On the other hand, Vag suppressed c-Fos expression in the proximal colon but had no effect in the distal colon. These results concerning c-Fos expression are consistent with the differences in the functional site of action between the vagus and pelvic nerves, as discussed above.

It is yet unclear whether c-Fos expression, which was induced by WAS-loading, is a direct or indirect response to pelvic efferent nerve activation. As a direct effect, c-Fos expression could be due to inputs from the preganglionic efferent fibers to the myenteric neurons<sup>[21,26]</sup>. As indirect effect, this may be due to mechanisms such as colonic motility and secretory responses, sensory neurons and interneurons in enteric nervous systems, or the release of gastrointestinal hormones and other factors that activate myenteric neurons. Although we did not clarify the



**Figure 5** Effects of vagotomy and rectal nerve transection on the induction of c-Fos-expressing cells in the colon after water avoidance stress. Using whole mount preparations of longitudinal muscle myenteric plexus in the proximal (A, C) and distal colons (B, D), numbers of c-Fos immunoreactive cells per ganglia in the proximal and distal colons of rectal nerve transection (RNT) rats (A, B) and vagotomy (Vag) rats (C, D) after water avoidance stress (WAS) treatment were counted and compared to those of sham stress rats. Each value represents the mean  $\pm$  SE ( $n = 6-12$  per group). <sup>a</sup> $P < 0.05$  vs sham stress rats; <sup>c</sup> $P < 0.05$  vs sham-operated (sham op) rats.

types of neurons that expressed c-Fos protein in these experiments, myenteric neurons are generally classified as motor neurons, sensory neurons, and interneurons based on histological and morphological properties<sup>[31,32]</sup>. A double-labeling study revealed that 24%-25% of the total peripheral-choline acetyltransferase-positive neurons, which are mostly motor neurons in the myenteric plexus<sup>[32]</sup>, were c-Fos positive in the proximal and distal colon after WAS<sup>[31]</sup>. Furthermore, the muscarinic receptor antagonist atropine inhibits the WAS-induced increase in fecal pellets without reducing the elevation of c-Fos-IR nuclei in the proximal and distal colonic LMMP<sup>[31,33]</sup>. Taken together, these results suggest that myenteric neurons in the distal and proximal colon are activated directly *via* the pelvic and vagal efferent, respectively, followed by acceleration of colonic transit and defecation.

The HPA-axis is also known as a key stress response pathway. HPA-axis activation ultimately induces corticosterone release from the adrenal gland<sup>[16]</sup>. In this study, the failure to release corticosterone by bilateral Adx did not affect the WAS-induced increase in fecal pellets. Furthermore, Lenz<sup>[22]</sup> showed that Adx or hypophysectomy did not alter the restraint stress-induced acceleration of colonic transit. These results indicate that the HPA-axis is not mainly involved in acute stress-induced acceleration of colorectal transit and defecation. Our results from adrenalectomized rats in this study also imply that the SAM-axis is of little importance in acute stress-induced colonic dysmotility because Adx inhibits the SAM-axis in addition to the HPA-axis<sup>[34]</sup>. It was shown recently that a corticoid-receptor antagonist inhibits chronic stress-

induced defecation, indicating that the HPA-axis could be involved in the acceleration of colonic motor function by stress<sup>[35]</sup>. It is not clear why this corticoid-receptor antagonist decreased stress-induced colonic motor function, but this might be due to the different mechanisms that are involved in acute and chronic stress. In fact, it has been reported that chronic exposure of the amygdala to corticosterone delayed colorectal transit after WAS<sup>[36]</sup>. The current study has only examined the involvement of the parasympathetic nerves in stress-induced acceleration of colonic transit and defecation. The colon and rectum also receives sympathetic inputs from the lumbar colonic nerves and hypogastric nerves. It has been shown that stimulation of sympathetic nerves inhibits rectal motility in guinea pigs and cats<sup>[37-39]</sup>. Furthermore, it has been reported that sympathetic nerve denervation increased the c-Fos immunoreactivity in the LMMP<sup>[40]</sup> and the rectal motility<sup>[27]</sup>. These results suggested that sympathetic nerves may negatively regulate the colonic motor function. In the stressed-condition, however, it remains to be determined whether sympathetic nerve activity participates in the acceleration of colorectal transit and defecation induced by stress. Further experimental investigations are needed to understand the involvement of sympathetic nerves in stress-induced colonic dysmotility.

In conclusion, we have shown that WAS-induced acceleration of distal colonic transit and defecation is mediated by the parasympathetic pelvic efferent. The pelvic nerve may convey stress stimuli from the brain to the distal colon and rectum, directly activating myenteric neurons and subsequently accelerating distal colonic motility.



In contrast, WAS-induced acceleration of colonic transit and activation of myenteric neurons in proximal colon is mediated by parasympathetic vagal efferent.

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## COMMENTS

### Background

Stress is a key factor in causing abnormal bowel habits, such as constipation and diarrhea, and has been known to exacerbate these symptoms. It has been described that various stressors stimulate colonic motor functions. Although it is reported that the activation of colonic motor function is mediated via the parasympathetic vagus nerve, there are few studies that have reported the involvement of the parasympathetic pelvic nerve in stress-induced gut dysmotility.

### Research frontiers

The pelvic efferent projects from the sacral parasympathetic nucleus to the distal colon and rectum. It is reported that water avoidance stress (WAS) activate the sacral parasympathetic nucleus, however, it is still unclear whether activation of the pelvic nerve is involved in altered colonic and rectal motilities under stressed conditions.

### Innovations and breakthroughs

This study demonstrated the importance of the pelvic nerve in the response to WAS-induced acceleration of distal colonic transit and increase of defecation. Furthermore, the results confirmed the involvement of vagus nerve to WAS-induced acceleration of proximal colonic transit. This is the first study to report the differences in the functional site of action between two nerves under stressed condition.

### Applications

By understanding how stress stimuli cause the colonic dysmotility, these results would be useful for the therapeutic applications of stress-related colonic motor dysfunction, such as irritable bowel syndrome.

### Terminology

Longitudinal muscle myenteric plexus (LMMP): LMMP is generally used as immunohistochemical study of the myenteric plexus; c-Fos: c-Fos protein is the product of immediate-early gene, c-fos, which is used as a marker of neural activation.

### Peer review

The authors clearly demonstrated involvement of pelvic nerve in the psychological stress-induced acceleration of distal colonic transit and defecation. Additionally this study suggested that pelvic nerve directly activate the myenteric neurons and subsequently accelerating distal colonic motility. This is an interesting study, that clarify the role of the pelvic and vagus nerve in the stress-induced colonic dysmotility.

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## Inhibition of pacemaker activity in interstitial cells of Cajal by LPS *via* NF- $\kappa$ B and MAP kinase

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of ICCs and this could be blocked by AH6809, a prostaglandin E2-EP2 receptor antagonist or NG-Nitro-L-arginine Methyl Ester, an inhibitor of nitric oxide (NO) synthase. Toll-like receptor 4, inducible NO synthase or cyclooxygenase-2 immunoreactivity by specific antibodies was detected on ICCs. Catalase (antioxidant agent) had no action on LPS-induced action in ICCs. LPS actions were blocked by nuclear factor  $\kappa$ B (NF- $\kappa$ B) inhibitor, actinomycin D (a gene transcription inhibitor), PD 98059 (a p42/44 mitogen-activated protein kinases inhibitor) or SB 203580 [a p38 mitogen-activated protein kinases (MAPK) inhibitor]. SB 203580 also blocked the prostaglandin E2-induced action on pacemaker currents in ICCs but not NO.

**CONCLUSION:** LPS inhibit the pacemaker currents in ICCs *via* prostaglandin E2- and NO-dependent mechanism through toll-like receptor 4 and suggest that MAPK and NF- $\kappa$ B are implicated in these actions.

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**Key words:** Interstitial cells of Cajal; Lipopolysaccharide; Mitogen-activated protein kinases; Nuclear factor  $\kappa$ B; Small intestine

### Abstract

**AIM:** To investigate lipopolysaccharide (LPS) related signal transduction in interstitial cells of Cajal (ICCs) from mouse small intestine.

**METHODS:** For this study, primary culture of ICCs was prepared from the small intestine of the mouse. LPS was treated to the cells prior to measurement of the membrane currents by using whole-cell patch clamp technique. Immunocytochemistry was used to examine the expression of the proteins in ICCs.

**RESULTS:** LPS suppressed the pacemaker currents

Zuo DC, Choi S, Shahi PK, Kim MY, Park CG, Kim YD, Lee J, Chang IY, So I, Jun JY. Inhibition of pacemaker activity in interstitial cells of Cajal by LPS *via* NF- $\kappa$ B and MAP kinase. *World J Gastroenterol* 2013; 19(8): 1210-1218 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i8/1210.htm>  
DOI: <http://dx.doi.org/10.3748/wjg.v19.i8.1210>

### INTRODUCTION

Many morphological or functional studies have disclosed the roles of interstitial cells of Cajal (ICCs) in many organs including gastrointestinal (GI) tract. An accumulation of

evidence from these studies has revealed ICCs has the distinct role in pacemaker activity<sup>[1-3]</sup> and as mediators of enteric motor neurotransmission in GI tract<sup>[4-6]</sup>. For development and maintenance, the ICCs express the receptor tyrosine kinase Kit<sup>[1,3,7]</sup>. And, it is well known that c-Kit or ANO1 can be a selective molecular marker for researching the distribution and function of ICCs<sup>[8]</sup>.

GI inflammation can be caused by inflammatory cytokines such as interleukins, chemical inflammatory mediators *etc* and lipopolysaccharide (LPS) from gram-negative bacteria is a major causative factor of GI inflammation<sup>[9]</sup>. Various *vivo* and *vitro* experiments showed LPS could play a major role in GI motility disorders<sup>[10-13]</sup>. Also, there are several reports that the morphological and functional changes of ICCs are involved in inflammation-induced GI motility<sup>[14-16]</sup>. These indicate ICCs may be important target for inflammation-induced motility disorders. Recently we reported that LPS inhibited the pacemaker currents in cultured ICCs from mouse small intestine. LPS-action was blocked by cyclooxygenase (COX)-2 inhibitor or nitric oxide (NO) synthase inhibitor, suggesting prostaglandins (PGs) and NO are involved in these actions<sup>[17]</sup>, can verify this.

Generally, the stimulation of LPS rouses regulatory pathways such as the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway, as well as reactive oxygen species (ROS) signaling cascades *via* a toll like receptor-4-mediated signaling pathway<sup>[18]</sup>. Furthermore, the stimulation of LPS results in the activation of kinases including extracellular signal-regulated kinases, c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (MAPKs) critical for growth and cytokine production that induces inflammatory-related substances, such as interleukin, PGs, inducible NO synthase (iNOS) *etc*<sup>[19-21]</sup>. Our previous report suggested production of inflammatory-related substances, such as prostaglandin E2 (PGE<sub>2</sub>) or NO are involved in LPS-induced action on ICCs<sup>[17]</sup>. However, the details of the PGs or iNOS induction in ICCs by LPS stimulation remain unclear.

In the present study, we aimed to clarify the involvement of NF- $\kappa$ B, ROS and MAPKs that contribute to PGs or iNOS induction in ICCs and changing of pacemaker activity in LPS-treated ICCs.

## MATERIALS AND METHODS

### Solutions and drugs

The cells were bathed in a solution containing 5 mmol/L KCl, 135 mmol/L NaCl, 2 mmol/L CaCl<sub>2</sub>, 10 mmol/L glucose, 1.2 mmol/L MgCl<sub>2</sub>, and 10 mmol/L HEPES, adjusted to pH 7.2 with Tris. NaCl was replaced with equimolar N-methyl-D-glucamine for making Na<sup>+</sup>-free solution, and CaCl<sub>2</sub> was omitted in the bath solution for Ca<sup>2+</sup>-free solution. The pipette solution contained 20 mmol/L K-aspartate, 120 mmol/L KCl, 5 mmol/L MgCl<sub>2</sub>, 2.7 mmol/L K<sub>2</sub>ATP, 0.1 mmol/L Na<sub>2</sub>GTP, 2.5 mmol/L creatine phosphate disodium, 5 mmol/L

4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), and 0.1 mmol/L EGTA, adjusted to pH 7.2 with Tris.

The drugs used were LPS (salmonella enteric serotype typhimurium), JNK inhibitor II, SN-50 (NF- $\kappa$ B inhibitor) and catalase from Calbiochem, (San Diego, CA, United States) and the other compounds were purchased from Sigma.

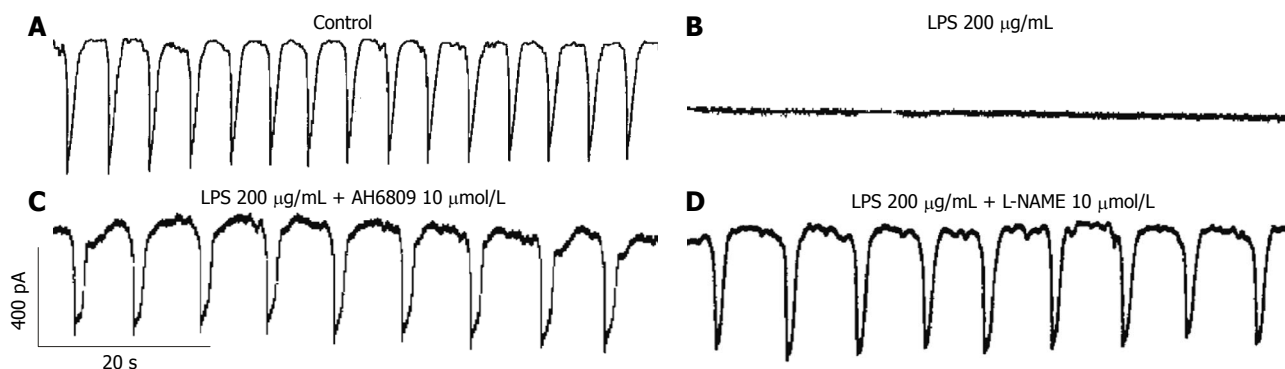
### Preparation of cells and tissues

Balb/C mice (3-8 d old) of either sex were anesthetized with ether and were sacrificed by cervical dislocation. The small intestines from 1 cm below the pyloric ring to the cecum were removed and opened along the mesenteric border. The luminal contents were washed away with Krebs-Ringer bicarbonate solution. The tissues were pinned to the base of a Sylgard dish and the mucosa was removed by sharp dissection. Small strips of intestinal muscle were equilibrated in Ca<sup>2+</sup>-free Hank's solution containing 5.36 mmol/L KCl, 125 mmol/L NaCl, 0.34 mmol/L NaOH, 0.44 mmol/L Na<sub>2</sub>HCO<sub>3</sub>, 10 mmol/L glucose, 2.9 mmol/L sucrose, and 11 mmol/L HEPES for 30 min, and the cells were dispersed with an enzyme solution containing 1.3 mg/mL collagenase (Worthington Biochemical Co, Lakewood, NJ, United States), 2 mg/mL bovine serum albumin (Sigma Chemical Co., St. Louis, MO, United States), 2 mg/mL trypsin inhibitor (Sigma) and 0.27 mg/mL ATP. Cells were plated onto sterile glass coverslips coated with poly L-lysine (2.5  $\mu$ g/mL, Sigma) in 35 mm culture dishes. The cells were then cultured at 37 °C in a 5% CO<sub>2</sub> incubator in SMGM (smooth muscle growth medium) (Clonetics Co., Walkersville, MD, United States) supplemented with 2% antibiotics/antimycotics (Gibco, Grand Island, NY, United States) and murine stem cell factor (SCF, 5 ng/mL, Sigma). ICCs were identified immunologically with the use of a monoclonal antibody for kit protein (ACK2) labeled with Alexa Fluor 488 (Molecular Probes, Eugene, OR, United States).

### Patch clamp experiments

The whole-cell configuration of the patch clamp technique was used to record membrane currents (voltage clamp) and membrane potentials (current clamp) from cultured ICC after 2-3 d in culture. We recorded from small clusters of ICC, as spontaneous inward currents from small groups of cells are more robust and regular than from single cells. Currents or potentials were amplified by Axopatch 200B (Axon Instruments, Foster, CA, United States). A command pulse was applied using an IBM compatible personal computer and pClamp software (version 9.2; Axon Instruments). The data were filtered at 5 kHz, and the recorded data were displayed on a computer monitor saved for data analysis and future references. Results were analyzed using the Clampfit program (Axon Instruments) and Graph Pad Prism (version 2.01) software. All experiments were performed at 30 °C.





**Figure 1** Effects of AH6809 or NG-Nitro-L-arginine Methyl Ester on lipopolysaccharide-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) at a holding potential of -70 mV in control condition; B: Pacemaker currents of ICCs incubated at 37 °C with 200 µg/mL of lipopolysaccharide (LPS) for 12 h; C: Pacemaker currents in ICCs pretreated with AH6809 (10 µmol/L) for 2 h prior to LPS incubation; D: Pacemaker currents in ICCs pretreated with NG-Nitro-L-arginine Methyl Ester (10 µmol/L) for 2 h prior to LPS incubation. LPS: Lipopolysaccharide; L-NAME: NG-Nitro-L-arginine Methyl Ester.

### Immunocytochemistry

Cultured cells were fixed in acetone (20 °C/5 min). Following fixation, preparations were washed for 60 min in phosphate buffered saline (PBS; 0.01 mol, pH 7.4). Cultured cells were then incubated in 10% goat serum containing 1% bovine serum albumin for 1 h at RT to reduce nonspecific antibody binding. For double immunostaining, cells were incubated overnight at 4 °C in a mixture of two primary antibodies raised in different species (a rat monoclonal c-Kit antibody, 1:200, Gibco-BRL, Gaithersburg, MD, United States; a goat polyclonal TLR4 antibody, 1:100, Santa Cruz Biotechnology, CA, United States; a goat polyclonal iNOS antibody, 1:100, Santa Cruz Biotechnology; a goat polyclonal COX-2 antibody, 1:100, Santa Cruz Biotechnology). The secondary antibodies were fluorescein isothiocyanate (FITC) for c-Kit and Texas Red for anti-TLR4, iNOS or COX-2, diluted 1:100 (all reagents from Vector Laboratories, Burlingame, CA). Immunoreactivity was detected using FITC-conjugated secondary antibody (FITC-anti-rat; Vector Laboratories, 1:100 in PBS, 1 h, room temperature). Control cultured ICCs were prepared in a similar manner, but, omitting ACK2 from the incubation medium. After washing with PBS, the mixture of labeled secondary antibodies was treated with FITC-conjugated donkey anti-rabbit IgG (1:100, Chemicon, Temecula, CA, United States) and Texas red-conjugated donkey anti-goat IgG (1:100, Jackson ImmunoResearch Laboratories, Baltimore, PA, United States). The incubation of labeled secondary antibodies was performed for 1 h at room temperature. Cells were examined with a FV300 confocal microscope (Olympus, Tokyo, Japan) with an excitation wavelength appropriate for FITC (488 nm) and Texas Red (568 nm). Final images were constructed with Flow View Software Program (Olympus, Tokyo, Japan).

### Ethics

All experiments were performed according to the Guiding Principles for the Care and Use of Animals approved by the Ethics Committee of Chosun University and the

National Institute of Health Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize both the number of animals used and the suffering of the animals.

### Statistical analysis

Data are expressed as the mean  $\pm$  SE. Differences in the data were evaluated by use of the Student's *t* test. *P* values less than 0.05 are considered a statistically significant difference. The *n* values reported in the text refer to the number of cells used in the patch clamp experiments.

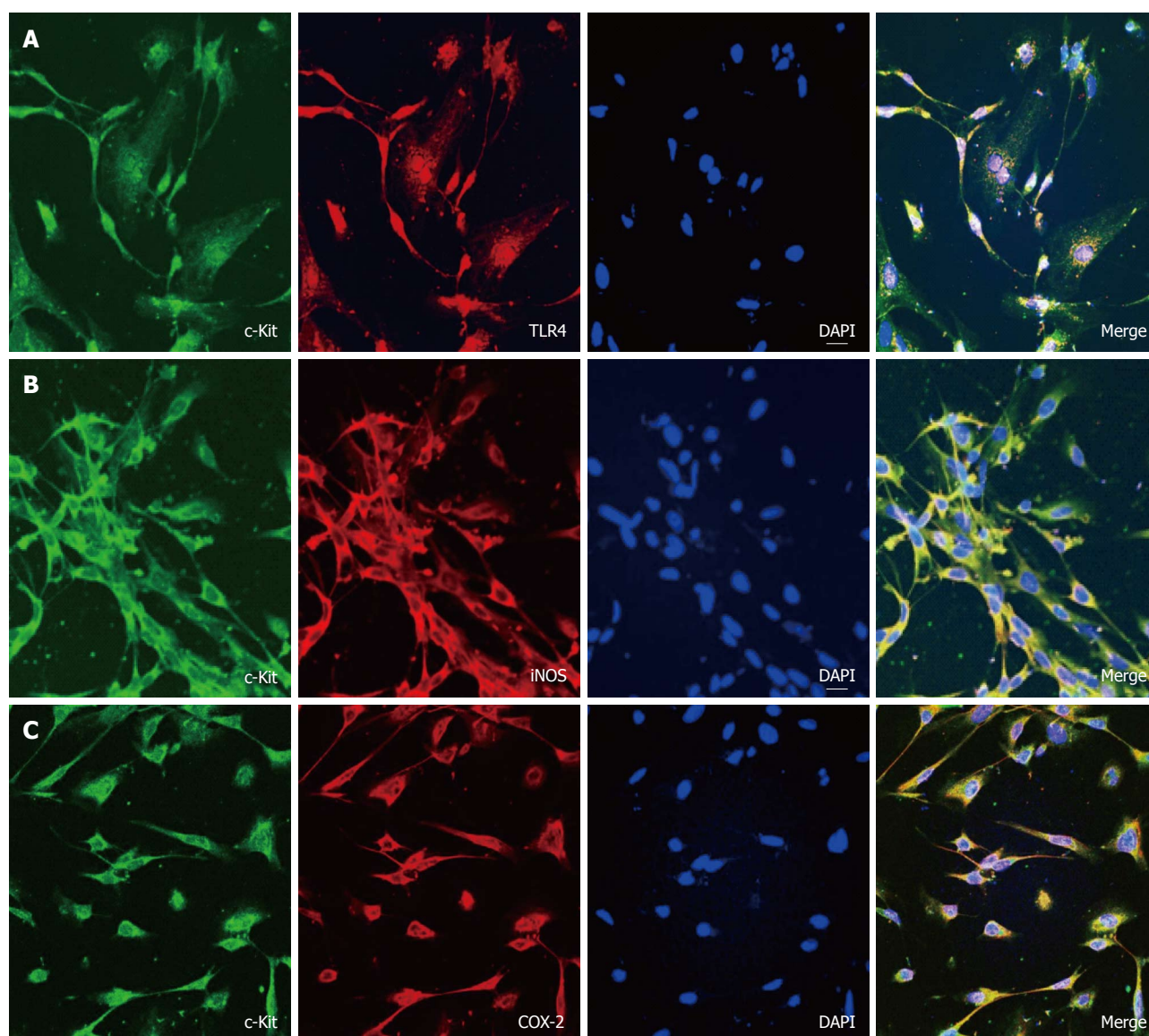
## RESULTS

### Confirmation of prostaglandin or NO production involvement on LPS-induced action in ICCs

First, we verified inhibitory action of LPS on ICCs and the involvement of PG or NO production on LPS-induced action. Recordings were made from cells within networks that had morphologies similar to the cells that were immunopositive for c-Kit. Under voltage clamp mode at a holding potential of -70 mV, ICCs showed spontaneous inward pacemaker currents (Figure 1A) and this inhibited by 200 µg/mL LPS incubation for 12 h (Figure 1B). And inhibitory action of LPS on pacemaker activity in ICCs was blocked by 10 µmol/L AH6809 (a PGE<sub>2</sub>-EP2 receptor antagonist) or 10 µmol/L NG-Nitro-L-arginine Methyl Ester (L-NAME) (an inhibitor of NO synthase) (*n* = 5, Figure 1C and D, bar graph not shown). These could make sure the involvement of PG or NO production on LPS-induced action in ICCs.

### Localization of toll like receptor-4, inducible NO synthase and COX-2 in ICCs

For searching the ability that can interact with TLR4 and produce NO or PGs in ICCs, we tried immunocytochemistry with specific antibody for TLR4, iNOS and COX-2 proteins. Double staining with anti-c-Kit and anti-TLR4, anti-iNOS or anti-COX-2 antibodies revealed TLR4, iNOS and COX-2 immunoreactivity in c-Kit immune-positive ICCs (Figure 2A-C).



**Figure 2** Expression of TLR4, iNOS and COX-2 in c-Kit positive cells. A: Double labeling of TLR4- and c-Kit-like immunoreactivity in cultured interstitial cells of Cajal (ICCs). Green (c-Kit) and red (TLR4) result in the mixed color yellow, indicating the colocalization of both peptides; B: Double labeling of iNOS and c-Kit-like immunoreactivity in ICCs. Green (c-Kit) and red (iNOS) result in the mixed color yellow, indicating the colocalization of both peptides; C: Double labeling of COX-2 and c-Kit-like immunoreactivity in ICCs. Green (c-Kit) and red (COX-2) result in the mixed color yellow, indicating the colocalization of both peptides (bar = 20  $\mu$ m).

### Effects of catalase on LPS-induced action in ICCs

For finding the involvement of ROS production on LPS-induced action, we used the antioxidant reagent, catalase. To investigate the effect of LPS, ICCs were incubated with 200  $\mu$ g/mL LPS for 12 h and we could reconfirm the disappearance of pacemaker currents (Figure 3A). Pretreatment with catalase (3000 unit/mL) for 2 h prior to 200  $\mu$ g/mL LPS incubation could not show any difference compared with LPS alone (Figure 3B). The frequency and amplitude values of pacemaker currents in catalase treatment with LPS were not significantly different compared with LPS alone ( $n = 4$ , Figure 3C and D).

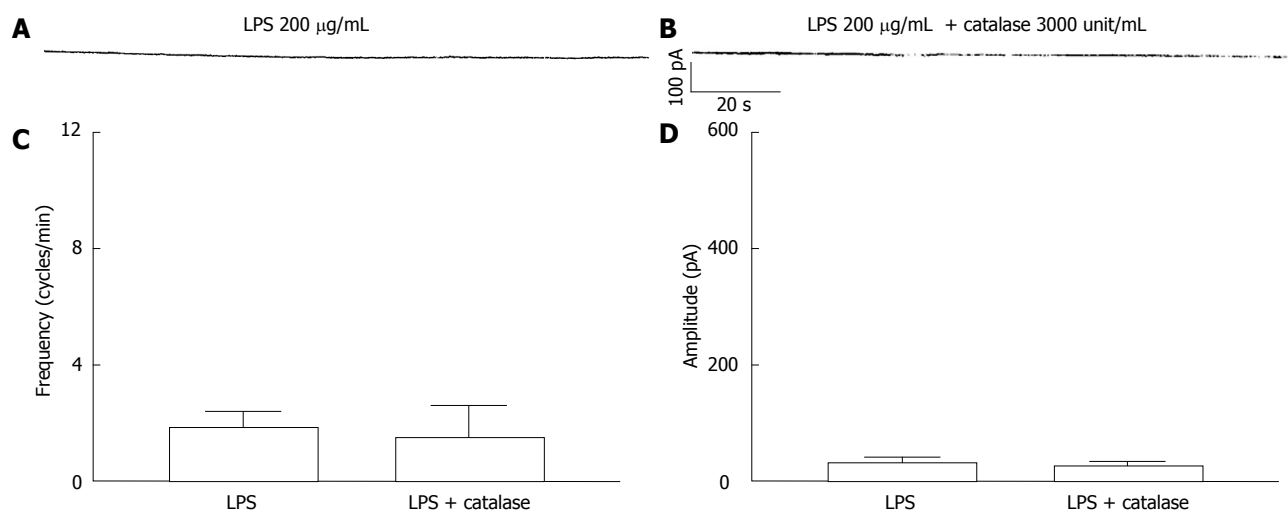
### Effects of SN-50 or actinomycin D on lipopolysaccharide-induced action in ICCs

For detecting the role for NF- $\kappa$ B or involvement of gene transcriptions on LPS-induced action, we used SN-50,

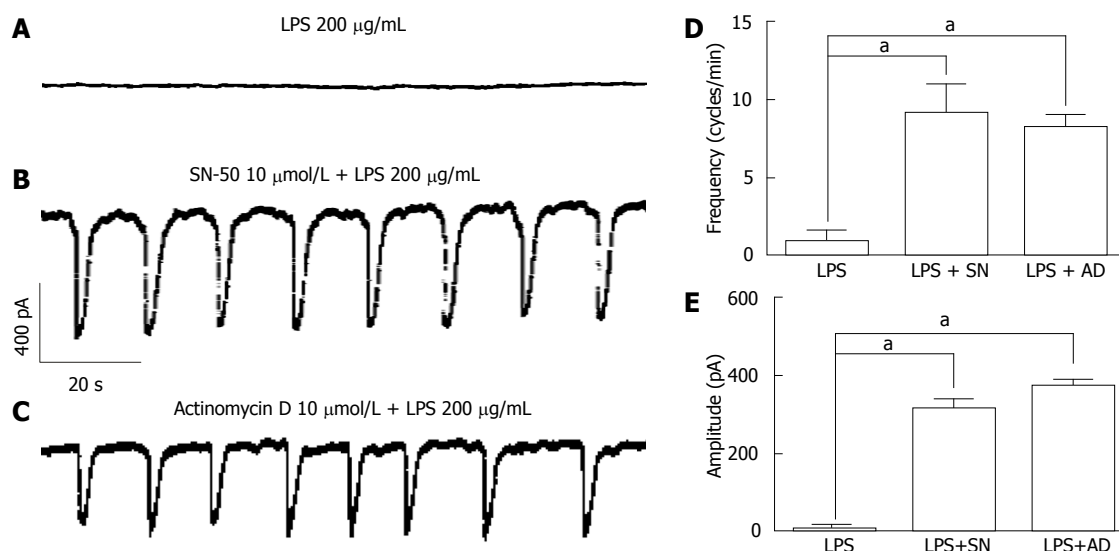
the NF- $\kappa$ B inhibitor and actinomycin D, the gene transcription inhibitor. The incubation with LPS 200  $\mu$ g/mL alone, the pacemaker currents were completely inhibited (Figure 4A). But when SN-50 (10  $\mu$ mol/L) was treated for 2 h prior to LPS incubation, LPS-induced action on pacemaker currents in ICCs was blocked (Figure 4B). Also, we could find that actinomycin D (10  $\mu$ mol/L) blocked the LPS-induced pacemaker currents suppression (Figure 4C). The values of frequency and amplitude induced by LPS in the presence of SN-50 or actinomycin D were significantly different from those obtained in the absence of NF- $\kappa$ B inhibitor ( $n = 5$ , Figure 4D and E).

### Effects of MAPK inhibitors on LPS-induced action in ICCs

For understanding the involvement of MAPKs on LPS-induced action, specific MAPKs inhibitors were used. When the pretreatments of ICCs with PD 98059 (a



**Figure 3** Effects of catalase on lipopolysaccharide-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) incubated at 37 °C with 200 µg/mL of lipopolysaccharide (LPS) for 12 h; B: Pacemaker currents in ICCs pretreated with catalase (3000 unit/mL) for 2 h prior to LPS incubation. The effects of catalase on the LPS-induced action in ICCs are summarized in C and D. Bars represent mean  $\pm$  SE values ( $n = 4$  per group).



**Figure 4** Effects of SN-50 or actinomycin D on lipopolysaccharide-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) incubated at 37 °C with 200 µg/mL of lipopolysaccharide (LPS) for 12 h at a holding potential of -70 mV; B: Pacemaker currents in ICCs pretreated with SN-50 (10 µmol/L) for 2 h prior to LPS incubation; C: Pacemaker currents in ICCs pretreated with actinomycin D (10 µmol/L) for 2 h prior to LPS incubation. The effects of SN-50 or actinomycin D on the LPS-induced action in ICCs are summarized in D and E. Bars represent mean  $\pm$  SE values ( $n = 5$  per group).  $^aP < 0.05$  vs LPS alone treatment. SN: SN-50, AD: Actinomycin D.

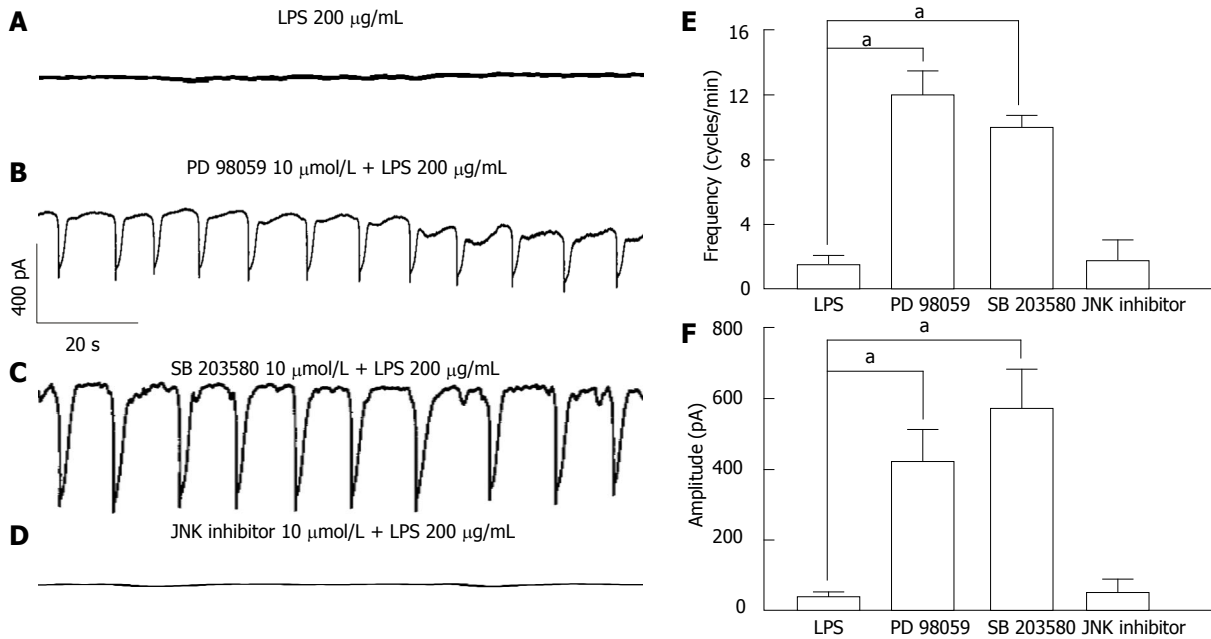
selective p42/44 inhibitor), SB 203580 (a selective p38 inhibitor) or JNK inhibitor II for 2 h prior to 200 µg/mL LPS incubation, the LPS-induced inhibitory action on pacemaker currents were blocked by PD 98059 (10 µmol/L), SB 203580 (10 µmol/L) but not by JNK inhibitor II (10 µmol/L) (Figure 5B-D). The values of frequency, amplitude and resting currents induced by LPS in the presence of PD 98059 or SB 203580 were significantly different from those obtained in the absence of these ( $n = 7$ , Figure 5E and F).

#### Effects of MAPK inhibitors on Prostaglandin E<sub>2</sub>-induced action in ICCs

For examining the involvement of MAPKs on PGE<sub>2</sub> pro-

duction by LPS stimulation in ICCs, we used p42/44 inhibitor and p38 inhibitor with PGE<sub>2</sub>. Because JNK inhibitor II did not show any influence on LPS-induced action in ICCs, we did not test with JNK inhibitor II in here. At first, we could see PGE<sub>2</sub> (5 µmol/L) itself has the inhibitory effect on pacemaker currents in ICCs (Figure 6A). Then, we examined the effects of PGE<sub>2</sub> on pacemaker currents in presence of PD 98059 or SB203580. The pretreatments of ICCs with PD 98059 (10 µmol/L) blocked the 5 µmol/L PGE<sub>2</sub>-induced inhibitory action on pacemaker currents but not SB 203580 (10 µmol/L) (Figure 6B and C). The values of frequency and amplitude currents induced by PGE<sub>2</sub> in the presence of PD 98059 were significantly different from those obtained in the absence





**Figure 5** Effects of Mitogen-activated protein kinases inhibitors on lipopolysaccharide-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) incubated at 37 °C with 200  $\mu$ g/mL of lipopolysaccharide (LPS) for 12 h at a holding potential of -70 mV; B: Pacemaker currents after pretreatment with PD 98059 (10  $\mu$ mol/L) for 2 h prior to LPS incubation; C: Pacemaker currents after pretreatment with SB 203580 (10  $\mu$ mol/L) for 2 h prior to LPS incubation; D: Pacemaker currents after pretreatment with c-Jun N-terminal kinase (JNK) inhibitor II (10  $\mu$ mol/L) for 2 h prior to LPS incubation. The effects of mitogen-activated protein kinases inhibitors on the LPS-induced action are summarized in E and F. Bars represent mean  $\pm$  SE values ( $n = 7$  per group).  $^aP < 0.05$  vs LPS.

of PD 98059 ( $n = 4$ , Figure 6D and E).

### Effects of MAPK inhibitors on NO-induced action in ICCs

For examining the role of MAPKs on NO production by LPS stimulation in ICCs, we also used p42/44 inhibitor and p38 inhibitor with ( $\pm$ )-S-nitroso-N-acetylpenicillamine (SNAP), a NO donor. In our previous report<sup>[22]</sup>, we already showed SNAP has inhibitory action on pacemaker currents in ICCs like as PGE<sub>2</sub>. In here, we also could see the inhibitory action of 100  $\mu$ mol/L SNAP on pacemaker currents (Figure 7A). We examined the effects of SNAP on pacemaker currents in presence of PD 98059 or SB 203580. The pretreatments of ICCs with PD 98059 (10  $\mu$ mol/L) or SB 203580 (10  $\mu$ mol/L) did not show any influence on 5  $\mu$ mol/L SNAP-induced inhibitory action on pacemaker currents (Figure 7B and C). The values of frequency and amplitude induced by SNAP in the presence of PD 98059 or SB 203580 were not significantly different from those obtained in the absence of PD 98059 or SB 203580 ( $n = 5$ , Figure 7E and F). Furthermore, we checked the reciprocal relation between PG and NO with PGE<sub>2</sub> and L-NAME. In presence of 10  $\mu$ mol/L L-NAME, 5  $\mu$ mol/L PGE<sub>2</sub> still inhibited the pacemaker current ( $n = 4$ , Figure 7D, bar graph not shown).

## DISCUSSION

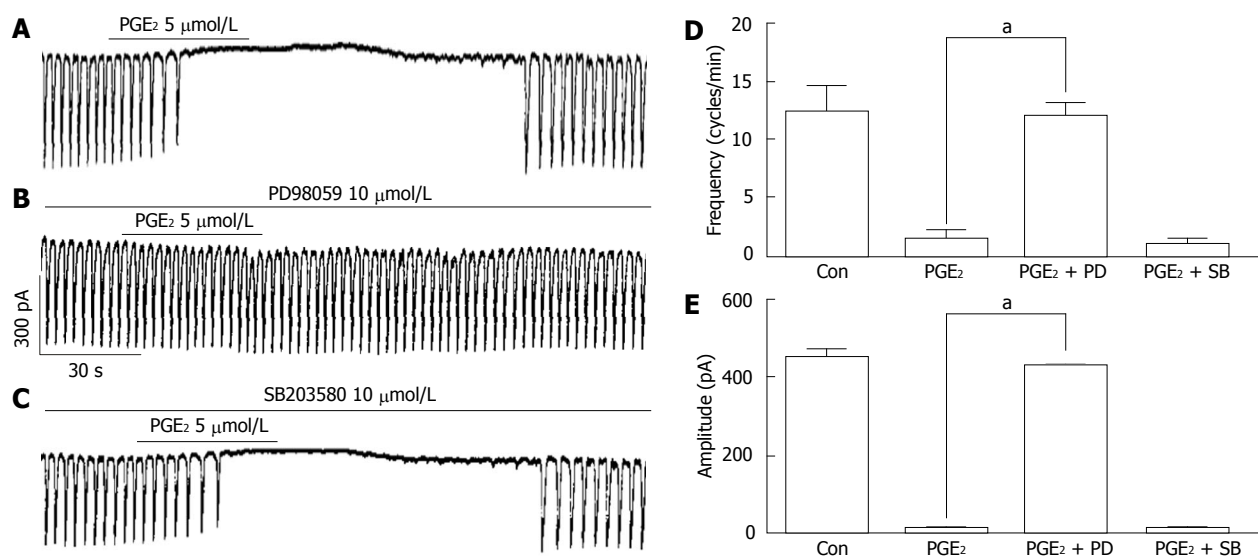
PGE<sub>2</sub> and NO are inhibitory mediators on intestinal motility and produced by COX (COX-1 or 2) and NOS

(nNOS/eNOS/iNOS), respectively. We have shown that LPS inhibited pacemaker currents of intestinal ICCs by activating ATP-sensitive K<sup>+</sup> channels through the release of PGE<sub>2</sub> and NO<sup>[17]</sup>. In this experiment, we further found that MAPKs are involving in LPS-induced inhibition of pacemaker currents through NF- $\kappa$ B-dependent mechanisms in ICCs.

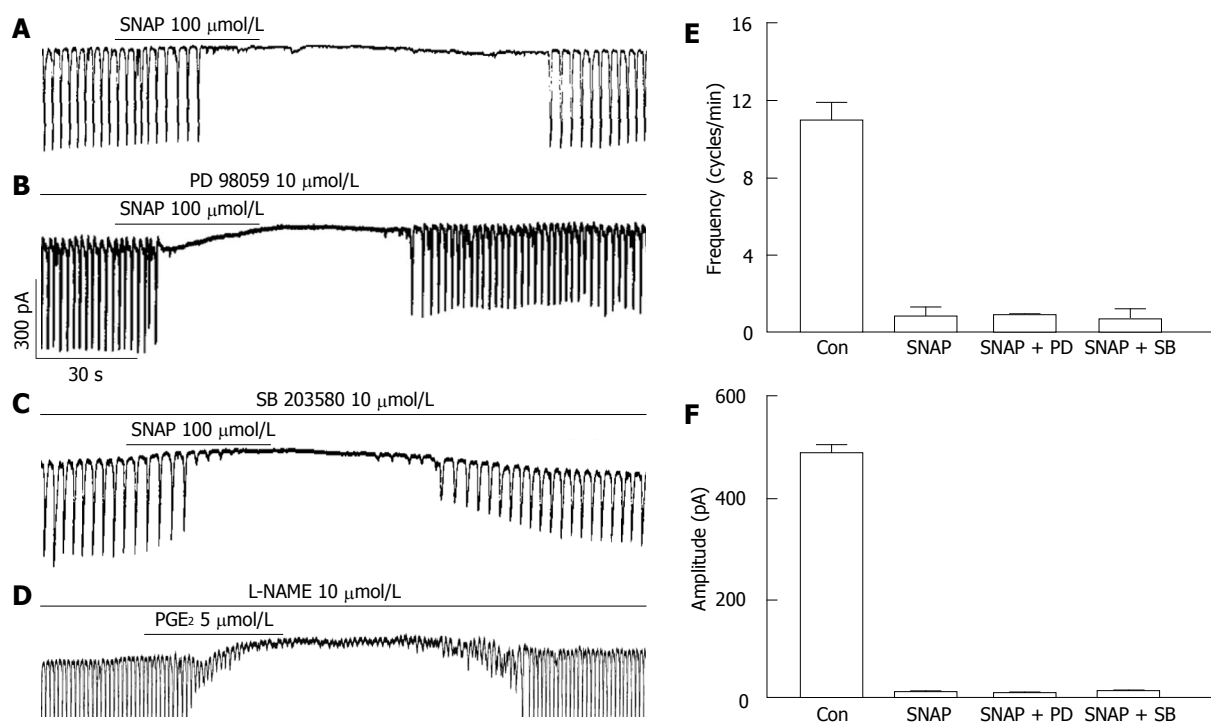
The binding of TLR4 with LPS activates NF- $\kappa$ B through myeloid differentiation primary response gene-88 (Myd88)-dependent pathway. LPS has been shown to induce NF- $\kappa$ B nuclear translocation in intestinal smooth muscle and myenteric plexus cells, but not in TLR4 knockout mice<sup>[23]</sup>. The activated NF- $\kappa$ B then induces various transcriptional genes including COX-2 and iNOS in GI tracts. In this study, LPS-induced inhibition of pacemaker currents was blocked by COX-2 inhibitor or iNOS inhibitor. And a specific NF- $\kappa$ B blocker or actinomycin D, a gene transcriptional blocker, suppressed LPS-induced inhibition of pacemaker currents. Furthermore, double staining with anti-c-Kit and anti-COX-2 antibodies or anti-iNOS antibodies revealed COX-2 or iNOS immunoreactivity in c-Kit immunopositive ICCs in cultured ICCs. These results suggest that NF- $\kappa$ B activation is essential to induce expression of COX-2 and iNOS exposed to LPS in ICCs.

MAPKs play an important role in the mediation of cellular responses, including transcriptional responses. Three types of MAPKs (p42/44 MAPK, p38 MAPK, and JNK) are involved in LPS-induced NF- $\kappa$ B or JNK activator protein-1 activation in various cells<sup>[24-27]</sup>. Recently, it was reported that three types of MAPKs were





**Figure 6** Effects of mitogen-activated protein kinases inhibitors on prostaglandin E<sub>2</sub>-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) treated with 5  $\mu$ mol/L prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) at a holding potential of -70 mV; B: Pacemaker currents after pretreatment with PD 98059 (10  $\mu$ mol/L) prior to PGE<sub>2</sub> treatment; C: Pacemaker currents after pretreatment with SB 203580 (10  $\mu$ mol/L) prior to PGE<sub>2</sub> treatment. The effects of mitogen-activated protein kinases inhibitors on the PGE<sub>2</sub>-induced action are summarized in D and E. Bars represent mean  $\pm$  SE values ( $n = 4$  per group). \* $P < 0.05$  vs PGE<sub>2</sub>. Con: Control, PD: PD 98059, SB: SB203580.



**Figure 7** Effects of mitogen-activated protein kinases inhibitors on ( $\pm$ )-S-nitroso-N-acetylpenicillamine-induced action in interstitial cells of Cajal. A: Pacemaker currents of interstitial cells of Cajal (ICCs) treated with 100  $\mu$ mol/L SNAP at a holding potential of -70 mV; B: Pacemaker currents after pretreatment with PD 98059 (10  $\mu$ mol/L) prior to SNAP treatment; C: Pacemaker currents after pretreatment with SB 203580 (10  $\mu$ mol/L) prior to SNAP treatment; D: Pacemaker currents after pretreatment with L-NAME (10  $\mu$ mol/L) prior to prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (5  $\mu$ mol/L) treatment. The effects of mitogen-activated protein kinases inhibitors on the ( $\pm$ )-S-nitroso-N-acetylpenicillamine-induced action are summarized in E and F. Bars represent mean  $\pm$  SE values ( $n = 5$  per group). Con: Control, PD: PD 98059, SB: SB203580; SNAP: S-nitroso-N-acetylpenicillamine; L-NAME: NG-Nitro-L-arginine Methyl Ester.

involved in the mechanism of LPS actions in rabbit ileum and the blockade of p38, JNK or p42/44 pathways suppressed the effects of LPS on the acetylcholine-induced contractions<sup>[28]</sup>. In our study, the LPS-induced effects in ICCs were blocked by PD98059 or SB20356,

but not by JNK inhibitor II, indicating that activation of p42/44 and p38 MAPKs are involved in LPS-induced activation of NF- $\kappa$ B in ICCs. These results are consistent with those of others showing that p42/44 and p38 MAPKs are involved in COX-2 and iNOS induction by

LPS in tracheal smooth muscle cells<sup>[26]</sup>. However, this study showed PGE<sub>2</sub>-induced inhibition of pacemaker currents is blocked by p42/44 MAPK inhibitor, but not by p38 MAPK inhibitor in intestinal ICCs and NO-induced inhibition of pacemaker currents was not blocked by both p42/44 MAPK inhibitor and p38 MAPK inhibitor. These results suggest that p42/44 MAPK and p38 MAPK play differential role in regulation of LPS-induced actions in ICCs. Thus, it seems that p38 MAPK may be involved in LPS-induced NF- $\kappa$ B activation to express COX-2 and iNOS, whereas p42/44 MAPK as a downstream pathway of NF- $\kappa$ B may be involved in PGE<sub>2</sub>-induced effects in ICCs not NO signaling. Our previous study could explain the possibility that NO-induced action on pacemaker currents is *via* activating ATP-sensitive K<sup>+</sup> channels through cGMP-dependent mechanism in ICCs but not involvement of MAPKs<sup>[22]</sup>.

LPS action was also mediated by ROS production through a nicotinamide adenine dinucleotide phosphate oxidase pathway. In rat aortic smooth muscles, LPS could induce the hydrogen peroxide signaling<sup>[29]</sup>. Also, we found that H<sub>2</sub>O<sub>2</sub> inhibited pacemaker currents by activating ATP-sensitive K<sup>+</sup> channels in ICCs, which was mediated through COX-2-dependent PGE<sub>2</sub> production<sup>[30]</sup>. Therefore, in order to investigate whether H<sub>2</sub>O<sub>2</sub> is involved in LPS-induced PGE<sub>2</sub> production, we treated catalase, a hydrogen peroxide scavenger. However, catalase did not block the LPS-induced inhibition of pacemaker currents. This result suggests that hydrogen peroxide do not involve in LPS-induced actions in intestinal ICCs.

Many reports are arguing the relation between the production of COX and NOS. NO production by NOS enzyme could stimulate or inhibit the COX enzyme induction<sup>[31,32]</sup>. And some reports suggested products by COX enzyme stimulate the NOS pathway<sup>[33,34]</sup>. In our opinion, the cross talk between NOS and COX may depend on the cell type or enzyme type. In here, we need to check whether inhibitory effect of PGE<sub>2</sub> on pacemaker activity is through induction of NO synthesis in ICCs. However, the proofs that PGE<sub>2</sub> alone could inhibit the pacemaker activity in ICCs in our previous study and L-NAME could not show any influence on PGE<sub>2</sub>-induced action in here could establish no-interaction between the products of COX and NOS by LPS stimulation in ICCs.

In summary, the current study provides evidence that LPS inhibited pacemaker currents in cultured ICCs *via* PGE<sub>2</sub> and NO-dependent pathways. NF- $\kappa$ B and MAP kinases are involved in this inhibitory action.

## COMMENTS

### Background

Interstitial cells of Cajal (ICCs) are the pacemaker cells present in the gastrointestinal tract (GI) that generate the pacemaker currents that regulate the GI motility. Lipopolysaccharides (LPS) are endotoxins and pro-inflammatory agents present in the cell wall of the Gram-negative bacteria responsible for various alterations of gastrointestinal functions including intestinal dysmotility.

### Innovations and breakthroughs

LPS altered GI motility through release of prostaglandins or nitric oxide (NO) by nuclear factor  $\kappa$ B (NF- $\kappa$ B) dependent gene transcription. This study showed LPS inhibited pacemaker currents of ICC. Prostaglandin E<sub>2</sub> and NO contributed to the inhibitory effects of LPS together with mitogen-activated protein kinases and NF- $\kappa$ B.

### Applications

LPS-induced inhibitory actions on pacemaker activities of ICC may be one possible mechanism to explain LPS-induced GI dysmotility.

### Terminology

ICCs refer to the several types of cells present on the musculature of the GI tract. ICCs act as pacemaker cells in gastrointestinal tracts, regulating rhythmicity by activating calcium activated chloride channel or nonselective cation channels.

### Peer review

The functional regulation of ICCs is still not fully understood and this study provides novel information on the TLR 4-mediated signaling pathways on LPS-suppressed pacemaker currents of ICCs.

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## NAFLD fibrosis score: A prognostic predictor for mortality and liver complications among NAFLD patients

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### Abstract

**AIM:** To study whether the severity of liver fibrosis estimated by the nonalcoholic fatty liver disease (NAFLD) fibrosis score can predict all-cause mortality, cardiac complications, and/or liver complications of patients with NAFLD over long-term follow-up.

**METHODS:** A cohort of well-characterized patients with NAFLD diagnosed during the period of 1980-2000 was identified through the Rochester Epidemiology Project. The NAFLD fibrosis score (NFS) was used to separate NAFLD patients with and without advanced liver fibrosis. We used the NFS score to classify the probability of fibrosis as  $< -1.5$  for low probability,  $> -1.5$  to  $< 0.67$  for intermediate probability, and  $> 0.67$

for high probability. Primary endpoints included all-cause death and cardiovascular- and/or liver-related mortality. From the 479 patients with NAFLD assessed, 302 patients (63%) greater than 18 years old were included. All patients were followed, and medical charts were reviewed until August 31, 2009 or the date when the first primary endpoint occurred. By using a standardized case record form, we recorded a detailed history and physical examination and the use of statins and metformin during the follow-up period.

**RESULTS:** A total of 302/479 (63%) NAFLD patients (mean age:  $47 \pm 13$  year) were included with a follow-up period of  $12.0 \pm 3.9$  year. A low probability of advanced fibrosis ( $NFS < -1.5$  at baseline) was found in 181 patients (60%), while an intermediate or high probability of advanced fibrosis ( $NFS > -1.5$ ) was found in 121 patients (40%). At the end of the follow-up period, 55 patients (18%) developed primary endpoints. A total of 39 patients (13%) died during the follow-up. The leading causes of death were non-hepatic malignancy ( $n = 13/39$ ; 33.3%), coronary heart disease (CHD) ( $n = 8/39$ ; 20.5%), and liver-related mortality ( $n = 5/39$ ; 12.8%). Thirty patients had new-onset CHD, whereas 8 of 30 patients (27%) died from CHD-related causes during the follow-up. In a multivariate analysis, a higher NFS at baseline and the presence of new-onset CHD were significantly predictive of death ( $OR = 2.6$  and  $9.2$ , respectively;  $P < 0.0001$ ). Our study showed a significant, graded relationship between the NFS, as classified into 3 subgroups (low, intermediate and high probability of liver fibrosis), and the occurrence of primary endpoints. The use of metformin or simvastatin for at least 3 mo during the follow-up was associated with fewer deaths in patients with NAFLD ( $OR = 0.2$  and  $0.03$ , respectively;  $P < 0.05$ ). Additionally, the rate of annual NFS change in patients with an intermediate or high probability of advanced liver fibrosis was significantly lower than those patients with a low probability of advanced liver fibrosis ( $0.06$  vs  $0.09$ ,  $P = 0.004$ ). The annual NFS change in patients who died was sig-



nificantly higher than those in patients who survived (0.14 *vs* 0.07,  $P = 0.03$ ). At the end of the follow-up, we classified the patients into 3 subgroups according to the progression pattern of liver fibrosis by comparing the NFS at baseline to the NFS at the end of the follow-up period. Most patients were in the stable-fibrosis (60%) and progressive-fibrosis (37%) groups, whereas only 3% were in the regressive fibrosis.

**CONCLUSION:** A higher NAFLD fibrosis score at baseline and a new onset of CHD were significantly predictive of death in patients with NAFLD.

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**Key words:** Nonalcoholic fatty liver disease fibrosis score; Prognostic predictor; Mortality; Liver complications

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## INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease in Western countries<sup>[1]</sup>. The prevalence of NAFLD is increasing and varies significantly with ethnicity, ranging from 24% in blacks to 33% in whites and 45% in Hispanics according to a recent study from the United States<sup>[2]</sup>. The mortality rate of NAFLD patients in the community was found to be higher than that of the general population in both the US and Sweden<sup>[3]</sup>. During an average 7.6 years of follow-up, 13% of the patients died, mainly from malignancy, coronary heart disease (CHD) and liver-related mortality<sup>[4]</sup>. Another study revealed that the survival of patients with nonalcoholic steatohepatitis (NASH) was reduced and that these patients died significantly more often from CHD and liver-related causes<sup>[3]</sup>. Patients with more advanced liver fibrosis tend to have more liver complications than those without liver fibrosis<sup>[4]</sup>. Liver biopsy is considered the gold standard for the diagnosis and assessment of fibrosis severity but has several limitations, such as sampling variability, invasiveness and expense<sup>[5]</sup>. Patients with NASH can have a significant progression of fibrosis within a few years<sup>[6-8]</sup>. Recently, a simple, noninvasive tool used for liver fibrosis assessment has been developed<sup>[9]</sup>. This new scoring system, the NAFLD fibrosis score (NFS), is a composite score of age, hyperglycemia, body mass index, platelet count, albumin, and aspartate aminotransferase and alanine aminotransferase (AST/ALT) ratio<sup>[9]</sup> and was found to independently identify NAFLD patients with and without advanced fibrosis at initial NAFLD diagnosis. Another clinical score com-

posed of body mass index (BMI)  $\geq 28$  kg/m<sup>2</sup>, AST/ALT ratio  $\geq 0.8$  and diabetes mellitus (the BARD score) has also been used to predict an increased chance of liver fibrosis<sup>[10]</sup>. However, the BARD score does not have the capacity to differentiate the severity of liver fibrosis among patients with a higher BMI or a higher ratio of AST/ALT, whereas the NFS takes into consideration the different ranges of BMI or AST/ALT ratios<sup>[9]</sup>. A study from Japan validated the NFS and found it to have an acceptable sensitivity, specificity, and positive and negative predictive values for advanced liver fibrosis of 100%, 83%, 63%, and 100%, respectively<sup>[11]</sup>. A longitudinal study of 103 NAFLD patients showed that the fibrosis stage progressed in 37%, remained stable in 34% and regressed in 29% of patients with a mean interval between liver biopsies of approximately 3 years<sup>[12]</sup>. The rate of fibrosis change ranged from -2.1 to 1.7 stages per year. The prognostic predictors for a higher rate of liver fibrosis were diabetes, a low initial fibrosis stage and a higher body mass index. In the current study, we aimed to determine whether the severity of liver fibrosis estimated by the NFS can predict a higher risk of overall mortality, cardiac complications, and/or liver complications among patients with NAFLD. Additionally, we aimed to determine the annual rate of the NFS change from the baseline to the end of the follow-up among NAFLD patients.

## MATERIALS AND METHODS

### Study subjects

In this a historical cohort study, patients residing in Olmsted County, Rochester, Minnesota, United States who had been diagnosed with NAFLD-fatty liver (HICDA Code 05710420), fatty liver hypertrophy (HICDA Code 05710421), fatty liver cirrhosis (HICDA Code 05710422), fatty liver steatohepatitis (HICDA Code 05710423) or NASH (HICDA Code 05710431), or fatty liver steatohepatitis (HICDA Code 05710-42-43) or steatosis (HICDA Code 02790-44-1) between January 1, 1980 and January 1, 2000 were drawn from the Rochester Epidemiology Project (REP) master diagnostic index. The REP index is a unique database system of medical diagnoses of the population living in Olmsted County, Minnesota<sup>[13]</sup>. Although fatty liver was recognized prior to 1980, this liver condition was better characterized in 1980<sup>[14]</sup>; therefore, we chose to identify patients after this date.

### Inclusion criteria

From the 479 patients with NAFLD assessed, 302 patients (63%) greater than 18 years old were included. All of these patients were followed, and their medical charts were reviewed, until August 31, 2009 or the date when the first primary endpoint occurred. By using a standardized case record form, we recorded a detailed history and physical examination and use of statins and metformin during the follow-up period.

### Exclusion criteria

We excluded NAFLD patients who lacked the data

needed for the NFS calculation, patients with pre-existing poor outcomes including overt CHD or overt liver complications at the time of NAFLD diagnosis and patients with duration of follow-up of less than 5 years. One hundred seventy-seven NAFLD patients were excluded due to missing the data needed for the NFS calculation ( $n = 95$ ), overt CHD confirmed at baseline ( $n = 63$ ), liver cirrhosis with complications confirmed at baseline ( $n = 11$ ) and duration of follow-up of less than 5 years ( $n = 8$ ).

### Definitions

The diagnosis of NAFLD was based on a liver biopsy showing steatosis in at least 5% of hepatocytes or fatty infiltration of the liver confirmed by imaging study (ultrasound, computed tomography, or magnetic resonance imaging) and the exclusion of liver disease of other etiologies, including alcohol-induced liver disease (history of excessive alcohol consumption greater than 20 gm/d), drug-induced liver disease, autoimmune or viral hepatitis and cholestatic or metabolic/genetic liver disease<sup>[1]</sup>. Cirrhosis was defined based on the pathological term for the chronic liver diseases<sup>[15]</sup>. In our study, 85% of patients ( $n = 256$ ) were diagnosed by liver imaging. Liver biopsy was performed in 46 patients (15% of 302 patients). The staging of fibrosis was divided into fibrosis stage 0 to stage 4 using the Brunt criteria<sup>[16]</sup>. The NAFLD patients with a histological liver fibrosis stage of 1-2 were classified as “mild liver fibrosis,” and those with a histological fibrosis stage of 3-4 were classified as “advanced liver fibrosis”<sup>[16]</sup>.

The primary endpoints were all-cause mortality, cardiac complications, and/or liver complications. Cardiac complications included new-onset CHD events as recorded in the medical records, defined as congestive heart failure, unstable angina, myocardial infarction, flow-limiting stenosis from angiography or angina requiring revascularization during the follow-up period and need for hospitalization<sup>[17]</sup>. Liver complications were diagnosed by clinical signs and symptoms<sup>[18]</sup>, including the presence of ascites, variceal bleeding, a severe grade of hepatic encephalopathy, liver failure or hepatocellular carcinoma, that occurred during the follow-up and required hospitalization with or without death<sup>[19]</sup>. All causes of death listed on the death certificates or pathological findings (underlying, intermediate, immediate and other major conditions) were recorded using the 10<sup>th</sup> revision of the International Classification of Diseases (ICD-10). The presence of metabolic syndrome (MetS) was defined by using the 2001 National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP III) criteria and the new definition, which requires the presence of at least three of the five features<sup>[20,21]</sup>. The NFS is composed of 6 variables, including age, hyperglycemia, BMI, platelet count, albumin, and AST/ALT ratio as independent indicators of advanced liver fibrosis<sup>[9]</sup>. NAFLD fibrosis score =  $-1.675 + 0.037 \times \text{age (year)} + 0.094 \times \text{BMI (kg/m}^2\text{)} + 1.13 \times \text{IFG/diabetes (yes = 1, no = 0)} + 0.99 \times \text{AST/ALT ratio} - 0.013 \times \text{platelet count (} \times 10^9/\text{L)} - 0.66 \times \text{albumin}$

(g/dL)<sup>[9]</sup>.

In this study, the NFS was used to classify advanced liver fibrosis into 2 categories<sup>[9]</sup>. NAFLD patients with a score less than -1.5 were classified as “low probability of advanced liver fibrosis,” and those patients with a score of at least -1.5 were classified as “intermediate or high probability of advanced liver fibrosis”<sup>[9]</sup>. Because the information required for determination of the NFS was not always available on the same day, the scores were calculated at the time of NAFLD diagnosis using data from the medical records from visits within 3 mo of the “true” NAFLD diagnosis date and the last follow-up date. If more than one assessment for a given variable was available in the medical records during this time period, the value closest to the “true” follow-up date was used for the NFS calculation<sup>[9]</sup>. According to the 3 subgroups of low, intermediate and high probability of fibrosis, we used parameters of NFS < -1.5 for low, NFS  $\geq$  -1.5 to NFS < 0.67 for intermediate and NFS  $\geq$  0.67 for high probability of fibrosis at baseline.

At the end of the follow-up period, we classified the patients into 3 subgroups according to the progression pattern of the liver fibrosis by comparing the NFS at baseline to the end of follow-up. The first group had stable fibrosis, defined as stability of the NFS during the follow-up. The second group had regression of fibrosis, defined as a reduction of the NFS to a milder stage of fibrosis during the follow-up. Lastly, the third group had fibrosis progression, defined as an increase of the NFS to a more advanced stage during the follow-up.

### Sample-size/statistical power considerations

We assumed that the overall mortality rate in NAFLD would be 12%, with liver-related complications being observed in at least 3% and cardiac complications in 11% of patients based on results from a previous study<sup>[4]</sup>. We anticipated that at least 150 patients, or 50% of the total cohort, would be classified as “low probability of advanced liver fibrosis” using the NFS, and at least 150 would be classified as “intermediate or high probability of advanced liver fibrosis”<sup>[12]</sup>. However, a portion of patients were expected to develop new CHD events, liver complications and death. We anticipated that 15% of patients would be identified as having experienced a primary endpoint. We assumed that, at most, 10% of patients with a low probability of advanced liver fibrosis would experience events within 5 years and at least 20% of patients with an intermediate or high probability of advanced liver fibrosis would experience events within 5 years to obtain a statistical power of at least 82%.

### Statistical analysis

Patients were categorized by the NFS into 2 groups of probability of advanced liver fibrosis. Differences between the primary endpoints of the two groups of low and high NFS were compared by using the  $\chi^2$  test. Differences between NAFLD patients with and without primary endpoints were tested by independent *t* tests for

**Table 1** Demographic data of 302 patients by nonalcoholic fatty liver disease fibrosis score at baseline *n* (%) (mean  $\pm$  SD)

Variable at baseline	Total ( <i>n</i> = 302)	Patients with a low probability of advanced liver fibrosis (NFS < -1.5) ( <i>n</i> = 181)	Patients with an intermediate or high probability of advanced liver fibrosis (NFS $\geq$ -1.5) ( <i>n</i> = 121)	<i>P</i> value
Age (yr)	47.3 $\pm$ 12.9	42.9 $\pm$ 11.1	53.8 $\pm$ 12.8	< 0.0001
Sex (% male)	132 (44)	92 (51)	40 (33)	0.002
Race, number (% White)	288 (95)	170 (94)	119 (97.5)	0.150
History of diabetes	48 (16)	5 (2.8)	43 (35.5)	< 0.0001
History of hypertension	125 (41)	55 (30.4)	70 (58)	< 0.0001
BMI (kg/m <sup>2</sup> )	33.6 $\pm$ 6.2	32.0 $\pm$ 5.2	36.0 $\pm$ 6.9	< 0.0001
Presence of obesity (BMI > 30 kg/m <sup>2</sup> )	221 (73)	121 (67)	100 (82.6)	0.002
Systolic blood pressure (mmHg)	136 $\pm$ 18	133 $\pm$ 17	139 $\pm$ 18	0.003
Diastolic blood pressure (mmHg)	83 $\pm$ 9	84 $\pm$ 8	81 $\pm$ 9	0.010
Cholesterol (mg/dL)	214 $\pm$ 48	215 $\pm$ 46	214 $\pm$ 50	0.780
Triglycerides (mg/dL)	221 $\pm$ 167	208 $\pm$ 123	242 $\pm$ 218	0.150
Glucose (mg/dL)	115 $\pm$ 41	103 $\pm$ 25	132 $\pm$ 54	< 0.0001
AST (U/L)	41.4 $\pm$ 21.9	40.8 $\pm$ 21.8	42.2 $\pm$ 28.9	0.620
ALT (U/L)	61.5 $\pm$ 43.3	69.7 $\pm$ 46	49.4 $\pm$ 35.7	< 0.0001
AST/ALT ratio	0.8 $\pm$ 0.4	0.7 $\pm$ 0.3	1.0 $\pm$ 0.6	< 0.0001
GGT (U/L)	131.9 $\pm$ 39.8	129.9 $\pm$ 32.9	134.2 $\pm$ 46.7	0.560
Platelets ( $\times 10^9$ /L)	240 $\pm$ 62	259 $\pm$ 60	212 $\pm$ 53	< 0.0001
Albumin (g/dL)	4.3 $\pm$ 0.4	4.4 $\pm$ 0.3	4.1 $\pm$ 0.3	< 0.0001
Alkaline phosphatase (U/L)	196 $\pm$ 88	186 $\pm$ 68	211 $\pm$ 111	0.030
Framingham Risk Score	8.4 $\pm$ 6.2	6.9 $\pm$ 6.4	10.5 $\pm$ 5.2	< 0.0001
Calculated CHD risk (%)	16.2 $\pm$ 14.6	14.1 $\pm$ 13.8	19.3 $\pm$ 15.2	0.003
NFS	-1.7 $\pm$ 1.4	-2.6 $\pm$ 0.8	-0.4 $\pm$ 0.9	< 0.0001

NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; GGT: Glutamyltransferase; CHD: Coronary heart disease; NFS: NAFLD fibrosis score.

continuous variables and were tested by the  $\chi^2$  test for proportions. Continuous outcomes were presented as the mean  $\pm$  SD, and categorical data were presented as numbers (percentage). Logistic regression analysis was used to identify the factors significantly associated with death among NAFLD patients. Only those variables with a *P* value < 0.1 by univariate analysis were included in multivariate analysis. To avoid overestimation of the model, we excluded those variables used as a part of the NFS calculation. We estimated receiver operating characteristics of related variables for the predicting of death to maximize the area under the curve (AUC). Two-sided *P* values < 0.05 were considered statistically significance. The rate of NFS change in each patient was calculated by the difference of NFS at the end of follow-up and at baseline divided by the duration of the follow-up time ( $\Delta$  NFS/ $\Delta$  time). Both phases of the study used the SPSS statistical software package (SPSS Version 15.0.1.1, Windows VISTA, July 3, 2007) for analysis. The study was approved by the Institutional Review Board (IRB) of the Mayo Clinic, Rochester, MN, United States, and all participants provided permission for their medical information to be used for research.

## RESULTS

### Baseline characteristic data of 302 patients with NAFLD

This study included three hundred two NAFLD patients aged  $47 \pm 13$  year (range 21–86 year), of which 95% were white, and 44% were male. Obesity was present in 73%, and a history of diabetes mellitus type II and hyperten-

sion was observed in 16% and 41%, respectively. Eighty-five percent of patients (*n* = 256) were diagnosed by liver imaging. Liver biopsy was performed in 46 patients (15%). Mild liver fibrosis (stage F 0–2) was found in 34 patients (74%), while advanced fibrosis (stage F 3–4) was found in 12 patients (26%). NAFLD patients who underwent a liver biopsy had a significantly lower diastolic blood pressure, a lower BMI and a higher AST level than those without a liver biopsy (*P* < 0.05). The characteristics of the 302 patients based on the degree of advanced liver fibrosis estimated by the NFS at baseline are shown in Table 1. A low probability of advanced liver fibrosis (NFS < -1.5) was found in 60% of patients, while an intermediate or high probability of advanced liver fibrosis (NFS  $\geq$  -1.5) was found in 40%. The mean  $\pm$  SD value of NFS in patients with a low probability of advanced liver fibrosis was  $-2.6 \pm 0.8$  and was lower than in patients with an intermediate or high probability of advanced liver fibrosis ( $-0.4 \pm 0.9$ , *P* < 0.0001). Patients with a low probability of advanced liver fibrosis had a lower CHD risk at baseline, as estimated by the Framingham risk score (FRS) calculation, compared to patients with an intermediate or high probability of advanced liver fibrosis (14% *vs* 19%, *P* = 0.003).

### Clinical outcomes of long-term follow-up

The mean follow-up of the total cohort was  $11.9 \pm 3.9$  years for a total of 3594 person-years. Approximately 47% of patients with a low probability of advanced liver fibrosis at baseline progressed to an intermediate or high probability of advanced liver fibrosis at the end of follow-up, while 94% of patients with an intermediate

**Table 2** Clinical parameters, laboratory features and clinical outcomes at the end of follow-up by nonalcoholic fatty liver disease fibrosis score at baseline *n* (%) (mean  $\pm$  SD)

Variable at the end of follow-up	Patients with a low probability of advanced liver fibrosis (NFS < -1.5) ( <i>n</i> = 181)	Patients with an intermediate or high probability of advanced liver fibrosis (NFS $\geq$ -1.5) ( <i>n</i> = 121)	<i>P</i> value
Clinical findings			
BMI (kg/m <sup>2</sup> )	32.9 $\pm$ 6.6	34.9 $\pm$ 7.6	0.02
Obesity (BMI $\geq$ 30 kg/m <sup>2</sup> )	119 (65.8)	91 (75.2)	0.08
NFS	-1.4 $\pm$ 1.3	0.4 $\pm$ 1.4	< 0.0001
NFS of intermediate or high probability of advanced liver fibrosis	85 (47)	114 (94)	< 0.0001
History of diabetes	54 (29.8)	83 (68.6)	< 0.0001
Use of metformin	32 (17.7)	48 (39.7)	< 0.0001
Use of glitazones	10 (5.5)	19 (15.7)	0.003
Use of aspirin	84 (46)	83 (69)	0.0001
History of hypothyroidism	19 (10.5)	31 (25.6)	0.0005
History of cholecystectomy	27 (15)	33 (27.3)	0.008
History of obstructive sleep apnea	33 (18.2)	34 (28.1)	0.04
Laboratory findings			
AST (U/L)	38.9 $\pm$ 30.6	33.2 $\pm$ 17.8	0.04
ALT (U/L)	53.9 $\pm$ 49.7	38.9 $\pm$ 21	0.0004
AST/ALT ratio	0.8 $\pm$ 0.5	1.0 $\pm$ 0.8	0.03
Hematocrit (%)	40.4 $\pm$ 4.4	38.6 $\pm$ 5.3	0.003
Platelets ( $\times 10^3$ /L)	259 $\pm$ 67	217 $\pm$ 74	< 0.0001
Albumin (g/dL)	4.1 $\pm$ 0.4	3.9 $\pm$ 0.6	< 0.0001
Cholesterol (mg/dL)	193 $\pm$ 40	178 $\pm$ 43	0.005
LDL-cholesterol (mg/dL)	109 $\pm$ 34	92 $\pm$ 30	< 0.0001
Glucose (mg/dL)	119 $\pm$ 42	131 $\pm$ 42	0.02
Clinical outcomes at the end of follow-up			
Lost to follow up	27 (15)	8 (7)	
Alive with continued follow-up	131 (72)	81 (67)	
Presence of primary endpoints	23 (13)	32 (26)	0.002
All-cause death	12 (6.6)	27 (22.3)	< 0.0001
New events of coronary heart disease	15 (8.3)	15 (12.4)	0.24
Liver complications	1 (0.6)	5 (4.1)	0.03

NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NFS: NAFLD fibrosis score; LDL: Low-density lipoprotein.

or high probability of advanced liver fibrosis remained in the same group. At the end of the follow-up, patients with an intermediate or high probability of advanced liver fibrosis had a significantly higher BMI, more frequent diabetes, and more frequent histories of hypothyroidism, cholecystectomy and obstructive sleep apnea. Furthermore, the patients with an intermediate or high probability of advanced liver fibrosis were more likely to use metformin, glitazones and aspirin compared to those with a low probability of advanced liver fibrosis ( $P < 0.05$ ).

A history of diabetes at baseline was found in 16%, while the proportion of patients with NAFLD who developed impaired fasting glycemia or diabetes during the follow-up was 89 patients (29%) with an average interval of  $6.3 \pm 4.2$  year (range 0.2-17.2) from the diagnosis of NAFLD. Patients who developed impaired fasting glycemia or diabetes during the follow-up were more frequent male, had less hypertension, and had a lower NAFLD Fibrosis Score at baseline compared to those with diabetes at baseline ( $P < 0.05$ ). The primary endpoints of both groups were not significantly different.

Patients with an intermediate or high probability of advanced liver fibrosis had a significantly higher glucose

and a higher AST/ALT ratio at the end of follow-up compared to those with a low probability of advanced liver fibrosis. Hematocrit, platelet count, AST, ALT, albumin, cholesterol and low-density lipoprotein (LDL)-cholesterol level were significantly lower in patients with an intermediate or high probability of advanced liver fibrosis compared to those with a low probability of advanced liver fibrosis (Table 2). At the end of follow-up, 55 patients developed primary endpoints, including death or the presence of CHD or liver complications. Thirty of these patients had new-onset CHD, whereas 8 of 30 patients (27%) died from CHD-related complications during the follow-up period (Table 2). Patients with new CHD events ( $n = 30$ ) were significantly older and had a higher systolic blood pressure (SBP), FRS at baseline, calculated % CHD risk at baseline, and NFS at the end of follow-up and exhibited a lower ALT than those patients without new CHD events ( $n = 272$ ) ( $P < 0.05$ ). In addition, the NFS at baseline was similar in patients with and without new CHD events ( $-1.2 \pm 1.6$  vs  $-1.8 \pm 1.4$ ,  $P = 0.07$ ). Liver complications occurred in 6 patients, 5 of whom (83%) died during the follow-up. The liver complications included massive ascites requiring abdomi-



**Table 3** Causes of mortality in 39 patients with nonalcoholic fatty liver disease

Causes of death	All causes mortality <i>n</i> (% of death)	All causes mortality (% of 302 patients)
Non-liver cancer	13 (33.3)	4.3%
Coronary heart disease	8 (20.5)	2.6%
Liver-related mortality (including hepatocellular carcinoma)	5 (12.8)	1.7%
Infection (including sepsis)	4 (10.3)	1.3%
Stroke	3 (7.7)	1.0%
Cardiac arrhythmia	2 (5.1)	0.7%
COPD and/or respiratory failure	2 (5.1)	0.7%
Other causes of death (GI bleeding, renal failure)	2 (5.1)	0.7%
Total	39 (100)	12.9%

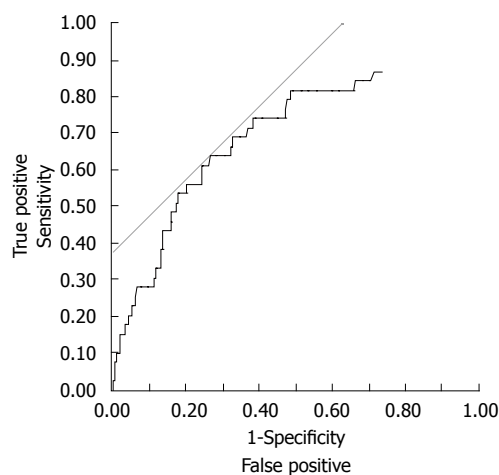
COPD: Chronic obstructive lung disease; GI: Gastrointestinal.

nal paracentesis ( $n = 3$ ), hepatopulmonary syndrome and hepatocellular carcinoma ( $n = 1$  each).

A total of 39/302 (13%) patients died during the follow-up period. The leading causes of death were non-hepatic malignancy ( $n = 13/39$ ; 33.3%), CHD ( $n = 8/39$ ; 20.5%), and liver related mortality ( $n = 5/39$ ; 12.8%). The other 13 patients (33.3%) died from various causes (Table 3). The primary types of cancers were gastric cancer ( $n = 2$ ), colon cancer ( $n = 2$ ), pancreatic cancer ( $n = 2$ ), breast cancer ( $n = 2$ ), leiomyosarcoma of uterus ( $n = 1$ ), diffuse B cell lymphoma ( $n = 1$ ), endometrial cancer ( $n = 1$ ), lung cancer ( $n = 1$ ) and unknown primary cancer with liver metastasis ( $n = 1$ ). Additionally, our study showed a significant, graded relationship between the NAFLD fibrosis score, classified into 3 subgroups (low, intermediate and high probability of liver fibrosis), and the occurrence of primary endpoints, as shown in Table 4.

### Predicting mortality

Patients who died ( $n = 39$ ) were significantly older with more frequent diabetes and had a higher SBP, NFS at baseline, FRS, glucose, and a lower diastolic blood pressure, ALT and albumin (Table 5). Moreover, they had greater NFS changes per year than those who survived ( $n = 263$ ,  $P < 0.05$ ). Three models of multivariate analysis were used to identify the best fit model for predictors of death and are illustrated in Table 6. In model 1, we added 9 variables, including gender, systolic blood pressure, diastolic blood pressure, NFS at baseline, use of metformin, use of simvastatin, use of aspirin, presence of new-onset CHD and new-onset liver complications, without interaction among these variables. Model 2 included 10 variables, gender, systolic blood pressure, diastolic blood pressure, NFS at baseline, NFS changes per year, use of metformin, use of simvastatin, use of aspirin, presence of new-onset of CHD and new-onset of liver complications without interaction among these variables. Finally, model 3 added the interaction between NFS at baseline and NAFLD NFS changes per year with the use of aspirin, metformin, and simvastatin into model 2. We did



**Figure 1** Presence of death estimated by the nonalcoholic fatty liver disease fibrosis score at baseline. Using the receiver operating characteristics curve, the nonalcoholic fatty liver disease fibrosis score at baseline of -0.9 was the best cutoff value for predicting death based on a sensitivity of 62%, specificity of 76%, positive predictive value of 28%, negative predictive value of 93% and area under the curve of 0.7.

not add the FRS into these models due to the repetition of several variables in the NFS and the FRS. Model 3 was the best fit model, which found that a higher NFS at baseline and more frequent new-onset CHD were significantly predictive of death (OR = 2.6 and 9.2, respectively;  $P < 0.0001$ ). The use of metformin or simvastatin for at least 3 mo during the follow-up were associated with fewer deaths in patients with NAFLD (OR = 0.2 and 0.03, respectively;  $P < 0.05$ ).

Table 7 showed the results of the comparison of NAFLD patients with and without death after excluding those with established type 2 diabetes at baseline ( $n = 254$ ). After excluding patients with established type 2 diabetes at baseline, we found that a higher NFS at baseline and higher alkaline phosphatase remained significantly predictive of death (OR = 1.9 and 1.006;  $P < 0.0001$  and 0.012, respectively) as shown in Table 8. Additionally, Table 9 showed that non-diabetic patients with intermediate or high probability of liver fibrosis had a significantly higher rate of primary end point and all-cause death than those patients with low probability of liver fibrosis.

Using the ROC curves to predict death, we found that a baseline NFS of -0.9 was the best cutoff value with a sensitivity of 62%, specificity of 76%, positive predictive value of 28%, negative predictive value of 93% and AUC of 0.7 (Figure 1).

The median rate of annual NFS change for all patients was 0.1 (IQR of 0.02, 0.13). The rate of annual NFS change in patients with an intermediate or high probability of advanced liver fibrosis was significantly lower than in patients with a low probability of advanced liver fibrosis (0.06 *vs* 0.09,  $P = 0.004$ ). The annual NFS change in patients who died was significantly higher than those in patients who survived (0.14 *vs* 0.07,  $P = 0.03$ ). At the end of the follow-up, we classified the patients into 3 subgroups according to the progression pattern of the

**Table 4 Association between the primary endpoint and the grading of the nonalcoholic fatty liver disease fibrosis score, classified into 3 subgroups (*n* = 302) *n* (%)**

Grading of the NAFLD fibrosis score ( <i>n</i> = 302)	Low prob. of advanced liver fibrosis ( <i>n</i> = 181)	Intermediate prob. of advanced liver fibrosis ( <i>n</i> = 108)	High prob. of advanced liver fibrosis ( <i>n</i> = 13)	<i>P</i> value
Presence of primary endpoint ( <i>n</i> = 55, 18.2%)	23/181 (12.7)	24/108 (22.2)	8/13 (61.5)	< 0.0001
All-cause death ( <i>n</i> = 39, 12.9%)	12/181 (6.6)	21/108 (19.4)	6/13 (46.2)	< 0.0001

NAFLD: Nonalcoholic fatty liver disease.

**Table 5 Comparison of nonalcoholic fatty liver disease patients alive *vs* deceased *n* (%) (mean  $\pm$  SD)**

Variables	NAFLD patients alive ( <i>n</i> = 263)	NAFLD patients deceased ( <i>n</i> = 39)	<i>P</i> value
At baseline			
Age (yr)	45.2 $\pm$ 11.5	61.1 $\pm$ 13.8	< 0.0001
Sex (% male)	120 (45.6)	12 (30.8)	0.08
History of diabetes	37 (14.1)	11 (28.2)	0.02
Systolic blood pressure (mmHg)	134 $\pm$ 17	143 $\pm$ 21	0.02
Diastolic blood pressure (mmHg)	83 $\pm$ 8	79 $\pm$ 10	0.03
Glucose (mg/dL)	112 $\pm$ 38.6	132.7 $\pm$ 54.3	0.03
AST (U/L)	42.2 $\pm$ 25.5	35.5 $\pm$ 20.0	0.06
ALT (U/L)	64.2 $\pm$ 44.6	43.6 $\pm$ 27.4	0.000
AST/ALT ratio	0.8 $\pm$ 0.4	1.0 $\pm$ 0.7	0.06
Albumin (g/dL)	4.3 $\pm$ 0.3	4.0 $\pm$ 0.4	< 0.0001
FRS	7.9 $\pm$ 6.2	11.4 $\pm$ 5.2	0.000
Calculated CHD risk (%)	15.3 $\pm$ 14.0	22.2 $\pm$ 17.1	0.02
NFS	-1.9 $\pm$ 1.3	-0.8 $\pm$ 1.7	0.0004
NFS of intermediate or high probability of advanced liver fibrosis (%)	94 (35.7)	27 (69.2)	< 0.0001
Presence of histologically advanced liver fibrosis	7/35 (20.0)	5/11 (45.5)	0.09
During the follow-up period			
Use of metformin	77 (29.3)	3 (7.7)	0.004
Use of aspirin	151 (57.4)	16 (41.0)	0.05
Use of simvastatin	107 (40.7)	3 (7.9)	< 0.0001
New events of coronary heart disease	16 (6.1)	14 (35.9)	< 0.0001
Liver complications	1 (0.4)	5 (12.8)	< 0.0001
At the end of follow-up			
BMI (kg/m <sup>2</sup> )	33.9 $\pm$ 6.9	31.8 $\pm$ 8.2	0.1
Hematocrit (%)	40.4 $\pm$ 4.1	34.5 $\pm$ 6.3	< 0.0001
Glucose (mg/dL)	122.0 $\pm$ 38.6	139.0 $\pm$ 62.3	0.12
AST/ALT ratio	0.9 $\pm$ 0.5	1.3 $\pm$ 1.0	0.01
Albumin (g/dL)	4.1 $\pm$ 0.3	3.3 $\pm$ 0.7	< 0.0001
Creatinine (mg/dL)	1.0 $\pm$ 0.5	1.7 $\pm$ 1.3	0.004
NFS	-0.9 $\pm$ 1.4	0.7 $\pm$ 2.3	< 0.0001
NFS change per year (Median; IQR)	0.07 (0.02, 0.12)	0.14 (0.01, 0.31)	0.03
NFS of intermediate to high probability of advanced liver fibrosis (%)	168 (63.9)	31 (79.5)	0.05

To avoid overestimation of the model, we excluded those variables used as a part of NAFLD fibrosis score calculation (age, history of diabetes, aspartate aminotransferase/alanine aminotransferase ratio, platelet count, albumin, body mass index and Framingham risk score). NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FRS: Framingham risk score; CHD: Coronary heart disease; NFS: NAFLD fibrosis score.

liver fibrosis by comparing the NFS at baseline to that at the end of follow-up. Most patients were in the stable-fibrosis (60%) and progressive-fibrosis (37%) groups, whereas only 3% were in the regressive-fibrosis group. The annual NFS change in the progressive-fibrosis group and the stable-disease group was  $0.20 \pm 0.02$  and  $0.05 \pm 0.08$ , respectively (*P* = not significant).

## DISCUSSION

In the current study, we found that a higher NFS at baseline and the presence of new-onset CHD were sig-

nificantly predictive of death. The use of noninvasive methods to predict poor clinical outcomes in NAFLD patients during follow-up is urgently needed. Studies of noninvasive markers used to identify steatohepatitis patients from NAFLD patients require validation before being widely used, as previously suggested<sup>[22]</sup>. Although liver biopsy is the currently recommended practice for identifying liver fibrosis, in NAFLD patients with risk factors that include older age, diabetes, severe obesity and metabolic syndrome, serial liver biopsies are invasive and are not applicable in clinical practice. Recently, Rafiq *et al*<sup>[22]</sup> showed that at least 3 risk factors, including type

**Table 6** Multivariate logistic regression model showing OR (95%CI) for predictors for death in 302 patients with non-alcoholic fatty liver disease

Multivariate analysis	P value	OR	95%CI
<b>Model 1</b>			
Presence of new-onset CHD	< 0.0001	9.0	2.9-28.4
NFS at baseline	< 0.0001	1.9	1.4-2.6
Use of metformin	0.02	0.2	0.04-0.8
Use of simvastatin	0.001	0.05	0.01-0.3
<b>Model 2</b>			
NFS changes per year	0.04	14.9	1.1-206.4
Presence of new onset of CHD	0.001	8.0	2.4-26.1
NFS at baseline	< 0.0001	2.1	1.5-2.9
Use of metformin	0.02	0.2	0.04-0.7
Use of simvastatin	0.001	0.06	0.01-0.3
<b>Model 3</b>			
Presence of new onset of CHD	< 0.0001	9.2	2.6-32.2
NFS at baseline	< 0.0001	2.6	1.7-3.9
Use of metformin	0.03	0.2	0.04-0.8
Use of simvastatin	0.001	0.03	0.003-0.2
Interaction between NFS at baseline and NFS change per year	0.004	0.06	0.008-0.4
NFS changes per year	0.6	2.2	0.07-67.8

Model 1 without interaction among 9 included variables: gender, systolic blood pressure, diastolic blood pressure, nonalcoholic fatty liver disease (NAFLD) fibrosis score at baseline, use of metformin, use of simvastatin, use of aspirin, presence of new-onset of coronary heart disease (CHD) and new onset of liver complications; Model 2 without interaction among 10 included variables: gender, systolic blood pressure, diastolic blood pressure, NAFLD fibrosis score (NFS) at baseline, NFS changes per year, use of metformin, use of simvastatin, use of aspirin, presence of new-onset coronary heart disease (CHD) and new onset of liver complications; Model 3 added variables of interaction between NFS at baseline and NFS changes per year, interaction among use of aspirin, metformin, aspirin and simvastatin into model 2.

2 diabetes, older age and low albumin level, were predictors of mortality and liver-related mortality. Most of these risk factors are components of the NFS<sup>[9]</sup>. Thus, a benefit of the current study is an extension of the clinical use of the NFS system for predicting death in patients with NAFLD.

Our results show that the annual NFS change in patients who died was two times higher than in survivors during the follow-up. Thus, it would be valuable to calculate NFS in newly diagnosed patients.

The annual rate of NFS change in the progressive-fibrosis group was approximately 4 times higher than that in the stable-disease group. Therefore, the median value of the annual rate of NFS change might be used as a surrogate marker for progression of liver fibrosis, but this measure requires further study to validate its benefit.

The long-term outcomes of patients with NAFLD are not uniform across the spectrum of the disease<sup>[3,22,23]</sup>. Poor outcomes are more frequent in patients with NASH, which was confirmed by our results, which showed patients with an intermediate or high probability of advanced liver fibrosis had an increased frequency of primary endpoints, all-cause deaths, and liver complications

compared to those with a low probability of advanced liver fibrosis.

Several previous studies have demonstrated a higher mortality rate (30%-45%) than observed in the current study (13%). This difference may be at least partly explained by a difference in patient selection. For instance, the prevalence of diabetes or impaired glucose tolerance, a well-known risk factor for increased mortality, was three times higher in a Swedish study than in the current study (53% *vs* 16%)<sup>[3]</sup>. In addition, previous studies did not exclude patients with known CHD or known liver complications at baseline<sup>[3,22,23]</sup>.

The use of metformin or simvastatin was found to be a protective factor against death in the present study. This finding is in line with a study by Ekstedt *et al*<sup>[24]</sup>, who showed a significant reduction in liver steatosis in NAFLD patients on statins *vs* those not on statins. A recent study found diabetes mellitus to be one of the important predictors for developing moderate to severe liver fibrosis<sup>[25]</sup>, and treatment with metformin improved liver histology and ALT levels in one-third of patients with NASH<sup>[26]</sup>. Two other studies suggested that metformin improved only the insulin sensitivity but did not improve liver histology in NASH patients<sup>[26,27]</sup>. Limited data exist concerning the efficacy of metformin in patients with NAFLD, and the result of the current study does not necessarily imply causality. Our results show that the use of simvastatin seems to improve the prognosis in patients with NAFLD. This improvement may relate to the effect on prevention of new-onset CHD, which accounted for 20% of deaths in our study. Recent data showed that the statins are safe and well-tolerated in patients with NAFLD<sup>[28-32]</sup>. Moreover, the use of statins was associated with a reduction of hepatic steatosis in NAFLD patients<sup>[24,32,33]</sup>. No study has assessed the efficacy of statins to reduce CHD mortality in NAFLD patients, although the benefits are well-recognized for both the primary and secondary prevention of CHD and the reduction of overall mortality in the general population<sup>[34,35]</sup>. Thus, the benefit of statins for CHD prevention in NAFLD patients with dyslipidemia and/or a high calculated risk of coronary heart disease by the FRS should be considered<sup>[36,37]</sup>.

The main strengths of our study are the inclusion of NAFLD patients from the community along with the long-term follow-up. All patients had complete data for calculation of the NFS at the time of NAFLD diagnosis and at the end of the follow-up. The exclusion of known CHD or liver cirrhosis with complications at baseline is important to reduce the overestimation of the incidence of primary endpoints or mortality rate during the follow-up period.

Our study has several limitations. First, only 6.6% of the patients with a low probability of advanced liver fibrosis died, which was less than expected (10%) by a sample size calculation and may affect the power of the study. Second, most of our patients in Olmsted County are white, and recent data showed that non-Caucasian

**Table 7 Comparison of nonalcoholic fatty liver disease patients with and without death after excluding those with established type 2 diabetes at baseline (*n* = 254) *n* (%) (mean  $\pm$  SD)**

Variables	NAFLD patients without death ( <i>n</i> = 226)	NAFLD patients with death ( <i>n</i> = 28)	<i>P</i> value
Age (yr)	44.5 $\pm$ 11.5	59.2 $\pm$ 14.3	< 0.0001
Sex (% male)	113 (50)	10 (35.7)	0.11
History of hypercholesterolemia	56 (24.8)	9 (32.1)	< 0.0001
Obesity	164 (72.6)	22 (78.6)	0.34
BMI (kg/m <sup>2</sup> )	33.5 $\pm$ 6.2	35.0 $\pm$ 6.6	0.25
Platelet count ( $\times 10^9$ /mm <sup>3</sup> )	241 $\pm$ 58	242 $\pm$ 87	0.98
Glucose (mg/dL)	215.7 $\pm$ 46.6	215.7 $\pm$ 46.6	
Cholesterol (mg/dL)	215 $\pm$ 48	219 $\pm$ 56	0.72
HDL-cholesterol (mg/dL)	42 $\pm$ 12	43 $\pm$ 20	0.76
AST (U/L)	42.0 $\pm$ 23.0	39.7 $\pm$ 21.0	0.62
ALT (U/L)	65.7 $\pm$ 45.0	50 $\pm$ 29	0.02
AST/ALT ratio	0.76 $\pm$ 0.38	0.93 $\pm$ 0.57	0.14
ALP(U/L)	184 $\pm$ 66	246 $\pm$ 179	0.09
GGT (U/L)	132 $\pm$ 41	142 $\pm$ 36	0.32
Albumin (g/dL)	4.3 $\pm$ 0.3	4.2 $\pm$ 0.4	< 0.0001
FRS	7.6 $\pm$ 6.2	10.5 $\pm$ 5.6	0.02
Calculated CHD risk (%)	15.3 $\pm$ 14.4	22.0 $\pm$ 18.8	0.076
NFS	-2.11 $\pm$ 1.15	-1.09 $\pm$ 1.61	0.003
NFS of intermediate or high probability of advanced liver fibrosis (%)	59 (26.1)	19 (67.9)	< 0.0001
B. During the follow-up periods			
Use of metformin	52 (23.0)	1 (3.6)	0.009
Use of aspirin	119 (52.7)	9 (32.1)	0.03
Use of simvastatin	90 (39.8)	3 (10.7)	< 0.0001
NFS change per year (median; IQR)	0.08 $\pm$ 0.08	0.12 $\pm$ 0.36	0.61

In order to avoid overestimation of the model, we excluded those variables used as a part of nonalcoholic fatty liver disease fibrosis score calculation (age, history of diabetes, aspartate aminotransferase/alanine aminotransferase ratio, platelet counts, albumin, body mass index and Framingham risk score). NAFLD: Nonalcoholic fatty liver disease; BMI: Body mass index; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; FRS: Framingham risk score; CHD: Coronary heart disease; NFS: NAFLD fibrosis score; ALP: Alkaline phosphatase; GGT: Glutamyltransferase; HDL: High-density lipoprotein.

**Table 8 Multivariate logistic regression model showing OR (95%CI) of predictors for death in 254 patients with non-alcoholic fatty liver disease after excluding those with established type 2 diabetes at baseline**

Multivariate analysis	<i>P</i> value	OR	95%CI
NAFLD fibrosis score at baseline	< 0.0001	1.92	1.4-2.7
Alkaline phosphatase	0.012	1.006	1.001-1.010

Model without interaction among 8 included variables; gender, nonalcoholic fatty liver disease fibrosis score at baseline, use of metformin, use of simvastatin, use of aspirin, history of hypercholesterolemia, alanine aminotransferase and alkaline phosphatase. NAFLD: Nonalcoholic fatty liver disease.

race was an important predictor of decreased survival<sup>[22]</sup>. With the relatively small sample size and the represented local population in the United States, our results are not necessarily applicable in other ethnic groups.

The current study is important because we extended the clinical use of the NFS system for predicting death or liver complications in NAFLD patients. A higher NFS at baseline and the presence of new-onset CHD can be used as prognostic predictors for mortality and liver complications among NAFLD patients. Further research is needed to validate the benefit of the NFS for predicting death or liver complications in NAFLD patients with

other ethnic groups. The NFS is simpler and less invasive than liver biopsy for the initial evaluation of the degree of liver fibrosis in patients with NAFLD. The NFS should be calculated for all patients with NAFLD at initial consultation to estimate the probability of advanced liver fibrosis.

## COMMENTS

### Background

Nonalcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease in Western countries. During an average 7.6 years of follow-up, 13% of the patients died, mainly from malignancy, coronary heart disease (CHD) and liver-related mortality.

### Research frontiers

Another study revealed that the survival of patients with nonalcoholic steatohepatitis was reduced and that these patients died significantly more often from CHD and liver-related causes. Patients with more advanced liver fibrosis tend to have more liver complications than those without liver fibrosis.

### Innovations and breakthroughs

A study from Japan validated the NAFLD Fibrosis Score and found it to have an acceptable sensitivity, specificity, and positive and negative predictive values for advanced liver fibrosis of 100%, 83%, 63%, and 100%, respectively.

### Peer review

In this manuscript, the authors intend to evaluate whether the NAFLD fibrosis score could act as a prognostic predictor for mortality and liver complications among NAFLD patients. It is suggested that the NAFLD fibrosis score is a simple, non-invasive method for predicting prognosis in patients with NAFLD. In general, this paper is significant and of clinical importance.



**Table 9** The association between the primary end point and the grading of the nonalcoholic fatty liver disease fibrosis score, classified into 2 subgroups, after excluding those with established type 2 diabetes at baseline *n* (%)

Grading of the NAFLD fibrosis score ( <i>n</i> = 254)	Low prob. of advanced liver fibrosis ( <i>n</i> = 176)	Intermediate prob. or High prob. of advanced liver fibrosis ( <i>n</i> = 78)	<i>P</i> value
Presence of primary end point ( <i>n</i> = 43; 17%)	20/176 (11.4)	23/78 (29.5)	< 0.0001
All-cause death ( <i>n</i> = 28; 11%)	9/176 (5.1)	19/78 (24.4)	< 0.0001

NAFLD: Nonalcoholic fatty liver disease.

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## ***Schistosoma japonicum* egg antigen up-regulates fibrogenesis and inhibits proliferation in primary hepatic stellate cells in a concentration-dependent manner**

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### **Abstract**

**AIM:** To investigate the effects of different concentrations of *Schistosoma japonicum* (*S. japonicum*) egg antigen on fibrogenesis and apoptosis in primary hepatic stellate cells (HSCs).

**METHODS:** A mouse model of schistosomiasis-associated liver fibrosis (SSLF) was established by infecting mice with schistosomal cercaria *via* the abdomen. HSCs were isolated from SSLF mice by discontinuous density gradient centrifugation, and their identity was confirmed by immunofluorescence double staining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and desmin. The growth inhibitory effect and 50% inhibitory concentration (IC<sub>50</sub>) of *S. japonicum* egg antigen for primary HSCs (24 h) were determined using a cell counting kit-8 (CCK-8) assay. The expression levels of  $\alpha$ -SMA, matrix metalloproteinase-9 (MMOL/LP-9) and tissue inhibitor

of metalloproteinases-1 (TIMP-1) in HSCs in response to different concentrations of *S. japonicum* egg antigen were detected by Western blotting and real-time reverse transcription-polymerase chain reaction. The levels of phospho-P38 (P-P38), phospho-Jun N-terminal kinase (P-JNK) and phospho-Akt (P-AKT) in HSCs were detected by Western blotting.

**RESULTS:** An SSLF mouse model was established, and primary HSCs were successfully isolated and cultured. *S. japonicum* egg antigen inhibited HSC proliferation in a concentration-dependent manner. The IC<sub>50</sub> of the *S. japonicum* egg antigen was  $244.53 \pm 35.26$   $\mu$ g/mL. *S. japonicum* egg antigen enhanced  $\alpha$ -SMA expression at both the mRNA and protein levels and enhanced TIMP-1 expression at the mRNA level in HSCs ( $P < 0.05$ ), whereas the expression of MMOL/LP-9 was attenuated at both the mRNA and protein levels in a concentration-dependent manner ( $P < 0.05$ ). A high concentration of *S. japonicum* egg antigen enhanced P-P38, P-JNK and P-AKT activation ( $P < 0.05$ ). The changes in  $\alpha$ -SMA and MMOL/LP-9 expression induced by *S. japonicum* egg antigen were closely correlated with P-P38 and P-JNK activation ( $P < 0.05$ ). The attenuation of MMOL/LP-9 was also correlated with P-AKT activation ( $P < 0.05$ ), but the increase in  $\alpha$ -SMA expression was not. TIMP-1 expression was not correlated with P-P38, P-JNK or P-AKT activation.

**CONCLUSION:** *S. japonicum* egg antigen promotes fibrogenesis, activates the P38/JNK mitogen-activated protein kinase and AKT/PI3K signaling pathways and inhibits proliferation in primary HSCs isolated from SSLF mice in a concentration-dependent manner.

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**Key words:** Schistosomiasis; Liver fibrosis; Hepatic stellate cells; Mitogen-activated protein kinase; Akt

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## INTRODUCTION

*Schistosomiasis* is a water-borne parasitic disease that plagues many tropical and subtropical regions. At least 200 million people in 76 countries are currently afflicted by this disease, and a further 500-600 million people are at risk of infection<sup>[1]</sup>. *Schistosoma japonicum* (*S. japonicum*), the Asian schistosome, causes schistosomiasis in China, Japan, the Philippines and Indonesia. A nationwide schistosomiasis survey carried out in 2003 indicated that there were still more than 800 000 people infected with *S. japonicum* in China<sup>[2]</sup>.

Despite recent progress in anti-schistosomal strategies, clinical management remains a challenge because schistosomiasis-associated liver fibrosis (SSLF) is a complex, multi-step and often fatal disease. Cercaria that are transmitted through the skin can lay a large number of eggs, which then pass through the sinusoidal endothelial vascular system, settle in the liver, release immol/Luno-competent products, interact with various liver cells and finally lead to liver fibrosis<sup>[3]</sup>. This liver fibrosis could develop into an irreversible advanced stage upon repeated exposure to the causative agents (*i.e.*, *S. japonicum* eggs). Primary hepatic stellate cells (HSCs) are believed to be the crucial contributors to the fibrotic process by producing extracellular matrix and interrupting the balance of fiber generation *vs* degradation<sup>[4]</sup>. Some studies have investigated the mechanism underlying the pathogenesis of SSLF. *S. mansoni* eggs can stimulate hepatic endothelial cell proliferation<sup>[5]</sup> and migration, promote angiogenesis<sup>[6]</sup>, induce fibroblast proliferation<sup>[7]</sup> and collagen synthesis<sup>[8]</sup> and down-regulate LX-2 activation and fibrogenesis<sup>[9]</sup>. However, little is known about the mechanisms that are active in HSCs during the development and progression of SSL<sup>[3,10]</sup>.

In this study, primary HSCs isolated from SSLF mice were exposed to different concentrations of *S. japonicum* egg antigen and then analyzed to better understand the interaction between HSCs and SSLF.

## MATERIALS AND METHODS

### Animals

Healthy 4- to 6-wk-old male BALB/C mice were obtained from a schistosomiasis control station in Hubei province, China.

### Reagents

*S. japonicum* egg antigen [0.01 g/mL in phosphate-

buffered saline (PBS)] was obtained from the Hubei schistosomiasis control station and diluted to the working concentration in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS) before use.

### Animal model development

The SSLF model was established by abdominal infection with schistosomal cercaria according to the method used in a previous study<sup>[11]</sup>. In brief, mice in the model group were percutaneously infected with *S. japonicum* by placing a glass slide carrying  $20 \pm 2$  cercariae in non-chlorinated water on the abdomen of each mouse for 15 min. Mice in the control group were treated with non-chlorinated water containing no cercariae. All mice were raised for 6 wk under pathogen-free conditions with free access to food and water. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Chinese Council on Animal Care. Liver samples were taken from 10 infected and 10 normal mice and fixed in 4% (v/v) paraformaldehyde (PFA) in PBS.

### Masson staining

Masson staining was performed using standard methods to observe collagen fiber deposition. About 5 middle-power fields were randomly selected from each sample for analysis, and the ratio of the area occupied by collagen fibers to the total area was quantified using Image Pro Plus 6.0 software (Media Cybernetics Inc., United States).

### Hepatic stellate cell isolation and culture

HSCs were prepared by the discontinuous density gradient centrifugation technique previously described by Schafer *et al*<sup>[12]</sup>, with minor modifications. Briefly, the liver was perfused with Solution I [137 mmol/L NaCl, 5.4 mmol/L KCl, 0.6 mmol/L NaH<sub>2</sub>PO<sub>4</sub>•2H<sub>2</sub>O, 0.8 mmol/L Na<sub>2</sub>HPO<sub>4</sub>•12H<sub>2</sub>O, 10 mmol/L HEPES, 0.5 mmol/L EGTA, 4.2 mmol/L NaHCO<sub>3</sub>, 5 mmol/L glucose, 100 U/mL penicillin and 100 U/mL streptomycin (Gibco, United States), pH = 7.4] and Solution II [137 mmol/L NaCl, 5.4 mmol/L KCl, 0.6 mmol/L NaH<sub>2</sub>PO<sub>4</sub>•2H<sub>2</sub>O, 0.8 mmol/L Na<sub>2</sub>HPO<sub>4</sub>•12H<sub>2</sub>O, 10 mmol/L HEPES, 3.8 mmol/L CaCl<sub>2</sub>•2H<sub>2</sub>O, 24.2 mmol/L NaHCO<sub>3</sub>, 5 mmol/L glucose, 600 mg/L collagenase IV (Gibco, United States), 400 mg/L pronase (Sigma, United States), 20 mg/L DNase (Gibco, United States), pH = 7.4] through the hepatic portal vein. The livers were removed and forced through a 200 gauge mesh. Parenchymal cells were separated by centrifugation at 20 *g* for 5 min. The supernatant was transferred to a 50 mL centrifuge tube and centrifuged at 500 *g* for 7 min. The pellet was resuspended in 15% OptiPrep (Sigma, United States), and 11% OptiPrep and 5 mL GBSS (120 mmol/L NaCl, 5 mmol/L KCl, 0.84 mmol/L Na<sub>2</sub>HPO<sub>4</sub>•2H<sub>2</sub>O, 0.22 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 1.9 mmol/L MgCl<sub>2</sub>•6H<sub>2</sub>O, 1.5 mmol/L CaCl<sub>2</sub>•2H<sub>2</sub>O, 27 mmol/L NaHCO<sub>3</sub>, 5 mmol/L glucose, pH = 7.4) were



then layered on the top of the 15% OptiPrep, and the gradient was centrifuged at 1400 *g* for 17 min. The interface between the 11% OptiPrep and the GBSS was collected and washed twice with GBSS. The collected cells were cultured in DMEM containing 10% FBS (Gibco, United States). The cell viability, as measured by a Trypan Blue exclusion assay, was approximately 90%. Primary HSCs from passages 7-8 were used in this study.

### Immunofluorescence staining

Immunofluorescence double staining of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and desmin was used to identify activated HSCs. Briefly, cell slides were fixed in 4% PFA and incubated with a blocking solution containing 0.1% Triton X-100 and 5% bovine serum albumin (BSA) in PBS, followed by incubation with anti-desmin (Abcam, United Kingdom) and anti- $\alpha$ -SMA (Boster, China) primary antibodies at 4 °C overnight. After three washes with PBS, the slides were incubated with secondary FITC-conjugated antibodies and Cy3-conjugated antibodies (Proteintech, United States) for 1 h at 37 °C and then with Hoechst 33258 for 5 min. The cells were observed and imaged using a TE-2000 Nikon Inverted fluorescence microscope, and the number of positive cells per 100 cells was analyzed.

### Cell counting Kit-8

The cell growth inhibitory effect and 50% inhibitory concentration (IC<sub>50</sub>) of *S. japonicum* egg antigen for primary HSCs were determined using a cell counting kit-8 (CCK-8) assay. HSCs were treated as follows: cultured cells ( $1 \times 10^4$ /well) in a 96-well plate were exposed to a range of concentrations of *S. japonicum* egg antigen (1, 5, 25, 125, 250, 625, 1250, 2500 and 3125  $\mu$ g/mL) for 24 h, and 10  $\mu$ L of water soluble tetrazolium-8 (WST-8) (Promoter, China) was then added to the medium containing the *S. japonicum* egg antigen. The cells were incubated then for 2 h. The absorbance of each well was read at 450 nm, and a blank well that contained only culture medium and was used for background correction. The percent inhibition was calculated according to the following formula: Inhibition (%) =  $[1 - (\text{treated}/\text{control})] \times 100$ <sup>[13,14]</sup>. The IC<sub>50</sub> was determined using Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., United States).

### Real-time reverse transcription-polymerase chain reaction

Cultured cells were incubated with various concentrations of *S. japonicum* egg antigen (0, 5, 10, 15, 50, 250  $\mu$ g/mL) for 24 h. Total RNA was isolated from HSCs using the TRIzol reagent (Invitrogen, United States) according to the protocol described by the manufacturer. RNA samples were quantified by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer, and all samples had an A<sub>260</sub>/A<sub>280</sub> ratio between 1.8 and 2.0, which indicated a high purity of the extracted RNA. The RNA concentrations were calculated based

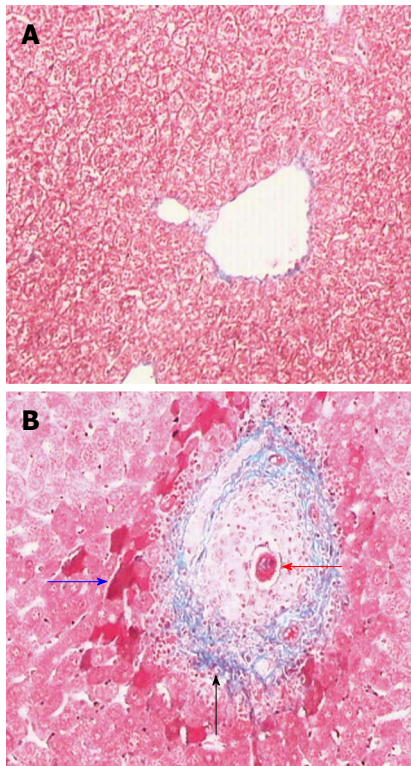
on the absorbance at 260 nm. Aliquots of total RNA (0.5  $\mu$ g) from each sample were reverse transcribed into cDNA according to the instructions provided with the first-strand cDNA synthesis kit (TaKaRa, Japan). Equal amounts of the reverse transcription products were subjected to polymerase chain reaction amplification using SYBR Green as a fluorescent indicator on an AB iCycler system (AB, United States). The levels of  $\alpha$ -SMA, matrix metalloproteinase-9 (MMOL/LP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1) mRNAs were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The fold change in the expression of target genes between the experimental and control samples was calculated using the  $2^{-\Delta\Delta C_T}$  method, as previously described<sup>[15]</sup>. The primers used in this study were synthesized by Invitrogen Ltd. (Invitrogen, United States):  $\alpha$ -SMA sense CGGGAGAAAATGACCCAGATT,  $\alpha$ -SMA antisense GGACAGCACAGCCTGAATAGC, MMOL/LP-9 sense ACAGCCAACTATGACCAGGAT, MMOL/LP-9 antisense CAGGAAGACGAAGGGGAAGAC, TIMP-1 sense CTGGTTCCCTGGCGTACTC, TIMP-1 antisense ACCTGATCCGTCCACAAACAG, GAPDH sense GGTGTCTCCTGCGACTTCA and GAPDH antisense GGGTGGTCCAGGGTTTCTTA.

### Western blotting

Western blot analysis was performed as described previously<sup>[16]</sup>. In brief, proteins from HSCs were extracted using RIPA lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH = 7.4) containing protease inhibitors. After the samples were boiled for 5 min at 95 °C in  $5 \times$  loading buffer, equal amounts (50  $\mu$ g) of cell homogenates were separated by 12% SDS-PAGE. The proteins were then electrophoretically transferred at 250 mA/h onto polyvinylidene fluoride membranes. The membranes were blocked with 5% nonfat dry milk or BSA in Tris-buffered saline-Tween 20 (TBST) and probed at 4 °C overnight with primary antibodies against  $\alpha$ -SMA (1:200, Boster, China), MMOL/LP-9 (1:200, Boster, China), TIMP-1 and  $\beta$ -actin (1:500, Santa Cruz, United States), phospho-P38 (P-P38) and total P38 (T-P38) (1:1000, Cell Signaling Technology, United States), phospho-JNK (P-JNK) and total-JNK (T-JNK) (1:1000, Cell Signaling Technology, United States), phospho-AKT (P-AKT) (1:1000, Cell Signaling Technology, United States) and total-AKT (T-AKT) (1:400, Santa Cruz, United States). The membranes were then washed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted 1:5000 or 1:2000. The membranes were washed, and all blots were visualized using an ECL detection system (Biouniquer, China). The bands were quantitated in grayscale using Image J software (NIH, United States).

### Statistical analysis

The results of multiple observations are presented as the means  $\pm$  SD of at least 3 independent experiments.



**Figure 1** Masson staining of 6 wk post-infect mice livers ( $\times 200$ ). A: Normal mice; B: Infected mice, the red arrow indicated the eggs deposited in the vein, the blue represented acidophilic necrosis, and the black was the collagen fiber deposited around the vein.

**Table 1** Ratio of collagen fiber deposited area to the total in the normal and infected mice livers (mean  $\pm$  SD)

Group	n	Ratio of deposited collagen fiber area against the total
Normal	10	5.18% $\pm$ 1.88%
Infected	10	14.53% $\pm$ 2.90% <sup>a</sup>

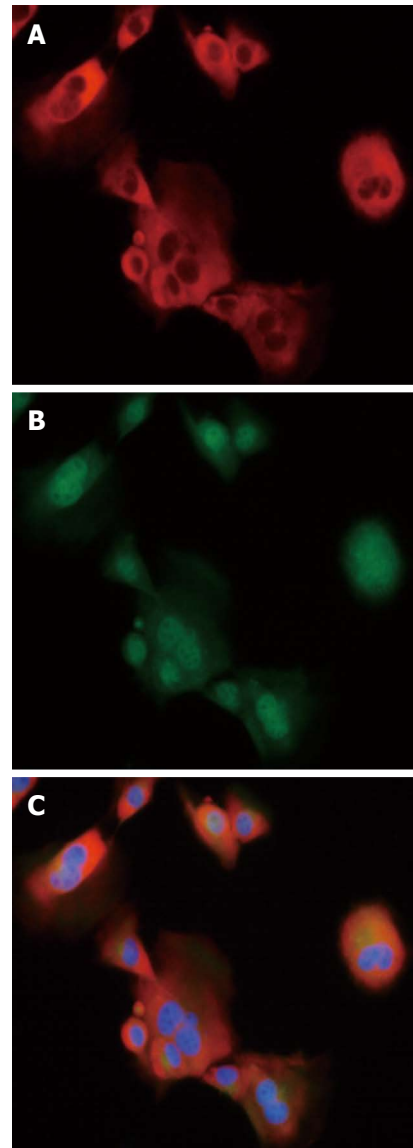
<sup>a</sup> $P < 0.05$  vs Normal.

Student's *t* test was used to evaluate the difference in the ratio of the collagen fiber-deposited area to the total area between infected and normal mouse livers. ANOVA was used to evaluate the differences between the *S. japonicum* egg antigen-treated group and the control group. Person linear correlation analysis was used to analyze the correlations between the  $\alpha$ -SMA, MMOL/LP-9 and TIMP-1 expression levels and the P-P38, P-JNK and P-AKT levels. All *P* values were two sided, and  $P < 0.05$  was considered statistically significant. Statistical analysis was performed using SPSS 16.0 software.

## RESULTS

### Establishment of the mouse model of SSLF

Masson staining showed that collagen fibers were deposited at the periphery of the eosinophilic granuloma and that eggs were deposited in the venae of mouse liver tissues at 6 wk post-infection; in contrast, there were no

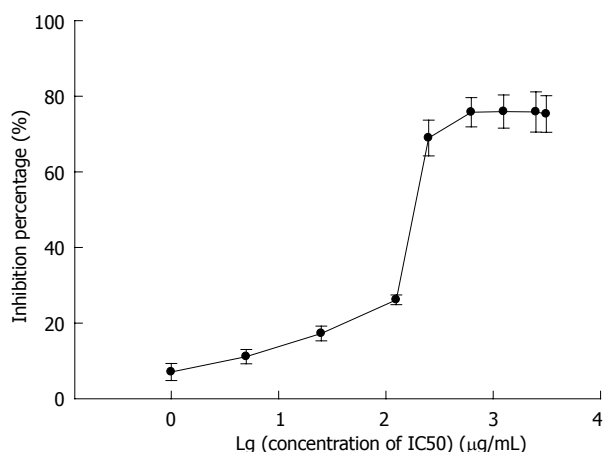


**Figure 2** Immunofluorescence staining in primary primary hepatic stellate cells ( $\times 400$ ). A:  $\alpha$ -Smooth muscle actin (red hue); B: Desmin (green hue); C: Overlay.

collagen fibers deposited in the normal mouse livers except in the vessels (Figure 1). In addition, the ratio of the collagen fiber-deposited area to the total area was significantly greater in the SSLF livers than in the normal livers (Table 1). The above results suggest that the SSLF mouse model was established successfully.

### Identification of isolated HSCs

Primary HSCs isolated from SSLF mice displayed a quiescent phenotype. When cultured in plastic culture dishes, these cells began to exhibit an activated phenotype. The expression levels of  $\alpha$ -SMA and desmin increased, and this feature was used to identify activated HSCs. Immunofluorescence double staining showed that  $\alpha$ -SMA and desmin were expressed in the cytoplasm of HSCs (Figure 2). Approximately 95% of the cells expressed both  $\alpha$ -SMA and desmin, suggesting that primary HSCs were



**Figure 3** Inhibitory proliferation of *Schistosoma japonicum* egg antigen in primary hepatic stellate cells. The black dots were quantification of 3 independent experiments.

isolated successfully.

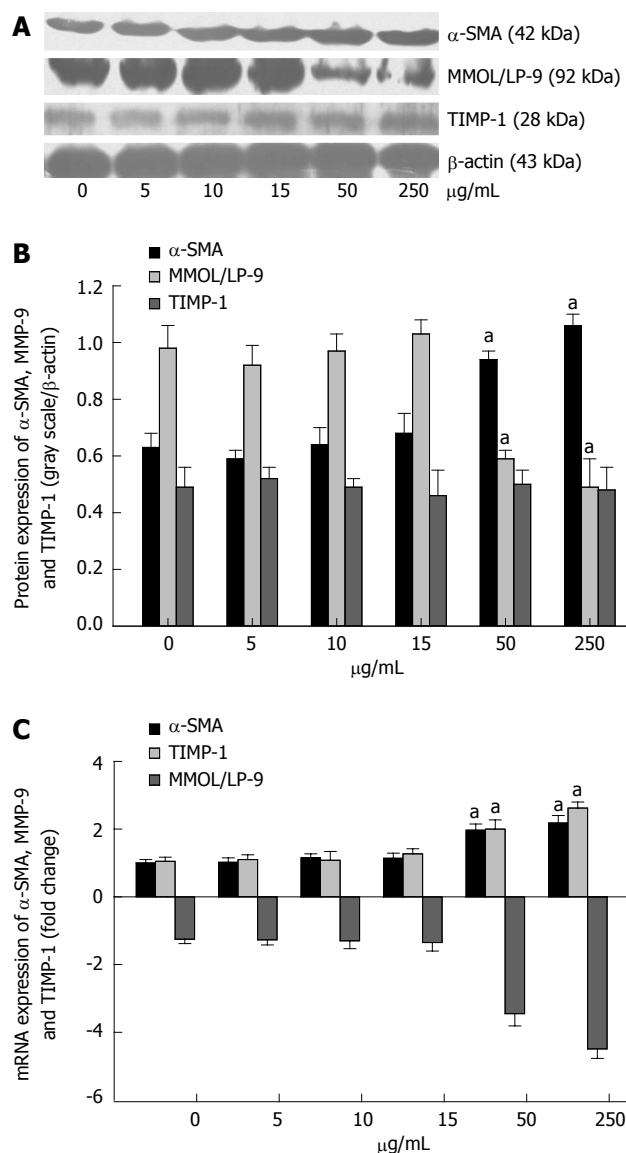
#### Inhibitory effect and IC<sub>50</sub> of *S. japonicum* egg antigen for primary HSCs

Figure 3 shows that *S. japonicum* egg antigen inhibited the proliferation of primary HSC in a concentration-dependent manner. The percent inhibition values for *S. japonicum* egg antigen were  $7.06\% \pm 2.26\%$  for 1  $\mu\text{g/mL}$ ,  $11.13\% \pm 1.90\%$  for 5  $\mu\text{g/mL}$ ,  $17.25\% \pm 1.95\%$  for 25  $\mu\text{g/mL}$ ,  $26.16\% \pm 1.28\%$  for 125  $\mu\text{g/mL}$ ,  $68.96\% \pm 4.73\%$  for 250  $\mu\text{g/mL}$ ,  $75.77\% \pm 3.87\%$  for 625  $\mu\text{g/mL}$ ,  $75.95\% \pm 4.40\%$  for 1250  $\mu\text{g/mL}$ ,  $75.84\% \pm 5.33\%$  for 2500  $\mu\text{g/mL}$  and  $75.30\% \pm 4.84\%$  for 3125  $\mu\text{g/mL}$ . The IC<sub>50</sub>, defined as the concentration of a substance that reduces cell survival by 50%, is a useful parameter to quantify the effect of a substance on cell survival. In this study, the IC<sub>50</sub> of *S. japonicum* egg antigen was calculated to be  $244.53 \pm 35.26 \mu\text{g/mL}$ .

#### Expression of $\alpha$ -SMA, MMP-9 and TIMP-1 in response to *S. japonicum* egg antigen in vitro

As shown in Figure 4, *S. japonicum* egg antigen at 50 or 250  $\mu\text{g/mL}$  enhanced  $\alpha$ -SMA expression in HSCs at both the mRNA and protein levels. At the mRNA level, the fold change in  $\alpha$ -SMA expression was  $1.97 \pm 0.18$  in response to 50  $\mu\text{g/mL}$  and  $2.18 \pm 0.22$  in response to 250  $\mu\text{g/mL}$  relative to the control ( $1.00 \pm 0.10$ ,  $P < 0.05$ ) (Figure 4C). At the protein level, the  $\alpha$ -SMA/ $\beta$ -actin ratio was  $0.94 \pm 0.03$  at 50  $\mu\text{g/mL}$  and  $1.06 \pm 0.04$  at 250  $\mu\text{g/mL}$ , compared with  $0.63 \pm 0.05$  for the control ( $P < 0.05$ , Figure 4A and B).

However, at the mRNA level,  $3.45 \pm 0.36$ -fold reduction in response to 50  $\mu\text{g/mL}$  and  $4.49 \pm 0.28$ -fold reduction in response to 250  $\mu\text{g/mL}$  in MMOL/LP-9 expression were observed after 24h with egg antigen treatment, compared with  $1.05 \pm 0.12$  for the control ( $P < 0.05$ , Figure 4C). At the protein level, the MMOL/LP-9/ $\beta$ -actin ratios were  $0.59 \pm 0.03$  at 50  $\mu\text{g/mL}$  and  $0.49 \pm 0.10$  at 250  $\mu\text{g/mL}$ , compared with  $0.98 \pm 0.08$  for the

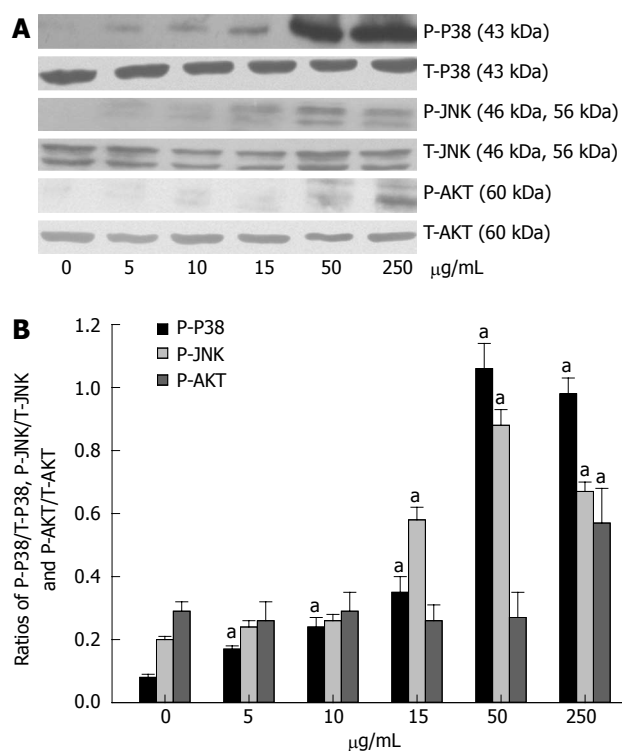


**Figure 4** Western blotting (A, B) and real-time polymerase chain reaction (C) of  $\alpha$ -smooth muscle actin, matrix metalloproteinase-9 and tissue inhibitor of metalloproteinases-1 induced by *Schistosoma japonicum* egg antigen in primary hepatic stellate cells. A: Western blotting of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), matrix metalloproteinase-9 (MMOL/LP-9) and tissue inhibitor of metalloproteinases-1 (TIMP-1), and representatives of 3 independent experiments; B: The histograms reported as ratio of respective target gray scale to that of  $\beta$ -actin; C: The histograms represented the fold change of target genes normalized to glyceraldehyde-3-phosphate dehydrogenase levels and quantified of 3 independent experiments.  $^*P < 0.05$  vs 0  $\mu\text{g/mL}$ .

control ( $P < 0.05$ , Figure 4A and B). In addition, *S. japonicum* egg antigen also enhanced TIMP-1 expression at the mRNA level. The fold change in TIMP-1 mRNA expression was  $2.00 \pm 0.27$  in response to 50  $\mu\text{g/mL}$  and  $2.62 \pm 0.18$  in response to 250  $\mu\text{g/mL}$  compared with  $1.05 \pm 0.13$  for the control ( $P < 0.05$ , Figure 4C). However, as shown in Figure 4A and B, *S. japonicum* egg antigen had no effect on TIMP-1 expression at the protein level.

Collectively, the above observations indicate that *S. japonicum* egg antigen promoted fibrogenesis in HSCs and participated in the extracellular matrix remodeling observed in SSLE.





**Figure 5** Western blotting of phospho-P38, phospho-Jun N-terminal kinase and phospho-Akt activation induced by *Schistosoma japonicum* egg antigen in primary hepatic stellate cells. A: Western blotting of phospho-P38 (P-P38), phospho-Jun N-terminal kinase (P-JNK) and phospho-Akt (P-AKT), and representatives of 3 independent experiments; B: The histograms respectively reported as ratio of P-P38/T-P38, P-JNK/T-JNK, and P-AKT/T-AKT. \* $P < 0.05$  vs 0 µg/mL.

### P38/JNK MAPK and AKT signaling pathway activation by *S. japonicum* egg antigen in vitro

As shown in Figure 5, the P-P38, P-JNK and P-AKT activation levels were enhanced by stimulation with the *S. japonicum* egg antigen. The P-P38/T-P38 ratio was  $1.03 \pm 0.08$  at 50 µg/mL and  $1.05 \pm 0.01$  at 250 µg/mL vs  $0.18 \pm 0.02$  for the control ( $P < 0.05$ ). The P-JNK/T-JNK ratio was  $0.58 \pm 0.04$  at 15 µg/mL,  $0.88 \pm 0.05$  at 50 µg/mL and  $0.67 \pm 0.03$  at 250 µg/mL vs  $0.20 \pm 0.01$  for the control ( $P < 0.05$ ). The P-AKT/T-AKT ratio was  $0.57 \pm 0.11$  at 250 µg/mL vs  $0.29 \pm 0.03$  for the control ( $P < 0.05$ ). The above results indicate that *S. japonicum* egg antigen activated the P38/JNK mitogen-activated protein kinase (MAPK) and AKT signaling pathways in a concentration-dependent manner.

### Relationships among the levels of the $\alpha$ -SMA, MMP-9 and TIMP-1 and P-P38, P-JNK and P-AKT in HSCs

$\alpha$ -SMA protein expression was positively correlated with the P-P38 ( $r = 0.669$ ,  $P = 0.002$ ) and P-JNK levels ( $r = 0.686$ ,  $P = 0.002$ ). MMOL/LP-9 expression was negatively correlated with the P-P38 ( $r = -0.976$ ,  $P = 0.000$ ), P-JNK ( $r = -0.86$ ,  $P = 0.000$ ) and P-AKT levels ( $r = -0.529$ ,  $P = 0.024$ ). However, TIMP-1 expression was not correlated with the P-P38, P-JNK or P-AKT level.  $\alpha$ -SMA expression was not correlated with the P-AKT

level.

## DISCUSSION

HSC activation is considered the key step in liver fibrogenesis, representing a transition from the quiescent state into a proliferative, fibrogenic and contractile state. HSCs reside in the space of disse and are recruited to areas of hepatic injury by chemokines, where they become activated<sup>[17]</sup>. During this process, HSCs are transformed from the quiescent to activated phenotype, a process that is accompanied by an increase in the expression levels of  $\alpha$ -SMA and desmin<sup>[18,19]</sup>, which are considered to be markers of HSC activation. The activated HSCs adopt a myofibroblast-like phenotype and secrete collagen-rich matrix into the extracellular space, leading to liver fibrosis<sup>[20]</sup>. HSCs have been implicated as the effector cells of hepatic fibrosis and cirrhosis associated with hepatitis B virus, hepatitis C virus, genetic hemochromatosis, biliary atresia, cystic fibrosis and alcoholic liver disease in humans<sup>[20-23]</sup>. HSCs also play a role in eosinophilic granulomatous inflammation and SSLF<sup>[24-26]</sup>. Bartley *et al.*<sup>[25]</sup> showed that activated HSCs could be detected at the edges of eosinophilic granulomas in *S. japonicum*-infected mice livers at 6 wk post-infection, and the localization of these cells was coincident with areas of collagen deposition. In this study, collagen fiber deposition was observed at the periphery of the eosinophilic granuloma, consistent with the previous reports<sup>[25]</sup>. In addition, we also found that *S. japonicum* egg antigen inhibited HSC proliferation in a concentration-dependent manner.

Activated HSC-secreted MMOL/LPs (*e.g.*, MMOL/LP-9) and their inhibitors (*e.g.*, TIMP-1) remodel the tissue matrix<sup>[22]</sup>, and an imbalance in MMOL/LP/TIMP expression has been associated with cumulative fibrosis<sup>[27]</sup>. The expression of  $\alpha$ -SMA is increased in the livers of SSLF patients and infected mice and is closely correlated with the degree of liver fibrosis<sup>[25,27]</sup>. In our experiment, the *S. japonicum* egg antigen attenuated MMOL/LP-9 expression and enhanced  $\alpha$ -SMA expression at both the mRNA and protein levels, and it also enhanced TIMP-1 expression at the mRNA level. However, the TIMP-1 protein level was not affected. The mRNA and protein expression levels are not always proportional due to the activities of various regulatory mechanisms, *e.g.*, post-transcriptional modulation by miRNAs. The above results imply that the *S. japonicum* egg antigen is involved in MMOL/LP-9-mediated matrix remodeling and thus collagen deposition, leading to liver fibrosis. The results of our study partially contradict to the previous report that *S. mansoni* eggs can down-regulate the activation of and fibrogenesis in the human hepatic stellate cell line LX-2<sup>[9]</sup>. One potential reason for this discrepancy is the different cell models and schistosomal egg antigens used in the two studies. *In vitro* exposure to schistosome eggs induces only limited activation of human dendritic cells (DCs) but strong activation of murine DCs<sup>[28]</sup>. Similarly, in this study, the same concentrations of egg antigen (5 µg/mL,



10 µg/mL, and 15 µg/mL) that attenuated  $\alpha$ -SMA expression in human LX-2 cells had no effect on primary mouse HSCs. In addition, *S. japonicum* and *S. mansoni* eggs release different antigens<sup>[29]</sup>, which may play a role in the discrepancy.

MAPKs are pivotal transmitters of extracellular signals such as hormones, cytokines, growth factors, and various environmental stress signals<sup>[30]</sup>. The MAPK family has three major subfamilies: ERK, P38, and JNK, and the P38 and JNK pathways are involved in HSC transformation<sup>[31]</sup> and in the regulation of the complex life cycle and host-parasite interactions of *S. japonicum*<sup>[32]</sup>. Many studies have shown that the regulation of MMOL/LP-9<sup>[33-36]</sup> and  $\alpha$ -SMA<sup>[37-39]</sup> in various cell models is dependent on the activation of the P38 and (or) JNK signaling pathways. In addition, AKT/PI3K activation contributes to liver<sup>[40]</sup>, heart<sup>[41]</sup> and pulmonary<sup>[42-44]</sup> fibrosis. To gain insight into the potential signaling mechanisms mediating the HSC responses induced by the *S. japonicum* egg antigen, we investigated the roles of the P38/JNK MAPK and AKT signaling pathways by assessing their phosphorylation status in primary HSCs after stimulation by the *S. japonicum* egg antigen. In this study, the P-P38 and P-JNK levels were significantly enhanced after the treatment. The increase in  $\alpha$ -SMA expression that was stimulated by the *S. japonicum* egg antigen was positively correlated with the P-P38 and P-JNK levels but not the P-AKT level. Attenuated MMOL/LP-9 expression was negatively correlated with the P-P38, P-JNK and P-AKT levels. TIMP-1 expression was not correlated with the P-P38, P-JNK or P-AKT level. We also noted that only *S. japonicum* egg antigen concentrations of 250 µg/mL or greater were associated with AKT activation, suggesting that AKT signaling may have a less important role than P38/JNK MAPK signaling in the induction of fibrogenesis by the *S. japonicum* egg antigen.

In conclusion, the results of this study suggest that the *S. japonicum* egg antigen promotes fibrogenesis and inhibits the proliferation of primary HSCs in a concentration-dependent manner. These effects of the *S. japonicum* egg antigen may be attributed to the activation of the P38/JNK signaling pathways in HSCs. Thus, inhibitors of the P38/JNK signaling pathways may serve as potential therapeutic treatments for SSLF.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Under the action of stimulus, hepatic stellate cells (HSCs) are activated and secrete extracellular matrix (ECM) into extracellular space, which leads to liver fibrosis. HSCs have been attributed as the effector cells for schistosomiasis-associated liver fibrosis (SLF). Continuous schistosome eggs deposition in

liver tissue was the chief factors leading to SSLF. So the study on the interaction between schistosome eggs antigen and HSCs is very important.

### Research frontiers

As the effector cells of liver fibrosis, HSC is usually used as the cell model to study the liver fibrosis *in vitro*. The research hotspot on SSLF is the effects induced by schistosome eggs in HSCs and the involved mechanisms, and finding out the targets of treatment on SSLF for future.

### Innovations and breakthroughs

The authors isolated primary HSCs which maintained the original features of HSC genes, and tried to explore the involved signaling pathways. The results of this study showed that *Schistosoma japonicum* (*S. japonicum*) eggs promoted the fibrogenesis and inhibited the proliferation of primary HSCs *in vitro*. P38/JNK MAPK signaling pathways might play an important role in the fibrogenesis induced by *S. japonicum* eggs, however AKT/PI3K signaling pathway might be not involved.

### Applications

The results of this study suggest that *S. japonicum* egg antigen induced-fibrogenesis is related with P38/JNK MAPK signaling pathway activation, and the inhibitors of the P38/JNK signaling pathways may serve as potential therapeutic treatments for SSLF.

### Terminology

Schistosomiasis is a water-borne parasitic disease that plagues many tropical and subtropical regions in world. There are 6 types schistosome which can infect humans: *Schistosoma haematobium*, *Schistosoma mansoni*, *S. japonicum*, *Schistosoma mekongi*, *Schistosoma intercalatum*, *Schistosoma malayensis*. *S. japonicum*, the Asian schistosome, causes schistosomiasis in China, Japan, the Philippines and Indonesia. Cercariae of schistosome parasitize in oncomelania that live in water and infect humans through bare skins, develop into imagoes in human bodies, and produce a large number of eggs which can deposit in liver tissues. With the eggs continuous deposition, eosinophilic granuloma and liver fibrosis, which are the pathological characteristics of schistosomiasis, are finally induced.

### Peer review

This is a good study in which authors describe the fibrogenesis and inhibitory proliferation effects induced by *S. japonicum* in primary HSCs, and analyzed the signaling pathways (e.g., P38/JNK MAPK) which may be involved in the above effects. The results are interesting and suggest that the inhibitors of the P38/JNK signaling pathways may serve as potential therapeutic treatments for SSLF in future.

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## Effect of silencing of high mobility group A2 gene on gastric cancer MKN-45 cells

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### Abstract

**AIM:** To investigate the effect of high mobility group A2 (HMGA2) gene silencing on gastric cancer MKN-45 cells *in vitro*.

**METHODS:** HMGA2 short hairpin RNA (shRNA) expression plasmids were constructed, including a pair of random scrambled sequences. Human gastric cancer cell line MKN-45 cells were divided into three groups: blank control group (non-transfected cells), transfected group (cells transfected with HMGA2 shRNA recombinant plasmid) and scrambled sequence group (transfected with random scrambled plasmid). Cells were transfected with HMGA2 shRNA recombinant plasmids and scrambled plasmid *in vitro*, and the cells transfection efficiency was assayed by fluorescence microscopy. The HMGA2 messenger RNA (mRNA) ex-

pression was detected by reverse transcription polymerase chain reaction, gastric cancer cells apoptosis was detected by flow cytometry, cell proliferation was detected by methyl thiazol tetrazolium, and the protein expression of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt), P27, caspase-9 and B-cell leukemia/lymphoma-2 (Bcl-2) were analyzed by Western blotting.

**RESULTS:** Compared with the blank control group and the scrambled sequence group, the levels of HMGA2 mRNA and protein expression in the transfected group were significantly reduced ( $P < 0.05$ ). The relative HMGA2 mRNA expression levels of the blank control group, transfected group and scrambled sequence group were  $0.674 \pm 0.129$ ,  $0.374 \pm 0.048$  and  $0.689 \pm 0.124$ , respectively. The relative HMGA2 protein expression levels of the blank control group, transfected group and scrambled sequence group were  $0.554 \pm 0.082$ ,  $0.113 \pm 0.032$  and  $0.484 \pm 0.123$ , respectively. Moreover, transfection with the scrambled sequence had no effect on the expression of HMGA2. After being transfected with shRNA for 24, 48 and 72 h, the cell apoptotic rates of the transfected group were  $21.65\% \pm 0.28\%$ ,  $39.98\% \pm 1.82\%$  and  $24.51\% \pm 0.93\%$ , respectively, which significantly higher than those of blank control group ( $4.72\% \pm 1.34\%$ ,  $5.83\% \pm 0.13\%$  and  $5.22\% \pm 1.07\%$ ) and scrambled sequence group ( $4.28\% \pm 1.33\%$ ,  $7.87\% \pm 1.43\%$  and  $6.71\% \pm 0.92\%$ ). After 24, 48 and 72 h, the cell proliferation inhibition rates in the transfected group were  $31.57\% \pm 1.17\%$ ,  $39.45\% \pm 2.07\%$  and  $37.56\% \pm 2.32\%$ , respectively; the most obvious cell proliferation inhibition appeared at 48 h after transfection. Compared with the blank control group and scrambled sequence group, after transfection of shRNA for 72 h, the protein expression levels of PI3K ( $0.042 \pm 0.005$  vs  $0.069 \pm 0.003$ ,  $0.067 \pm 0.05$ ), Akt ( $0.248 \pm 0.004$  vs  $0.489 \pm 0.006$ ,  $0.496 \pm 0.104$ ) and Bcl-2 ( $0.295 \pm 0.084$  vs  $0.592 \pm 0.072$ ,  $0.594 \pm 0.109$ ) were significantly reduced. The protein expression levels of P27 ( $0.151 \pm 0.010$  vs  $0.068$



$\pm 0.014$ ,  $0.060 \pm 0.013$ ) and caspase-9 ( $0.136 \pm 0.042$  vs  $0.075 \pm 0.010$ ,  $0.073 \pm 0.072$ ) were significantly upregulated.

**CONCLUSION:** *HMGA2* shRNA gene silencing induces apoptosis and suppresses proliferation of MKN-45 cells.

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**Key words:** Gastric cancer cells; RNA interference; High mobility group A2; Proliferation; Apoptosis

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## INTRODUCTION

Gastric cancer is one of the most common malignancies, being the fourth most common cancer and the second leading cause of cancer related deaths (740 000 deaths in 2008), with nearly one million newly diagnosed cases each year<sup>[1]</sup>. Up to now, the exact molecular mechanisms involved in carcinogenesis and progression of gastric cancer have remained unclear. Therefore, investigating the cell proliferation and apoptosis involved in these processes is essential for gastric cancer research.

The high mobility group A2 (*HMGA2*) gene is a member of the high mobility group AT-hook (HMGA) gene family. *HMGA2* plays a critical role in several cellular biological processes, including cell transformation, growth, differentiation, senescence and cycle control<sup>[2-4]</sup>. Normally, *HMGA2* is abundantly expressed during embryogenesis, whereas its expression is low or absent in normal adult differentiated tissues<sup>[5,6]</sup>. Evidence shows that *HMGA2* is overexpressed in breast cancer<sup>[7]</sup>, pancreatic cancer<sup>[8,9]</sup>, esophageal squamous cell carcinoma<sup>[10]</sup>, non-small cell lung cancer<sup>[11]</sup>, oral squamous cell cancer<sup>[12]</sup>, bladder cancer<sup>[13]</sup>, ovarian cancer<sup>[14,15]</sup> and colorectal cancer<sup>[16,17]</sup>. Its expression is correlated with malignant degree, pathological type and metastasis<sup>[18]</sup>. Thus, *HMGA2* might play a central role in the disease progression of many types of tumors. Overexpression of *HMGA2* has also been described in gastric cancer<sup>[19]</sup>. The study showed that the overexpression of *HMGA2* in gastric cancer patients was significantly correlated with poor prognosis and low overall survival. However, the exact mechanism of *HMGA2* in gastric cancer is not clear.

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway plays a central role in many human cancers. Akt regulates cell proliferation, differentiation and apoptosis *via* activation or inhibition of downstream target proteins, such as P27, caspase-9 and B-cell leukemia/lymphoma-2 (Bcl-2)<sup>[20,21]</sup>.

The aims of the present study were to investigate the effect of *HMGA2* expression on gastric cancer cell

proliferation and apoptosis. Moreover, the expressions of PI3K, Akt, P27, caspase-9 and Bcl-2 were detected to exploit the possible mechanism.

## MATERIALS AND METHODS

### Construction of *HMGA2*-shRNA expression plasmid

The human *HMGA2* was treated as the target gene (Accession Number: NM-003484) and four pairs of short hairpin RNA (shRNA) oligonucleotides were designed and synthesized (Cyagen Biosciences Inc., United States). A pair of random scrambled sequences, which have no homologous relation with any human gene sequences and whose shRNAs would not interfere with human mRNA were also designed (Table 1). The complementary form was obtained by annealing, which was then cloned and inserted into vector pLLU2 to construct a recombinant plasmid. The plasmid was transformed into *stb13* strain. Finally, the plasmid was identified by restriction enzyme digestion and sequenced (Cyagen Biosciences Inc., United States). According to the preliminary experimental results, the first pair shRNA oligonucleotides (Table 1) was the best interfering sequence, and the recombinant plasmid harboring it was used for subsequent experiments.

### Cell culture and transfection

Gastric cancer MKN-45 cells (ATCC, United States) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Solarbio Science and Technology Co., Ltd., Beijing, China) containing 10% placental bovine serum. After 24 h incubation at 37 °C with 5% CO<sub>2</sub>, logarithmic growth phase cells in good condition were digested by 0.25% trypsin + ethylenediaminetetraacetic acid. After calculating the cell number, cells were seeded into 6-well plates at  $1 \times 10^5$  cells per well. When the cells reached more than 80% confluence (usually the next day), plasmid and lipofectamine were added dropwise to form a mixture of 1:1 with mixing. After culturing in serum-free and antibiotic-free DMEM for 6 h, the culture medium was replaced by DMEM and the culture was continued for 24 h. The cells were then transfected using a Lipofectamine<sup>TM</sup>2000 Transfection Kit (Invitrogen, United States) according to the manufacturer's instructions. Cells were randomly counted in 10 microscope fields at 24, 48 and 72 h after transfection. The ratio of the number of cells with green fluorescent emission and the total cells number indicated the transfection efficiency. Non-transfected cells from the same batch were collected as the blank control group. The cells transfected with the random scrambled sequence plasmid served as the scrambled sequence group.

### Reverse transcription polymerase chain reaction analysis

Total RNA was isolated using TRIzol (Sangon Biotech Co., Ltd., Shanghai, China). A Reverse Transcriptase Kit (Fermentas China Co., Ltd.) was used to create cDNA, according to the manufacturer's instructions: 1 µg RNA, Oligo dT<sub>18</sub> as the primer, and 42 °C for 60 min followed by 70 °C for 5 min. Reverse transcriptase polymerase

**Table 1** Short hairpin RNA oligo sequences targeting high mobility group A2

Oligo name	Oligo sequence 5' to 3'
shHMGA2-1-F	TAGTCCCTCTAAAGCAGCTCAACTCGAGTTGAGCTGCTTTAGAGGGACTTTTTC
shHMGA2-1-R	TCGAGAAAAAGTCCCTCTAAAGCAGCTCAACTCGAGTTGAGCTGCTTTAGAGGGACTA
shHMGA2-2-F	TAGGAGGAAACTGAAGAGACATCTCGAGATGTCTCTTCAGTTTCCCTCTTTTTC
shHMGA2-2-R	TCGAGAAAAAGGAGGAAACTGAAGAGACATCTCGAGATGTCTCTTCAGTTTCCCTCTA
shHMGA2-3-F	TCTCACAAGAGTCTGCCGAAGACTCGAG TCTTCGGCAGACTCTTGAGAGTTTTC
shHMGA2-3-R	TCGAGAAAAACTCACAAGAGTCTGCCGAAGACTCGAGTCTTCGGCAGACTCTTGAGAGA
Scrambled-F	TGCGCGCTTTGTAGGATTCGCTCGAGCGAATCCTACAAAGCGCGCTTTTTC
Scrambled-R	TCGAGAAAAAGCGCGCTTTGTAGGATTCGCTCGAGCGAATCCTACAAAGCGCGCA

shHMGA2: Short hairpin high mobility group A2; F: Forward; R: Reserve.

chain reaction (RT-PCR) assays were carried out using on an ABI 2720 (Applied Biosystems, Foster City, CA, United States). The PCR primers (synthesized by Sangon Biotech Co., Ltd., Shanghai, China) used to detect HMGA2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: HMGA2, upstream primer 5'-TACTCTGTCTCTGCCTGTGC-3', downstream primer 5'-GGAGTGAATTGTGTCCCTTGA-3' (product length 228 bp); GAPDH, upstream primer 5'-GAAGGTGAAGGTCCGAGTC-3', downstream primer 5'-TCACACCCATGACGAACAT -3' (product length 401 bp). The 25  $\mu$ L PCR amplification reaction mixture contained: cDNA, 1.5  $\mu$ L; Taq Mix, 12.5  $\mu$ L; 10  $\mu$ mol/L; each primer, 1  $\mu$ L; RNase-free Water, 9  $\mu$ L. Amplification reaction program: initial denaturation at 95  $^{\circ}$ C for 5 min; 35 cycles of denaturation at 94  $^{\circ}$ C for 30 s, annealing at 58  $^{\circ}$ C for 35 s, extension at 72  $^{\circ}$ C for 30 s; and final extension at 72  $^{\circ}$ C for 7 min. The PCR products were analyzed on agarose gels; gel analysis software was used to analyze the bands. The gray value ratio of the objective band and the  $\beta$ -actin band indicated the relative expression levels of HMGA2 mRNA.

#### Western blotting analysis of the protein expression levels of HMGA2, PI3K, Akt, P27, caspase-9 and Bcl-2

Cells were lysed in RIPA cell lysis buffer (Sangon Biotech Co., Ltd., Shanghai, China). The ratio of lysate and loading buffer was 4:1, degeneration and proteins were extracted. About 80  $\mu$ g of proteins were fractionated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and electroblotted onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% nonfat milk for 2 h at room temperature and incubated overnight with the primary antibody (Santa Cruz Biotechnology, inc., United States). The membrane was washed with Tris-Buffered-Saline with Tween (TBST) three times for 10 min each, and then incubated for 2 h at room temperature with the secondary antibody (Zhongshanjinjiao Biotechnology Co., Ltd., Beijing, China). The membranes were washed with TBST 3 times for 10 min each, before being developed and fixed.  $\beta$ -actin (Zhongshanjinjiao Biotechnology Co., Ltd., Beijing, China) was used as an internal control. Gel analysis software was used to scan and analyze the bands. The gray value ratio of objective band and internal loading control band was used to de-

termine the objective proteins expression levels.

#### Flow cytometric analysis of cell apoptosis

Each group of cells was digested into a single cell suspension using 0.25% trypsin. One hundred  $\mu$ L of buffer from the Annexin v-FITC and PE Apoptosis Detection Kit (BD Co., United States), 5  $\mu$ L ADD and 5  $\mu$ L PE were added to the cells, which were incubated without shaking at room temperature for 15 min in the dark. About 400  $\mu$ L of buffer was then added, then the apoptosis rate of each group of cells was assayed by flow cytometry.

#### In vitro cell proliferation assay

A suspension of each group of cells was obtained by 0.25% trypsin digestion. The cells were centrifuged (1000 g, 5 min), resuspended in 1 mL culture medium and counted. A cell suspension containing  $2 \times 10^4$ /200  $\mu$ L cells was distributed into 96-well plates: six sets of parallel holes were used for each group. The cells were then incubated at 37  $^{\circ}$ C in 5% CO<sub>2</sub>. One 96-well plate was removed daily and 20  $\mu$ L per well of a fresh new methyl thiazol tetrazolium (MTT) solution (5 mg/mL) was added, and the plate incubated at 37  $^{\circ}$ C for 4 h. The supernatants were removed, and 150  $\mu$ L of dimethyl sulfoxide was added to each well and the plates were oscillated for 10 min. The optical density value (*A* value) of the cells in each well was measured by an enzyme-labeling instrument at a wavelength of 570 nm, and a growth curve for the cells was drawn. The cell proliferation inhibition rate (CPIR) was calculated according to the formula: CPIR (%) =  $(1 - A_{\text{experimental group}} / A_{\text{control group}}) \times 100\%$ .

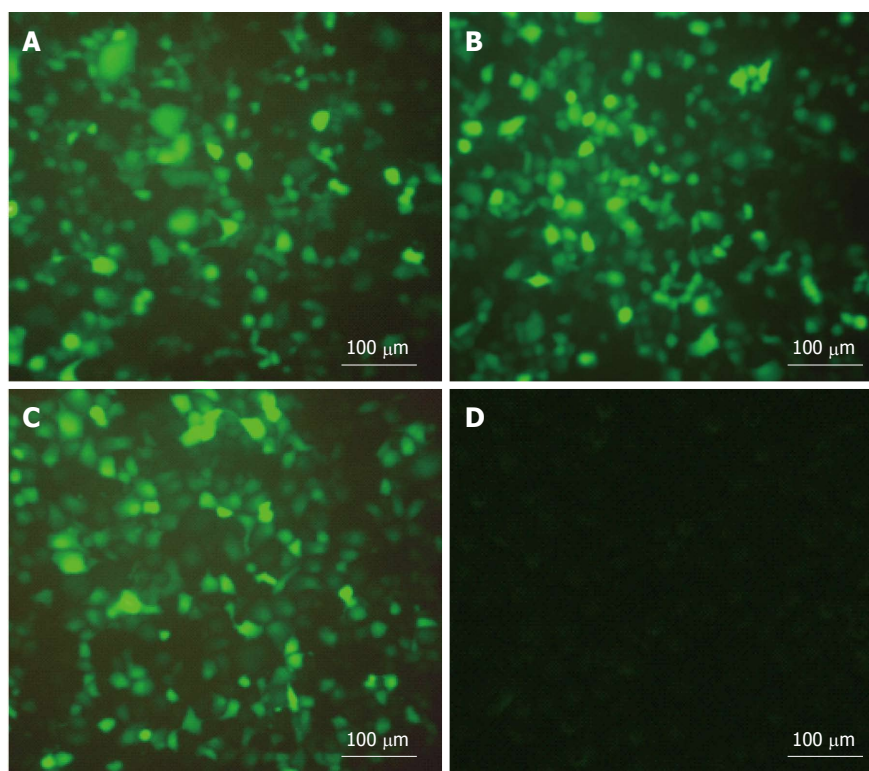
#### Statistical analysis

SPSS 13.0 software (Chicago, United States) was used to perform the statistical analysis. Data were expressed as mean  $\pm$  SD. One-way analysis of variance was used to analyze the correlation of data among three or more groups, and an *SNK* test was used for pairwise comparison between groups. *P* < 0.05 was considered significant.

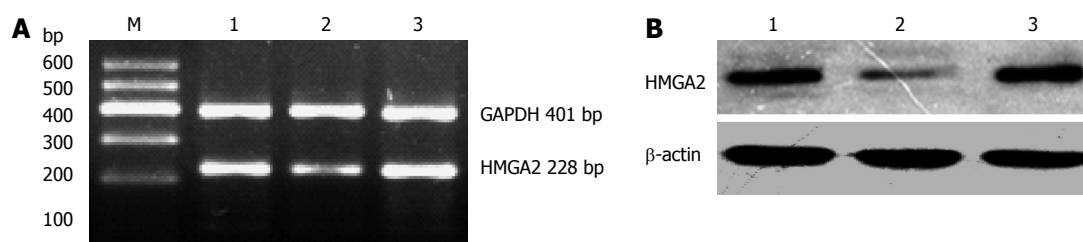
## RESULTS

#### Fluorescence microscopy analysis of cell transfection

The result showed that the transfection efficiency of the



**Figure 1** Cell transfection under the fluorescence microscope (magnification,  $\times 400$ ). A: Transfected group 24 h after transfection; B: Transfected group 48 h after transfection; C: Transfected group 72 h after transfection; D: Scrambled sequence group 48 h after transfection.



**Figure 2** Expression of high mobility group A2 messenger RNA and high mobility group A2 protein. A: Expression of high mobility group A2 (HMGA2) messenger RNA (mRNA); B: Expression of HMGA2 protein. M: Marker; 1: Blank control group; 2: Transfected group; 3: Scrambled sequence group. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

transfected group cells was 60%-70% after transfection for 48 h and was significantly higher than that at 24 h. Compared with 48 h, there was no significant change at 72 h. The scrambled sequence group cells were not transfected at any time (Figure 1).

#### Expression of HMGA2 mRNA in each group of cells

Compared with the blank control group and the scrambled sequence group, the expression of HMGA2 mRNA in the transfected group was significantly reduced ( $0.374 \pm 0.048$  vs  $0.674 \pm 0.129$  and  $0.689 \pm 0.124$ ,  $P < 0.05$ ); however, the difference between the blank control group and the scrambled sequence group was not statistically significant ( $P > 0.05$ ) (Figure 2). The results showed that the RNA interference had a targeted inhibition affect on the HMGA2 gene.

#### Expression of HMGA2 protein in each group of cells

Compared with the blank control group and scrambled sequence group, the HMGA2 the protein expression was

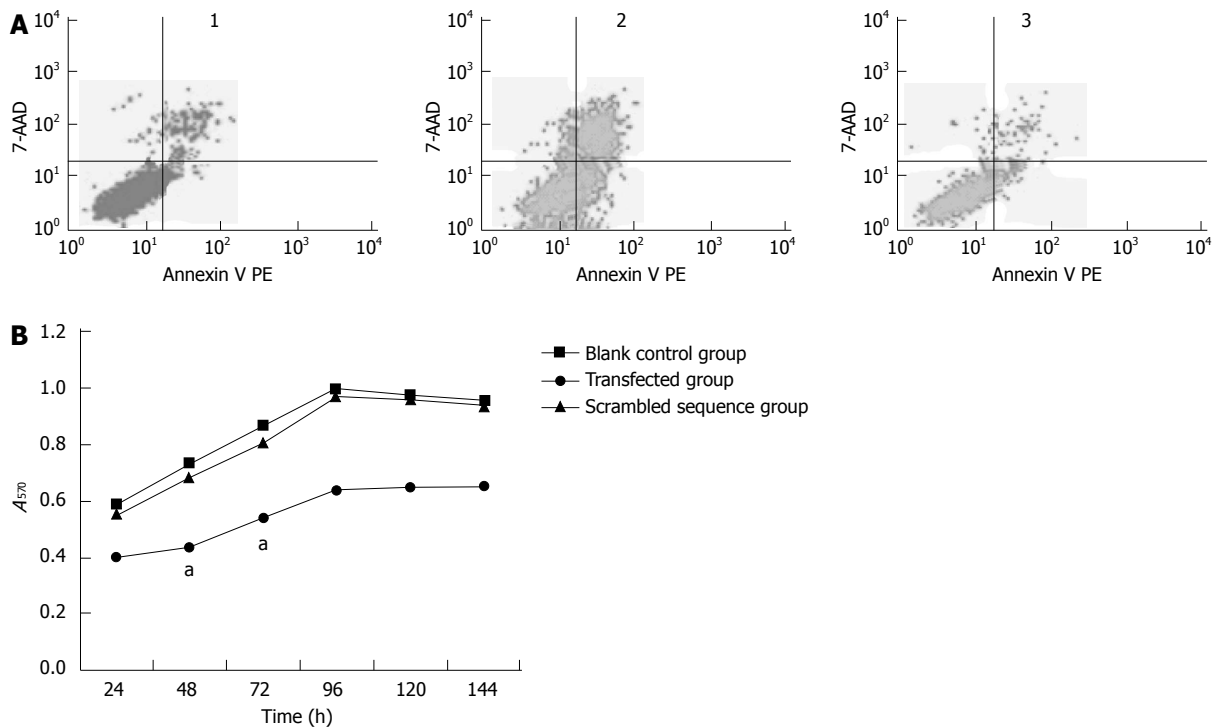
significantly decreased, the difference was statistically significant ( $0.113 \pm 0.032$  vs  $0.554 \pm 0.082$  and  $0.484 \pm 0.123$ ,  $P < 0.05$ ); however, the difference between the blank control group and the scrambled sequence group was not statistically significant ( $P > 0.05$ ) (Figure 2). The results showed that transfection of HMGA2 shRNA expression vector had significantly inhibited the protein expression from the target gene.

#### Apoptosis analysis of gastric cancer cells

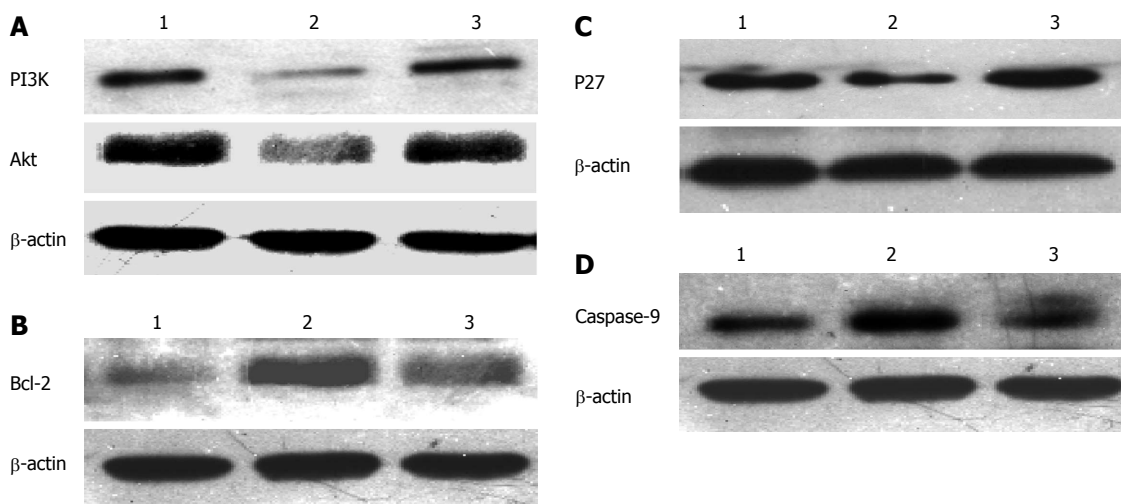
The cell apoptotic rate of the transfected group was significantly higher than that of the blank control group and scrambled sequence group; the difference was statistically significant ( $P < 0.05$ ). The highest cell apoptotic rate appeared at 48 h after transfection. The difference between the blank control group and the scrambled sequence group was not statistically significant ( $P > 0.05$ ) (Figure 3).

#### Cell proliferation assay

The MTT assay revealed that there were remarkable dif-



**Figure 3 Apoptosis and cell proliferation analysis.** A: Flow cytometry. The cell apoptotic rate of the transfected group was significantly higher than those of the blank control group and the scrambled sequence group; B: Cell proliferation analysis. <sup>a</sup> $P < 0.05$  vs blank control group. 1: Blank control group; 2: Transfected group; 3: Scrambled sequence group.



**Figure 4 Western blotting analysis of protein expression.** A: Phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt); B: B-cell leukemia/lymphoma-2 (Bcl-2); C: P27; D: Caspase-9. 1: Blank control group; 2: Transfected group; 3: Scrambled sequence group.

ferences in cell proliferation at 24, 48 and 72 h after transfection with shRNA. The most obvious cell proliferation inhibition appeared at 48 h after transfection in the transfected group ( $P < 0.05$ ). There was no significant difference in the inhibition rate of the blank control group and the scrambled sequence group ( $P > 0.05$ ) (Figure 3).

#### Expression of PI3K, Akt, P27, caspase-9 and Bcl-2 proteins

Compared with the blank control group and scrambled sequence group, after transfection of shRNA for 72 h, the protein expression levels of PI3K ( $0.042 \pm 0.005$  vs

$0.069 \pm 0.003$  and  $0.067 \pm 0.05$ ,  $P < 0.05$ ), Akt ( $0.248 \pm 0.004$  vs  $0.489 \pm 0.006$  and  $0.496 \pm 0.104$ ,  $P < 0.05$ ) and Bcl-2 ( $0.295 \pm 0.084$  vs  $0.592 \pm 0.072$  and  $0.594 \pm 0.109$ ,  $P < 0.05$ ) were significantly reduced. Difference between the blank control group and the scrambled sequence group were statistically significant ( $P > 0.05$ ) (Figure 4A and B). The protein expression levels of P27 ( $0.151 \pm 0.010$  vs  $0.068 \pm 0.014$  and  $0.060 \pm 0.013$ ,  $P < 0.05$ ) and caspase-9 ( $0.136 \pm 0.042$  vs  $0.075 \pm 0.010$  and  $0.073 \pm 0.072$ ,  $P < 0.05$ ) were significantly upregulated. The difference between the blank control group and the



scrambled sequence group was not statistically significant ( $P > 0.05$ ) (Figure 4C and D).

## DISCUSSION

Activation of oncogenes, inactivation of suppressor genes and abnormal expression of apoptosis-related genes are closely related to carcinogenesis and progression of gastric cancer. HMGA2 is a transcription factor that is involved in transcriptional regulation of various tumor-related genes<sup>[22]</sup>. Several studies<sup>[14,15,23]</sup> have showed that HMGA2 plays a critical role in the processes of many tumors, including enhancing cell growth and invasion. Moreover, the prognosis of patients with high HMGA2 expression is worse than those with low HMGA2 expression<sup>[24,25]</sup>. The clinical association of HMGA2 with gastric cancer has been reported<sup>[19]</sup>. The study showed that overexpression of HMGA2 appeared in human gastric cancer, and might be involved in cancer invasion and metastasis. However, the exact mechanism of HMGA2's involvement in gastric cancer is not clear, especially in relation to signaling pathways and the molecular mechanism.

In the present study, fluorescence microscopy revealed that the transfection efficiency of the transfected group cells can reach about 60%-70%, and that recombinant plasmid was effectively expressed in cells. HMGA2 shRNA transfection could effectively inhibit the expression of *HMGA2* gene. The inhibition was achieved without off-target effects, which was confirmed at the mRNA and protein levels by RT-PCR and Western blotting. According to the results of MTT and flow cytometry, cell proliferation was inhibited and apoptosis was increased after transfection. Therefore, we conclude that HMGA2 shRNA gene silencing can effectively induce proliferation inhibition and apoptosis of MKN-45 cells *in vitro* without off-target effects.

The PI3K/Akt signaling pathway plays important roles in cell survival and resistance to apoptosis<sup>[26]</sup>; it has been shown to be activated in many cancers and activated Akt acts to phosphorylate P27, caspase-9 and Bcl-2 to promote the resistance of cancer cells to apoptosis<sup>[20,21]</sup>. A study showed that activation of the PI3K/Akt signaling pathway is induced by overexpression of HMGA2<sup>[27]</sup>. In this study, western blotting showed that the expressions of PI3K and Akt protein were reduced in the transfected group. Thus, decreased expression of HMGA2 inhibits the activity of the PI3K/Akt signaling pathway.

P27 is a cyclin-dependent kinase (CDK) inhibitor that functions as a fail-safe mechanism in DNA repair and apoptosis<sup>[28]</sup>. A report showed that the *P27* gene reduced cell mitosis division and inhibited cell generation<sup>[29]</sup>. Overexpression of P27 inhibits CDK activation and entry into the S phase of the cell cycle<sup>[30]</sup>. The expression level of P27 was positively correlated with cell differentiation, and its loss of function may subsequently contribute to tumorigenesis<sup>[31]</sup>. Caspase-9, a member of the protease family, is intimately associated with the initiation of

apoptosis, and is thought to be activated, while Akt is inhibited<sup>[32]</sup>. Activated caspase-9 is able to cleave caspase-3, leading to apoptosis<sup>[33]</sup>. Bcl-2 is a key anti-apoptotic protein involved in the regulation of apoptosis and is overexpressed in many tumors<sup>[34-36]</sup>. A previous study showed that Bcl-2 delays cell cycle entry by inhibiting Myc activity through the elevation of p27. Bcl-2 can be induced to express *via* the PI3K/Akt signaling pathway and inhibit cell apoptosis<sup>[21,37]</sup>.

In our study, western blotting revealed that the protein expression levels of Bcl-2 were reduced, whereas the protein expression level of P27 and caspase-9 were upregulated. Thus, decreased expression of HMGA2 inhibits the activity of the PI3K/Akt signaling pathway, and then inhibits cells proliferation and increases apoptosis *via* upregulation of the protein expression of P27 and caspase-9, and decreased protein expression of Bcl-2.

In summary, our results demonstrated that HMGA2 shRNA gene silencing could induce apoptosis and suppress proliferation of MKN-45 cells *in vitro*. The PI3K/Akt signaling pathway, P27, caspase-9 and Bcl-2 may play important roles in this HMGA2-mediated effect. We suggest that HMGA2 shRNA gene silencing may be suitable for gastric cancer therapy by suppressing the proliferation and inducing apoptosis of gastric cancer cells.

## COMMENTS

### Background

The newly discovered oncogene, high mobility group A2 (*HMGA2*), is an endonuclear chromatin protein, which is highly expressed in many malignant tumors. The mechanism by which *HMGA2* causes tumors remains unclear, particularly in gastric cancer.

### Research frontiers

RNA interference can specifically mediate degradation of *HMGA2* messenger RNA, block the expression of the gene, inhibit cell proliferation and increase apoptosis, which could be exploited for gastric cancer gene therapy.

### Innovations and breakthroughs

This study demonstrated that RNA interference (RNAi) specifically downregulated the expression of *HMGA2*, inhibited cell proliferation and increased apoptosis. The mechanism appears to be that the decreased expression of *HMGA2* inhibits the activity of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway. Inhibition of cell proliferation and increased apoptosis is associated with upregulated protein expression of P27 and caspase-9, and decreasing expression of B-cell leukemia/lymphoma-2 (Bcl-2).

### Applications

The aims of this study were to investigate the effect of *HMGA2* expression on gastric cancer cell proliferation and apoptosis. RNAi was used regulate the expression of *HMGA2*. Moreover, the expression of PI3K, Akt, P27, caspase-9 and Bcl-2 were detected to investigate the possible mechanism. This will help enrich the theoretical research into the pathogenesis of gastric cancer, and provide an experimental basis for clinical diagnosis and gene therapy of gastric cancer.

### Peer review

This is a well-written manuscript and the results are interesting. It has potential application in gastric cancer gene therapy.

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## Predictors of colorectal cancer testing using the California Health Inventory Survey

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### Abstract

**AIM:** To identify key variables associated with colon cancer testing using the 2009 California Health Inventory Survey (CHIS).

**METHODS:** The CHIS has been conducted biennially since 2001 using a two-stage, geographically stratified random-digit-dial sample design to produce a representative sample of the entire State. For this study we used survey data from 2001-2009 inclusive. We restricted our analysis to White, Black, and Hispanic/Latinos aged 50-80 years. Weighted data was used to calculate the proportion of participants who underwent some form of colon cancer testing (colonoscopy, flexible sigmoidoscopy or fecal occult blood testing) within the previous 5 years stratified by race/ethnicity. For inferential analysis, bootstrapping with replacement was performed on the weighted sample to attain variance estimates at the 95%CI. For mean differences among categories we used *t*-tests and for comparisons of categorical data we used Pearson's  $\chi^2$ . Binary logistic regression was used to identify independent variables associated with undergoing some form of testing. Trend analysis was performed to determine rates of testing over the study period stratified by race.

**RESULTS:** The CHIS database for 2009 had 30 857 unique respondents corresponding to a weighted sample size of 10.6 million Californians. Overall, 63.0% (63.0-63.1) underwent a colon cancer test within the previous 5 years; with 70.5% (70.5%-70.6%) of this subset having undergone colonoscopy. That is 44.5% (44.4%-44.5%) of all individuals 50-80 underwent colonoscopy. By multivariable regression, those tested were more likely to be male (OR = 1.06; 95%CI: 1.06-1.06), Black (OR = 1.30; 95%CI: 1.30-1.31), have a family member with colon cancer (OR = 1.71; 95%CI: 1.70-1.72), and have health insurance (OR = 2.71; 95%CI: 2.70-2.72). Progressive levels above the poverty line were also associated with receiving a test (100%-199%: 1.21; 1.20-1.21), (200%-299%: 1.41; 1.40-1.42), (> 300%: 1.69; 1.68-1.70). The strongest variable was physician recommendation (OR = 3.90; 95%CI: 3.88-3.91). For the Hispanic/Latino group, additional variables associated with testing were success of physician-patient communication (OR = 2.44; 95%CI: 2.40-2.48) and naturalized citizenship status (OR = 1.91; 95%CI: 1.89-1.93). Trend analysis demonstrated increased colon cancer testing for all racial/ethnic subgroups from 2001-2009 although the rate remained considerably lower for the Hispanic/Latino subgroup.

**CONCLUSION:** Using CHIS we identified California citizens most likely to undergo colon cancer testing. The strongest variable associated with testing for all groups was physician recommendation.

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**Key words:** California Health Inventory Survey; Colon cancer testing; Colonoscopy; Hispanic

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## INTRODUCTION

Colorectal cancer is the fourth leading cause of new cancer cases diagnosed in the United States and is the second leading cause of cancer death<sup>[1]</sup>. Population-based surveys such as the Behavior Risk Factor Surveillance System (BRFSS) and the National Health Interview Survey (NHIS) suggest that colon cancer screening tests are underutilized in the United States<sup>[2]</sup>. Based on Surveillance, Epidemiology and End Results (SEER) data, adults over the age of 50 demonstrate a low prevalence of colon cancer screening<sup>[2-5]</sup>. One study reported that only 59% of adults aged  $\geq 50$  years were screened for colon cancer<sup>[6]</sup>.

Colon cancer can be prevented in most cases by strict adherence to accepted colon cancer screening guidelines<sup>[7]</sup>. A position paper co-authored by several specialty societies listed flexible sigmoidoscopy, Computed tomography colonography, barium enema every 5 years, or colonoscopy every 10 years in average risk adults  $\geq 50$  years of age as acceptable for the detection of adenomatous polyps and cancer<sup>[7-10]</sup>. Annual fecal occult blood testing (FOBT), preferably with immunochemical methodology, as an acceptable alternative method for cancer screening<sup>[7,8]</sup>. Cancer screening using stool DNA remains under investigation<sup>[8,10]</sup>. Despite a lack of evidence from randomized controlled trials, colonoscopy has been shown to be a more sensitive test for the detection of adenomas and colon cancer than flexible sigmoidoscopy +/- FOBT<sup>[11,12]</sup>.

Factors identified as positive predictors of colon cancer screening participation have included older age, male gender, married status, negative smoking history, higher education, white non-Hispanic race, family history of colon cancer, regular medical care, physician recommendation, participation in other preventive health care services, and health insurance coverage<sup>[2,4,13-26]</sup>.

The California Health Inventory Survey (CHIS) is the nation's largest state health survey. A random-dial telephone survey is conducted every two years on a wide range of health topics. CHIS data gives a detailed picture of the health care needs of California's large and diverse population. More than 50 000 Californians including adults, teenagers and children, are surveyed<sup>[27]</sup>. The specific aim of this study was to explore the CHIS database to identify demographic, socioeconomic, health and behavioral factors associated with participation in colon cancer testing. In addition, another aim was to perform a detailed analysis of the Latino population.

## MATERIALS AND METHODS

CHIS is a collaborative project of the University of California at Los Angeles Center for Health Policy Research, the California Department of Health Services, and the

Public Health Institute. (<http://www.chis.ucla.edu/methodology.html>). The survey has been conducted biennially since 2001 using a two-stage, geographically stratified random-digit-dial sample design to produce a representative sample of the State. At the first stage, telephone numbers are drawn within predefined geographic areas or "strata". Telephone numbers are screened to determine if they are households and thus eligible for the survey. At the second stage, one adult is randomly selected among all adults living in a household.

Questionnaires in CHIS include, among others, health status, health conditions, physical disability, mental health, health behaviors, women's health, cancer history and prevention, food environment, neighborhood and housing, access to and use of health care, health insurance, public program eligibility, interpersonal violence, employment, income and demographics including preferred spoken language, language of TV, radio, and newspaper. Participant citizenship, immigration status, country of birth and English language proficiency is also ascertained.

### Data processing

For this study CHIS data from 2001-2009 was downloaded in SPSS v19 (IBM, Armonk, NY, United States) format. Each survey was individually weighted using the final sample weight (RAKEDW0). RAKEDW0 accounts for sample selection probabilities and statistical adjustments for possible under coverage and nonresponse biases. It is essential to weigh the data for valid variance estimation otherwise it will be underestimated.

### Statistical analysis

We first analyzed the group aged 50-80 years from the most recent survey, 2009. Racial/ethnic analysis was limited to White, Black, and Hispanic/Latino groups as classified by the UCLA CHPR definition. For descriptive and inferential analyses, bootstrapping with replacement (iterations = 1000) was performed to attain variance estimates at the 95%CI<sup>[28,29]</sup>. This was performed using the bootstrapping module for SPSS. For mean differences among categories we used *t*-tests and for comparisons of categorical data we used Pearson's  $\chi^2$ . We performed multivariable logistic regression using colon cancer testing (colonoscopy, flexible sigmoidoscopy or FOBT) as the dependent variable. Independent predictor variables entered into the model were those judged to be of clinical importance in univariate analysis. The output was expressed as odds ratios and their bootstrapped 95% confidence intervals. We subsequently stratified our sample and repeated the regression analysis for those in the Hispanic/Latino sub-group.

We used weighted CHIS data for 2001-2009 to calculate the proportion of participants who underwent some form of colon cancer testing within the previous 5 years stratified by race/ethnicity. The proportion was derived by dividing the number of individuals age 50-80 in each race who underwent testing in a given year by the total number of 50-80 year olds in that particular racial/ethnic

**Table 1** Characteristics of California Health Inventory Survey participants ages 50-80 years (bootstrap 95%CI)

	Total <i>n</i> = 10 596 208	Colonoscopy/Flexible sigmoidoscopy/ Fecal occult blood test <i>n</i> = 6 678 773	Colonoscopy <i>n</i> = 4 711 189	No colonoscopy/Flexible sigmoidoscopy/Fecal occult blood test <i>n</i> = 3 917 435
Mean age, yr	63.33 (63.32-63.33)	64.19 (64.18-64.20)	64.69 (64.68-64.70)	61.86 (61.85-61.87)
Males, %	46.6 (46.5-46.6)	46.8 (46.7-46.8)	47.2 (47.1-47.2)	46.3 (46.2-46.3)
Body mass index, kg/m <sup>2</sup> , %				
< 18.5	1.7 (1.6-1.7)	1.5 (1.5-1.5)	1.5 (1.5-1.6)	2.0 (1.9-2.0)
18.5-24.99	36.1 (36.0-36.1)	35.1 (35.0-35.1)	36.3 (36.3-36.4)	37.8 (37.8-37.9)
25.0-29.99	37.2 (37.1-37.2)	38.6 (38.6-38.7)	37.8 (37.8-37.9)	34.7 (34.7-34.8)
> 30.0	25.1 (25.1-25.1)	24.8 (24.8-24.9)	24.3 (24.3-24.3)	25.5 (25.5-25.6)
Ethnicity <sup>1</sup> , %				
White	60.8 (60.8-60.9)	63.7 (63.7-63.8)	66.1 (66.1-66.2)	55.9 (55.8-56.0)
African American	6.1 (6.1-6.1)	6.3 (6.3-6.3)	6.0 (6.0-6.0)	5.8 (5.8-5.8)
Latino	14.8 (14.8-14.9)	12.2 (12.2-12.3)	10.6 (10.6-10.7)	19.2 (19.2-19.3)
Asian	11.3 (11.2-11.3)	10.7 (10.7-10.7)	10.5 (10.4-10.5)	12.2 (12.2-12.2)
Other	7.0 (7.0-7.0)	7.0 (7.0-7.0)	6.7 (6.6-6.7)	5.8 (5.7-5.8)
Smoking status, %				
Current	11.1 (11.1-11.2)	8.2 (8.2-8.3)	8.0 (8.0-8.0)	16.1 (16.1-16.1)
Former	35.9 (35.8-35.9)	37.8 (37.8-37.9)	38.4 (38.3-38.4)	32.5 (32.5-32.6)
Never	53.0 (52.9-53.0)	53.9 (53.9-54.0)	53.6 (53.6-53.7)	51.3 (51.3-51.4)
Did not complete high school, %	15.7 (15.7-15.8)	12.9 (12.9-13.0)	10.7 (10.7-10.8)	20.4 (20.4-20.5)
Marital status, %				
Married	65.0 (65.0-65.0)	67.9 (67.8-67.9)	68.8 (68.7-68.8)	60.1 (60.0-60.1)
Living w/partner	4.1 (4.1-4.1)	3.7 (3.7-3.8)	3.6 (3.6-3.6)	4.6 (4.6-4.7)
Widow/separated/divorced	25.1 (25.1-25.2)	23.7 (23.6-23.7)	23.2 (23.2-23.2)	27.7 (27.6-27.7)
Never married	5.8 (5.8-5.8)	4.7 (4.7-4.7)	4.4 (4.4-4.4)	7.6 (7.6-7.7)
Household income	73 671	79 191	81 798	64 260
(United States \$)	(73 630-73 720)	(79 151-79 238)	(81 728-81 876)	(64 157-64 336)
Income relative to poverty level, %				
0-99	10.8 (10.8-10.8)	7.7 (7.7-7.8)	7.1 (7.1-7.1)	16.5 (16.4-16.5)
100-199	17.1 (17.1-17.2)	14.9 (14.8-14.9)	14.3 (14.3-14.3)	21.3 (21.2-21.3)
200-299	13.7 (13.6-13.7)	13.2 (13.2-13.3)	13.5 (13.5-13.5)	14.4 (14.4-14.5)
> 300	58.4 (58.4-58.4)	64.2 (64.1-64.2)	65.1 (65.1-65.2)	47.8 (47.6-47.9)
Dwelling <sup>2</sup> , %				
Urban	39.7 (39.6-39.7)	39.5 (39.4-39.5)	37.6 (37.6-37.7)	40 (40.0-40.1)
Secondary city	20.3 (20.2-20.3)	19.5 (19.5-19.6)	19.4 (19.3-19.4)	21.4 (21.4-21.5)
Suburban	25.1 (25.1-25.1)	26.9 (26.9-27.0)	28.2 (28.2-28.3)	22.1 (22.0-22.1)
Rural	15.0 (14.9-15.0)	14.1 (14.0-14.1)	14.8 (14.8-14.8)	16.4 (16.4-16.5)
Insurance status, %				
Uninsured	9.5 (9.5-9.5)	4.6 (4.6-4.6)	3.3 (3.3-3.3)	17.8 (17.8-17.9)
Medicare and medicaid	8.9 (8.9-9.0)	8.0 (8.0-8.0)	8.5 (8.5-8.6)	10.5 (10.5-10.5)
Medicare and others	27.2 (27.2-27.2)	31.8 (31.8-31.9)	33.9 (33.9-34.0)	19.3 (19.3-19.4)
Medicare only	3.4 (3.4-3.4)	3.1 (3.1-3.1)	3.4 (3.4-3.4)	3.8 (3.8-3.8)
Medicaid	3.6 (3.6-3.6)	2.3 (2.3-2.3)	2.1 (2.0-2.1)	5.8 (5.7-5.8)
Employer-based	41.1 (41.0-41.1)	43.6 (43.6-43.6)	42.2 (42.1-42.2)	36.7 (36.7-36.7)
Privately purchased	4.6 (4.6-4.6)	4.6 (4.6-4.6)	4.6 (4.6-4.6)	4.6 (4.5-4.6)
Other	1.8 (1.8-1.8)	2.0 (2.0-2.0)	2.1 (2.0-2.1)	1.5 (1.5-1.5)
Alcohol use, %				
Never	93.3 (93.2-93.4)	93.5 (93.4-93.6)	93.5 (93.4-93.6)	93.0 (93.0-93.1)
Yes	6.7 (6.2-6.8)	6.6 (6.5-6.9)	6.5 (6.5-6.8)	6.9 (6.8-7.0)
Working status, %				
Employed full time	41.4 (41.4-41.4)	39.9 (39.8-39.9)	38.1 (38.1-38.2)	44.0 (43.9-44.0)
Employed part time	7.1 (7.1-7.1)	7.0 (7.0-7.1)	7.2 (7.2-7.2)	7.1 (7.1-7.2)
Mod-Severe work impaired	6.1 (6.1-6.1)	5.4 (5.4-5.4)	5.3 (5.3-5.4)	7.4 (7.4-7.5)
Spouse Employed F/P Time	32.6 (32.4-32.6)	33.6 (33.5-33.6)	33.4 (33.4-33.6)	30.8 (30.7-30.8)

<sup>1</sup>UCLA CHPR Definition; <sup>2</sup> By census tract.

category for that year. A two-tailed *P* value < 0.05 was considered significant for hypothesis testing.

## RESULTS

### Patients

There were 47 614 individual participants in the 2009 CHIS survey. This corresponded to a full weighted sam-

ple size of 27.6 million individuals from the State of California. There were 30 857 unique respondents between the ages of 50-80 years corresponding to a weighted sample of 10.6 million citizens. Table 1 categorizes these participants as to whether they received at least one colon cancer test within the previous 5 years and further identifies those who received colonoscopy. Overall, 63.0% (63.0-63.1) underwent a colon cancer test; with 70.5%

**Table 2 Health screening issues and compliance as related to receiving a colonoscopy for California Health Inventory Survey participants  $\geq 50$  years old (bootstrap 95%CI)**

	Colonoscopy <i>n</i> = 4 711 189	No colonoscopy <i>n</i> = 5 885 019
Number doctor visits in past year, %	4.22 (4.22-4.22)	3.28 (3.28-3.28)
Site of health provider, %		
Doctor's office	78.1 (78.1-78.2)	65.1 (65.0-65.1)
Community/government clinic	18.1 (18.1-18.2)	21.4 (21.3-21.4)
Emergency room	0.2 (0.2-0.2)	0.8 (0.8-0.8)
Random source/none	0.5 (0.5-0.5)	12.7 (12.6-12.7)
General health, %		
Excellent	17.8 (17.7-17.7)	16.7 (16.6-16.7)
Very good	31.0 (31.0-31.1)	28.2 (28.2-28.3)
Good	29.0 (28.9-29.1)	29.0 (28.9-29.0)
Fair	16.3 (16.3-16.4)	19.2 (19.2-19.3)
Poor	5.8 (5.8-5.8)	6.9 (6.9-6.9)
Medical health, %		
Elevated cholesterol	12.1 (12.1-12.2)	10.0 (9.9-10.0)
Hypertension	43.8 (43.8-43.9)	35.9 (35.8-35.9)
Diabetes	15.9 (15.9-15.9)	15.0 (14.9-15.0)
Heart disease	14.2 (14.1-14.2)	10.8 (10.8-10.8)
Mammograms in past 6 yr, <i>n</i>	2.23 (2.23-2.34)	1.64 (1.63-1.64)
PSA ever tested, %	34.3 (34.3-34.4)	22.1 (22.0-22.1)
Physician recommended	67.3 (67.3-67.4)	39.6 (39.5-39.6)
"colon test", %		
Reason for colonoscopy, %		
Routine screening	75.4 (75.3-75.4)	
Because of a problem	16.8 (16.7-16.8)	
Other	7.9 (7.9-7.9)	
Reason no colonoscopy in 10 yr, %		
Never thought about it		11.9 (11.9-12.0)
Doctor did not recommend		10.0 (10.0-10.1)
No problems/not necessary		9.6 (9.5-9.6)
Too expensive/no insurance		4.5 (4.5-4.6)
Procrastination		3.8 (3.8-3.8)
Too painful/embarrassing		2.9 (2.9-2.9)
Other		57.3 (57.3-57.4)
Family history of CRC, %		
Sibling	2.8 (2.8-2.8)	1.6 (1.6-1.6)
Father	4.1 (4.1-4.1)	1.8 (1.8-1.8)
Mother	3.8 (3.8-3.8)	1.4 (1.4-1.4)
Total	10.7 (10.7-10.7)	4.8 (4.8-4.8)

CRC: Colorectal cancer; PSA: Prostate specific antigen.

(70.5-70.6) of this subset having undergone colonoscopy. That is 44.5% (44.4-44.5) of all individuals 50-80 underwent colonoscopy. The mean age of CHIS participants between the ages of 50-80 years was 63.33 (63.32-63.33). Those who underwent colonoscopy were approximately three years older. Whites comprised the largest racial group at 60.8% (60.8-60.9) followed by Hispanic/Latinos 14.8% (CI 14.8-14.9). Hispanic/Latinos were least likely to undergo a colon cancer test. There were minimal differences in body weight among the test categories; however current smokers were far less likely to undergo a colon cancer test. There appeared to be no association between alcohol use and testing status.

There was an association between educational attainment and colon cancer testing. Those who underwent a colonoscopy were nearly twice as likely [20.4% (20.4-20.5) *vs* 10.7% (10.7-10.8)] to have graduated high school. Marital status and household income also appeared to be

associated with testing. Those tested were far more likely to be living above the poverty line threshold as defined by the United States Department of Labor. Among those who underwent a test, 4.6% (4.6-4.6) were uninsured. In contrast, among those who did not undergo a test, 17.8% (17.8-17.9) were uninsured. Factors associated with colonoscopy (Table 2). Respondents who underwent colonoscopy reported more frequent doctor visits in the year prior to the survey. They also reported receiving most of their medical care in a doctor's office setting compared to those who did not [78.1 (78.1-78.2) *vs* 65.1 (65.0-65.1)]. Those who underwent a colonoscopy had a higher prevalence of medical problems such as diabetes and were more likely to participate in health screening tests such as mammograms for females and prostate specific antigen (PSA) tests for males. The prevalence of having a family history of colorectal cancer (CRC) in a first degree relative, usually a parent, was two-fold higher in respondents who underwent colonoscopy [10.7 (10.7-10.7) *vs* 4.8 (4.8-4.8)]. Participants who underwent a colonoscopy were far more likely to receive a recommendation for this test from their physician. A substantial majority were performed for "routine screening". Characteristics of Hispanic/Latino CHIS Participants (Table 3).

The largest number of Hispanic/Latino CHIS participants were of Mexican origin. The prevalence of testing was higher for naturalized citizens compared to those born in the United States. Non-citizens were least likely to undergo testing. Those who did not undergo testing lived within the United States longer than those who were tested.

The ability to speak English and the language spoken at home was associated with testing status. The prevalence of testing in those who spoke "very good" or "good" English compared to their less proficient counterparts was [37.2% (37.0-37.3) *vs* 26.2% (26.1-26.4)]. Those tested were more likely to speak English in their home. In addition, the language of TV/radio/newspaper was also associated with testing. Among Hispanic/Latino respondents who underwent colon testing, rates were higher for those who listened and watched media in English. Testing rates were lowest for those who listened and watched only in Spanish. Respondents were considerably more likely to have undergone a colon test if their doctor conducted the interview in English [41.0% (40.8-41.1) *vs* 21.5% (21.4-21.6)]. Lastly, participants who did not have a test were twice as likely to have "Had hard time understanding their doctor at the last visit" [7.3 (7.2-7.4) *vs* 3.5 (3.5-3.6)]. Multivariable analysis (Table 4).

The output from the regression model demonstrated that Blacks had an odds ratio 30% higher than Whites for undergoing a colon cancer test. Male gender, family history of colon cancer, and progressive levels above the poverty line were also factors independently associated with testing. Having health insurance and a physicians' recommendation were the strongest predictors of testing. For the Hispanic/Latino group, additional independent factors associated with testing included citizenship sta-

**Table 3** Language and demographic characteristics of Latino/Hispanic California Health Inventory Survey Participants ages 50-80 years related to colon cancer screening status (bootstrap 95%CI)

	Total Weighted <i>n</i> = 1 487 447	Colonoscopy/Flexible sigmoidoscopy/ Fecal occult blood test Weighted <i>n</i> = 801 832	Colonoscopy Weighted <i>n</i> = 492 646	No Colonoscopy/Flexible sigmoidoscopy/Fecal occult blood test Weighted <i>n</i> = 685 615
Latino/Hispanic sub-type, %				
Mexican	80.6 (80.5-80.6)	79.8 (79.7-79.9)	79.2 (79.1-79.4)	81.4 (81.3-81.5)
Salvadoran	8.2 (8.1-8.2)	6.4 (6.4-6.5)	8.0 (7.9-8.1)	10.3 (10.3-10.4)
Guatemalan	2.4 (2.3-2.4)	2.0 (2.0-2.0)	1.6 (1.6-1.7)	2.7 (2.7-2.8)
Central American	2.8 (2.7-2.8)	3.5 (3.4-3.5)	3.0 (2.9-3.0)	1.9 (1.9-1.9)
South American	2.1 (2.0-2.1)	2.7 (2.7-2.8)	2.3 (2.3-2.3)	1.4 (1.3-1.4)
Other Latino	3.9 (3.8-3.9)	5.6 (5.5-5.6)	5.9 (5.9-6.0)	2.3 (2.3-2.4)
Citizen status, %				
United states born citizen	31.2 (31.1-31.2)	37.1 (37.0-37.2)	39.3 (39.1-39.4)	24.2 (24.1-24.3)
Naturalized citizen	43.5 (43.4-43.6)	47.4 (47.3-47.6)	50.2 (50.1-50.3)	38.9 (38.8-39.0)
Non-citizen	25.3 (25.2-25.4)	15.4 (15.4-15.5)	10.5 (10.4-10.6)	36.9 (36.8-37.0)
Year lived in United States, %				
≤ 1	0.2 (0.2-0.2)	0.0 (0.0-0.1)	0.0 (0.0-0.2)	0.5 (0.4-0.5)
2-4	1.4 (1.3-1.4)	1.1 (1.1-1.1)	0.8 (0.8-0.8)	1.6 (1.6-1.7)
5-9	1.0 (1.0-1.1)	0.5 (0.5-0.5)	0.3 (0.2-0.3)	1.6 (1.6-1.7)
10-14	3.9 (3.8-3.9)	3.1 (3.1-3.2)	1.3 (1.2-1.3)	4.8 (4.7-4.8)
≥ 15	62.4 (62.3-62.4)	58.1 (58.1-58.3)	58.4 (58.3-58.6)	67.3 (67.2-67.5)
Not ascertained	31.2 (31.2-31.3)	37.2 (37.2-37.3)	39.2 (39.1-39.4)	24.2 (24.1-24.5)
Ability to speak English, %				
Very good	13.0 (12.9-13.1)	15.5 (15.4-15.5)	17.7 (16.9-17.1)	10.1 (10.1-10.2)
Good	19.1 (19.0-19.1)	21.7 (21.6-21.8)	26.9 (26.7-27.0)	16.1 (16.0-16.2)
Not good	33.4 (33.3-33.5)	29.6 (29.5-29.8)	23.1 (23.0-23.3)	37.8 (37.7-37.9)
Not at all	18.8 (18.7-18.8)	14.6 (14.5-14.7)	15.3 (15.2-15.5)	23.6 (23.5-23.7)
Language of TV/radio/newspaper, %				
English	17.2 (17.1-17.3)	19.9 (19.8-20.0)	24.3 (24.2-24.5)	14.0 (14.0-14.2)
English and Spanish	33.0 (32.9-33.1)	32.9 (32.7-33.0)	31.1 (30.9-31.2)	33.2 (33.1-33.3)
Only Spanish	34.1 (34.0-34.2)	28.7 (28.6-28.8)	27.6 (27.5-27.8)	40.4 (40.3-40.6)
Not ascertained	15.7 (15.7-15.7)	18.6 (18.5-18.7)	17.0 (16.9-17.1)	12.3 (12.2-12.4)
Language spoken at home, %				
English	15.7 (15.7-15.8)	18.6 (18.5-18.7)	17.0 (16.9-17.1)	12.3 (12.2-12.4)
English and Spanish	46.6 (46.5-46.7)	48.4 (48.3-48.5)	54.0 (53.9-54.3)	44.5 (44.4-44.7)
Only Spanish	37.0 (36.9-37.1)	32.2 (32.2-32.3)	28.1 (27.9-28.2)	42.6 (42.5-42.8)
Other	0.7 (0.7-0.7)	0.8 (0.8-0.9)	0.9 (0.8-0.9)	0.6 (0.6-0.7)
Doctor conducted last interview in, %				
English	32.0 (31.9-32.1)	41.0 (40.8-41.1)	46.4 (46.2-46.5)	21.5 (21.4-21.6)
Spanish	39.7 (39.6-39.8)	34.5 (34.4-34.6)	29.6 (29.4-29.7)	45.7 (45.6-45.9)
Not ascertained	28.3 (28.2-28.4)	24.5 (24.4-24.6)	24.0 (23.9-24.3)	32.8 (32.7-32.9)
Hard time understanding your doctor, %				
Yes	5.3 (5.2-5.3)	3.5 (3.5-3.6)	4.0 (3.9-4.1)	7.3 (7.2-7.4)
No	87.0 (87.0-87.1)	94.1 (94.0-94.2)	92.4 (92.3-92.5)	78.8 (78.7-78.9)
Not ascertained	7.7 (7.7-7.8)	2.4 (2.2-2.6)	3.6 (3.5-3.7)	13.9 (13.8-14.0)

tus and ability to understand their physician during their most recent office visit.

Colon Cancer Testing Trend Data (Figure 1). Figure 1 displays the proportion of CHIS participants age 50-80 who underwent colon cancer testing (colonoscopy, flexible sigmoidoscopy or FOBT) within the previous 5 years stratified by race/ethnicity. The proportion tested increased in all groups between 2001 and 2007 and then appeared to plateau. Overall, the prevalence of testing was nearly identical among Whites and Blacks. The proportion of Hispanic/Latinos who underwent a test rose from approximately 35% in 2001 to 50% in 2009.

## DISCUSSION

According to the 2009 CHIS survey, approximately

63.0% of participants aged 50-80 had a colon cancer test such as FOBT, flexible sigmoidoscopy or colonoscopy within the previous 5 years. Of these, approximately 70% underwent colonoscopy as the colon cancer test. Regression analysis demonstrated that those who underwent at least one colon cancer test were more likely to be male, Black, have a family history of CRC, and have health insurance. Progressive levels above the poverty line were also associated with receiving a test. The strongest variable associated with undergoing a test was physician recommendation. For the Hispanic/Latino group, those at increased risk for no testing were those born in the United States and those who "Had hard time understanding their doctor at the last visit".

Trend analysis demonstrated that the proportion of Blacks in California undergoing a colon cancer test is in-



**Table 4** Multivariable logistic regression (dependent variable is colon cancer test in previous 5 years)

Variable	n	All participants adjusted OR <sup>1</sup> (95%CI)	White adjusted OR <sup>1</sup> (95%CI)	Black adjusted OR <sup>1</sup> (95%CI)	Hispanic adjusted OR <sup>1</sup> (95%CI)
Gender					
Female	5 658 375	1.00	1.00	1.00	1.00
Male	4 937 833	1.06 (1.06-1.06)	1.03 (1.03-1.04)	1.07 (1.06-1.08)	1.25 (1.24-1.26)
High school educated					
No	1 663 605	1.00	1.00	1.00	1.00
Yes	8 932 603	1.01 (1.00-1.01)	0.96 (0.95-0.99)	1.03 (0.98-1.05)	1.28 (1.27-1.29)
Family history of colon cancer					
No	9 809 630	1.00	1.00	1.00	1.00
Yes	786 578	1.71 (1.70-1.72)	1.88 (1.87-1.89)	1.54 (1.53-1.56)	1.29 (1.26-1.33)
Income relative to poverty level					
0%-99%	1 144 390	1.00	1.00	1.00	1.00
100%-199%	1 811 952	1.21 (1.20-1.21)	1.33 (1.30-1.36)	1.14 (1.12-1.15)	0.88 (0.87-0.89)
200%-299%	1 451 680	1.41 (1.40-1.42)	1.44 (1.40-1.48)	1.31 (1.27-1.40)	1.50 (1.49-1.52)
> 300%	6 188 185	1.69 (1.68-1.70)	1.77 (1.72-1.86)	1.66 (1.64-1.69)	1.48 (1.46-1.50)
Health insured					
No	1 006 640	1.00	1.00	1.00	1.00
Yes	9 589 568	2.71 (2.70-2.72)	2.88 (2.87-2.89)	2.14 (2.11-2.23)	3.52 (3.48-3.56)
Physician Recommended colon test					
No	5 095 110	1.00	1.00	1.00	1.00
Yes	5 501 098	3.90 (3.88-3.91)	3.77 (3.74-3.80)	3.99 (3.96-4.02)	4.37 (4.32-4.42)
Citizenship status <sup>1</sup>					
United States born citizen	464 083				1.00
Naturalized citizen	647 039				1.91 (1.89-1.93)
Years lived in United States <sup>1</sup>					
≤ 1	29 749				1.00
2-4	20 824				0.98 (0.96-1.02)
5-9	14 874				1.38 (1.33-1.44)
10-14	58 010				0.78 (0.75-0.82)
≥ 15	928 167				1.57 (1.54-1.62)
Hard time understanding physician <sup>1</sup>					
Yes	78 835				1.00
No	1 294 079				2.44 (2.40-2.48)

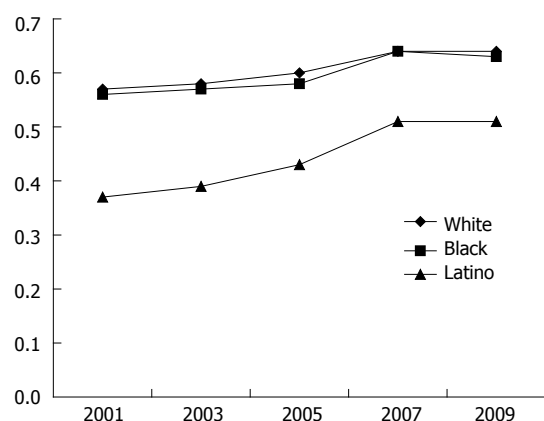
<sup>1</sup>Numbers for Hispanic/Latino only. OR: Odds ratio.

creasing in parallel with other racial/ethnic groups. This is encouraging as prior studies had identified white, non-Hispanic race as a predictor of colon cancer testing<sup>[29]</sup>. The increased participation by Blacks may be due to the more widespread use of colonoscopy as a colon cancer test. One survey revealed that this is the preferred colon cancer test by this group<sup>[13]</sup>. We were surprised to find no association between colon cancer testing and educational level. Prior studies suggested educational attainment was a positive predictor of colon cancer screening<sup>[27,30]</sup>. Similar to prior large population-based studies, our study also identified males, a family history of colon cancer, having health insurance, progressive levels above the poverty line, and having undergone screening with PSA or mammography as predictors of adherence to colon cancer testing<sup>[21]</sup>. Most importantly, our study confirms physician recommendation as the strongest variable associated with testing.

Several population-based studies looking at predictors of colorectal cancer screening participation have been performed<sup>[30]</sup>. BRFSS is a nationwide population-based telephone survey designed to measure preventive health practices, such as adherence to colon cancer testing and risk factors for adherence or non-adherence. A study of 52 754 respondents over age 50 in 1997 demonstrated

low participation in colon cancer screening protocols with 19.8% for FOBT, 30.5% for sigmoidoscopy and 41.1% for either<sup>[21]</sup>. Respondents who underwent other preventive testing such as mammography or PAP smear demonstrated a higher adherence to screening<sup>[21]</sup>. Similarly, responses of 64 084 BRFSS participants over the age of 50 in 1999 demonstrated that colon cancer screening was reported by 43.4%, with 22.8% undergoing colonoscopy or sigmoidoscopy and 9.9% undergoing FOBT<sup>[30]</sup>. Negative predictors of screening included < college education and lack of health care coverage<sup>[30]</sup>. An examination of the 2002 BRFSS demonstrated a disparity in colon cancer testing between Hispanic and non-Hispanic Whites. Only, 41.9% of Hispanic respondents aged > 50 underwent colon cancer testing in the past year by either FOBT or colonoscopy/sigmoidoscopy compared to 55.2% of non-Hispanic whites<sup>[19]</sup>.

The NHIS is an in-person survey conducted by the Centers for Disease Control and Prevention. The 2000 NHIS survey revealed that less than half of the United States population > 50 years underwent colon cancer screening at the recommended time intervals<sup>[23]</sup>. The strongest predictor of adherence was a physician visit within the previous year. A usual source of care, having undergone recent health preventive services such as a



**Figure 1** Proportion of individuals aged 50-80 undergoing a colon cancer test in the previous 5 years stratified by race/ethnicity.

mammogram or PAP smear, and physician recommendation were also important predictors<sup>[23]</sup>.

An analysis of health behaviors of Medicare enrollees living in the United States was conducted using the SEER database<sup>[3]</sup>. A random sample of Medicare claims from 14 states for the period 1995 to 2003 were reviewed and demonstrated increased use of colonoscopy for colon cancer testing as well as a decline in the use of FOBT and sigmoidoscopy. The transition to use of colonoscopy was more rapid in Whites than non-Whites<sup>[3]</sup>. A similar study of Medicare claims in SEER regions from 1996-2005 showed a persistence of racial and ethnic disparities in the use of colonoscopy for screening as Whites were more likely to undergo this test as the physician preferred modality than Blacks or Hispanics<sup>[29]</sup>. Data collected in 2003 from 11 SEER regions demonstrated racial/ethnic disparities in colon cancer screening that varied significantly by geography<sup>[5]</sup>. A telephone survey of Medicare consumers living in three urban counties in North Carolina and 17 urban and rural counties in South Carolina was performed in 2001<sup>[17]</sup>. Overall, 56.8% of Whites adhered to Medicare-covered intervals for colon cancer testing compared to only 39.1% of Blacks<sup>[17]</sup>. Blacks preferred to undergo a colonoscopy rather than FOBT.

A major strength of our study is the sampling methodology incorporated into CHIS. Once weighted, the sample is representative of the entire population of the State of California including recent immigrants. Bootstrap methodology with resampling allows for accurate variance estimates. The repeated nature of the survey allows for the identification of trends in the use of health care services for the population. The incorporation of health behaviors, income, education, insurance coverage and other integral variables allows for adjusted estimates of healthcare utilization.

There are important weaknesses of our results. First, the indication for colon cancer testing is not available for every biennial survey. Other than 2009 (Table 2), we were not able to determine whether testing was performed for screening or was indicated for a sign/symptom of colonic disease. Therefore we were careful to use the terms

“testing” and “screening” separately in our analysis and discussion. The dependent variable in this manuscript is clearly colon cancer testing. Erroneous conclusions about the presented trend data may be drawn if this is not kept in mind. For example, Blacks may be undergoing more colon tests over time because they are more willing to see their doctor for blood in their stools.

Another important weakness is that medical records are unavailable to confirm testing. Without records, we are left with the possibility of recall bias by the participants which could lead to misclassification. Examples include inability to remember that a doctor recommended a colon cancer test, or even that the participant had a test. Another shortcoming without records is that we are unable to separate whether patients underwent a sigmoidoscopy or FOBT as these results are reported in aggregate. Finally, without personal knowledge of the physician-patient interaction, we cannot further explain the variable “Had hard time understanding their doctor at the last visit”. According to CHIS documentation for 2009, only 1.67% of those responding yes to this question answered that this was due to language differences. Other possibilities may include visual, auditory, or intellectual disabilities.

In conclusion, approximately 63% of California citizens between the ages of 50 and 80 have some form of colon cancer testing. Males, Blacks, insured, those with a family history of colorectal cancer, and those living above the poverty line were more likely to undergo a test. The strongest variable associated with testing was physician recommendation. For Hispanic/Latinos, those born in the United States and those with difficulty understanding their doctor are at increased risk for no testing. Our data is encouraging in that the level of participation in colon cancer testing appears to be increasing over the past decade for all studied racial/ethnic groups.

## COMMENTS

### Background

Colorectal cancer is the fourth leading cause of new cancer cases diagnosed in the United States and is the second leading cause of cancer death. Population-based surveys such as the Behavior Risk Factor Surveillance System (BRFSS) and the National Health Interview Survey (NHIS) suggest that colon cancer screening tests are underutilized in the United States. Colon cancer can be prevented in most cases by strict adherence to accepted colon cancer screening guidelines. Despite a lack of evidence from randomized controlled trials, colonoscopy has been shown to be a more sensitive test for the detection of adenomas and colon cancer than flexible sigmoidoscopy +/- fecal occult blood test (FOBT). The California Health Inventory Survey (CHIS) is the nation's largest state health survey. CHIS data gives a detailed picture of the health care needs of California's large and diverse population. The specific aim of this study was to explore the CHIS database to identify demographic, socioeconomic, health and behavioral factors associated with participation in colon cancer testing. In addition, another aim was to perform a detailed analysis of the Latino population.

### Research frontiers

Colorectal cancer is one of the leading causes of cancer diagnosed in the United States. Prior population-based studies reported positive and negative predictors of adherence to colon cancer testing with mixed results. This large population-based study seeks to clarify these predictors and to provide a detailed analysis of the Latino population.

### Innovations and breakthroughs

A major strength of our study is the sampling methodology incorporated into

CHIS. Once weighted, the sample is representative of the entire population of the State of California including recent immigrants. Bootstrap methodology with resampling allows for accurate variance estimates. The repeated nature of the survey allows for the identification of trends in the use of health care services for the population. The incorporation of health behaviors, income, education, insurance coverage and other integral variables allows for adjusted estimates of healthcare utilization.

### Applications

The study indicates approximately 63% of California citizens between the ages of 50 and 80 have some form of colon cancer testing. Males, Blacks, insured, those with a family history of colorectal cancer, and those living above the poverty line were more likely to undergo a test. The strongest variable associated with testing was physician recommendation. For Hispanic/Latinos, those born in the United States and those with difficulty understanding their doctor are at increased risk for no testing. Author's data is encouraging in that the level of participation in colon cancer testing appears to be increasing over the past decade for all studied racial/ethnic groups. Most importantly, the data can be used to remove barriers to colon cancer testing amongst the general population and minorities such as the Latino population.

### Terminology

CHIS is the nation's largest state health survey. A random-dial telephone survey is conducted every two years on a wide range of health topics. CHIS data gives a detailed picture of the health care needs of California's large and diverse population. Colon cancer testing includes annual FOBT, flexible sigmoidoscopy +/- FOBT, and/or colonoscopy.

### Peer review

This large population-based study provides insight into predictors of undergoing colon cancer testing. This data can be used to reduce the prevalence of colorectal cancer in the United States by removing barriers to colon cancer testing in the general population which includes minorities.

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## Magnetic resonance imaging: Is there a role in clinical management for acute ischemic colitis?

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### Abstract

**AIM:** To validate the utility of magnetic resonance imaging (MRI) for the clinical management of acute ischemic colitis (IC).

**METHODS:** This is a magnetic resonance (MR) prospective evaluation of 7 patients who were proved to have acute IC on the basis of clinical, endoscopic and computed tomography (CT) findings and who were imaged in our institution between February 2011 and

July 2012. The mean age of the patients was 72.28 years. Abdominal CTs were obtained using a 64-detector row configuration for all patients with un-enhanced and contrast-enhanced scans, in the late arterial phase (start delay 45-50 s) and in the portal venous phase (start delay 70-80 s). The MR examinations were performed using a 1.5T superconducting magnet, using Fast Imaging Employing Steady State Acquisition and T2-weighted fast-recovery fast-spin echo sequences in axial and coronal plane. CT and MRI examinations were analysed for the presence of colonic abnormalities and associated findings.

**RESULTS:** Segmental involvement was seen in 6 patients (85.71%), with a mean length of involvement of 412 mm (range 145.5-1000 mm). Wall thickness varied between 6 mm and 17.5 mm (mean 10.52 mm) upon CT examinations and from 5 to 15 mm (mean 8.8 mm) upon MR examinations. The MRI appearance of the colonic wall varied over the time: Type I appearance with a 3 layer sandwich sign was seen in 5 out of 12 examinations (41.66%), patients underwent MR within a mean of 36 h (ranging from 1 to 54 h) after the CT examination. Type II and III appearance with a 2 layer sign, was seen in 4 examinations (33.33%), patients underwent MR within a mean of 420.5 h (ranging from 121 to 720 h) after the CT examination. In the remaining three MRI examinations, performed within a mean of 410 h (ranging from 99.5 to 720 h) the colonic wall appeared normal.

**CONCLUSION:** MRI, only using precontrast images, may be used as a substitute for invasive procedures in diagnosis and follow-up of acute IC.

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**Key words:** Ischemic colitis; Magnetic resonance imaging; Medical management; Colon; Computed tomography

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## INTRODUCTION

Ischemic colitis (IC) is a relatively common disease<sup>[1]</sup> and it is considered the most frequent form of intestinal ischemia and the second most frequent cause of lower gastrointestinal bleeding<sup>[2]</sup>. It represents the consequence of an acute or, more commonly, chronic decrease or blockage in the colonic blood supply, which may be either occlusive or non-occlusive in origin<sup>[3]</sup>. The original insult causing the ischemic event can rarely be established, but frequently occurs in elderly patients with diffuse disease in small segmental vessels and various co-morbidities. Today, with the introduction of new therapies, pharmacological causes could also be considered<sup>[4,5]</sup>. The anatomic damage results in ischemic necrosis of variable severity that can range from superficial mucosal involvement to full-thickness transmural necrosis<sup>[6]</sup>. The treatment depends on the acuteness and severity of the presentation<sup>[3]</sup>. Most cases of IC are transient and resolve spontaneously and such patients do not require specific therapy, instead very mild cases can be managed on an outpatient basis with a liquid diet, close observation and antibiotics<sup>[7]</sup>. IC rarely presents itself in a gangrenous form (acute fulminant IC).

The incidence of IC is underestimated because it often has a mild transient nature, clinical presentation can be nonspecific and highly variable, therefore, the diagnosis largely depends on clinical suspicion. In this context the role of imaging techniques remains controversial<sup>[8]</sup>. Standard radiology yields non-specific and late findings, while computed tomography (CT), the main technique for the noninvasive diagnosis of mesenteric ischemia, is well suited to confirm the clinical suspicion of IC, to suggest IC when it is unsuspected and to diagnose complications, however it requires the use of radiation and an iodinated contrast agent, limiting the possibility to use this technique in a short term follow-up<sup>[9-14]</sup>. Recently Iacobellis *et al.*<sup>[15]</sup> has proposed magnetic resonance imaging (MRI) as a substitute for invasive procedures in diagnosing and grading acute IC, allowing for the early identification of pathological findings and by defining the evolution of ischemic lesions with 7T magnetic resonance imaging (7T-MRI) on an animal model with acute IC. The purpose of this study was to validate the utility of MRI in the clinical management of acute IC. In particular to show our experience in daily practice, focusing the attention both on the diagnosis and follow-up of this pathological condition.

## MATERIALS AND METHODS

### Patients population

All human procedures were approved by our Institutional Care Committee.

This is a magnetic resonance (MR) prospective evaluation of 11 patients who were proved to have acute intestinal ischemia on the basis of clinical, endoscopic and CT findings and who were imaged in our institution between February 2011 and July 2012. Among the initial 11 patients, 7 were included in the study because they were identified as having IC. Two patients with extensive small intestinal infarction because of the occlusion of superior mesenteric artery and 2 patients with the occlusion of superior mesenteric vein were not included.

Of the 7 patients, 2 were men and 5 women, with a mean age of 72.28 years (range, 54-97 years). Clinical charts, CT and MR examinations were reviewed. Imaging evaluation was requested because of the following clinical findings: sudden-onset of abdominal pain in all the patients ( $n = 7$ ) (100%), bloody diarrhoea or bright red rectal blood in 4 patients (57.14%), nausea and vomiting in 1 patient (14.29%), elevated white blood cells (WBC) in 5 patients (71.42%), elevated Lactate dehydrogenase (LDH) levels in 6 patients (85.71%).

Five (71.42%) of these patients had arteriosclerotic cardiovascular disease on the basis of the findings from a clinical evaluation or cardiac work-up. Their mean age was 77 years. One patient (14.29%, 54 years old), during treatment of Lenalidomide for a relapsed multiple myeloma and one patient (14.29%, 80 years old) had hypotensive episodes associated with a recent exacerbation of chronic pancreatitis.

The diagnosis of IC was suspected on the basis of the clinical presentation and CT findings and was confirmed in all the patients: in 5 patients, an endoscopic procedure alone (sigmoidoscopy or colonoscopy) was performed, alternatively an endoscopic procedure and biopsy was performed on 1 patient; and 1 patient, underwent endoscopy and surgery with histological specimen evaluation.

### Multidetector-row computed tomography imaging protocol

Abdominal CTs were obtained using a 64-detector row configuration for all patients (Discovery CT 750HD, General Electric Healthcare, Milwaukee, WI, United States). In all patients the examination was performed with a spiral technique in a cranio-caudal direction (from the base of the lungs to the pelvic brim) and supine position. All patients underwent un-enhanced and contrast-enhanced CT, in the late arterial phase (start delay 45-50 s) and in the portal venous phase (start delay 70-80 s) with an intravenous injection of 2 mL/kg of non-ionic contrast material (Iopamiro 370; Bracco Diagnostics, Milan, Italy), followed by 40 mL of saline solution using a peristaltic semiautomated power injector (4 mL/s flow

rate, SIAS 757, Bologna, Italy) with an 18-gauge needle in the antecubital vein. Oral medium contrast was not given to any of the patients; rectal air or rectal contrast material was not administered. The following technical parameters were used: effective slice thickness of 3.75 mm for plain acquisition, 1.25 mm in the late arterial phase and 2.5 mm in the portal venous phase; beam pitch of 0.938, reconstruction interval of 0.8 mm, tube voltage of 120-140 KVp and reference mAs of 250/700 mA. Automatic tube current modulation was used to minimise radiation exposure. Standard reconstruction algorithm was used. Patients were instructed not to breath during helical imaging to avoid motion artefacts.

### MRI techniques

The MR examination was performed using a 1.5T superconducting magnet (SIGNA HD 1.5, General Electric Healthcare, Milwaukee, WI, United States), using the following sequences: Fast Imaging Employing Steady State Acquisition (FIESTA, TR/TE, 3.8/1.6; matrix size, 192 × 320; section thickness, 6 mm; intersection gap, 1 mm; field of view, 480 mm × 480 mm; NEX, 1; breath-hold) on coronal plane and T2-weighted fast-recovery fast-spin echo sequence (FRFSE: TR/TE, 6000/70, matrix size, 320 × 256; section thickness, 4mm; intersection gap, 0.4 mm; field of view, 480 mm × 480 mm; NEX, 4; respiratory triggering) in axial and coronal plane. An eight channel body phase-array surface coil was employed. Oral and intravenous medium contrast were not given to any of the patients.

### Image analysis and comparison

CT scans and MR images were evaluated by two Radiologists (Mazzei MA and Mazzei FG) experienced in gastrointestinal imaging, reaching a consensus agreement. The following were assessed: the location and length of the colonic segment involved; the appearance and degree of wall thickening; the presence of a double-halo or target configuration (two or three concentric rings); pericolic streakiness, peritoneal fluid or blood, presence of intramural, mesenteric, or portal venous gas, and free intraperitoneal air or other relevant abdominal findings were also recorded.

The bowel wall was considered thickened if it measured more than 3 mm in diameter. Right side colonic involvement was defined as abnormalities affecting a segment or the entire ascending colon including the hepatic flexure. Left-side involvement was defined as abnormalities starting at or distal to the splenic flexure. Finally, the gross appearance of the affected colonic wall at CT was divided into three morphologic groups according to Balthazar *et al*<sup>[14]</sup>: (1) Type I CT (acute IC), wall thickening with heterogeneous enhancement and zones of low attenuation compatible with severe colonic edema; there was enhancement of the mucosa consistent with an acute process, a shaggy contour, a loss of colonic haustra, with varying degrees of pericolic streakiness; (2) Type II CT (subacute IC), the CT appearance showed concentric and

symmetric mild mural thickening and homogeneous attenuation of the wall of the colon with a sharply defined contour and with or without minimal pericolic streakiness; and (3) Type III CT (gangrenous IC): there was circumferential intramural air consistent with pneumatosis coli.

A similar classification was realised by the analysis of MR images and a correlation between the gross appearance of the affected colonic wall on both CT and MRI and the time from the onset.

## RESULTS

Patient demographic information (age and sex), pre-existing disease, number of CT and MR examinations during hospitalisation, diagnostic proof (S = surgery, B = biopsy and E = endoscopy) and status at the time of discharge (D = died; L = living) for the 7 patient are summarised in Table 1. The diagnostic CT examinations were performed within a mean of 45.5 h (range, 2-168 h) after the clinical onset of symptoms, while the first MR examinations were performed in 4 patients within 48 h (mean of 14.8 h; range, 1-40 h) after the date of the CT examination, in 5 patients between 48 h and 15 d (mean of 137.5 h, range 54-240 h), and in 3 patients after 15 d from the CT examinations (mean of 524 h, range 384-720 h). Three patients underwent only one MRI examination, 3 patients were studied twice and 1 patient was studied three times, for a total of 12 MRI examinations. The duration of the MRI follow-up of the 7 patients considered varied from 1 to 30 d from the date of the CT examination.

Among the 7 patients, 6 (85.71%) exhibited a segmental involvement of the colon and 1 patient (14.29%) had the entire colon involved. The length of involvement in the patients with a segmental distribution, obtained using 2D reformat reconstructions on CT images, ranged from 145.5 to 1000 mm, with a mean length of 412 mm. The thickness of the wall of the colon in the affected segments varied from 6 to 17.5 mm, with a mean bowel wall thickness of 10.52 mm on CT images, and from 5 to 15 mm, with a mean bowel wall thickness of 8.8 mm, on MR images. Two out of 6 patients (33.33%) with a segmental involvement of the colon exhibited a left-sided and splenic flexure colitis, 1 (16.66%) a left-sided colitis only, 1 (16.66%) a left-sided colitis and IC of the transverse colon and splenic flexure, 1 (16.66%) an IC of the transverse colon only, and 1 (16.66%) an IC of the sigmoid colon. In the 1 patient with ischemic pancolitis, the diagnosis was confirmed at colonoscopy and total colectomy.

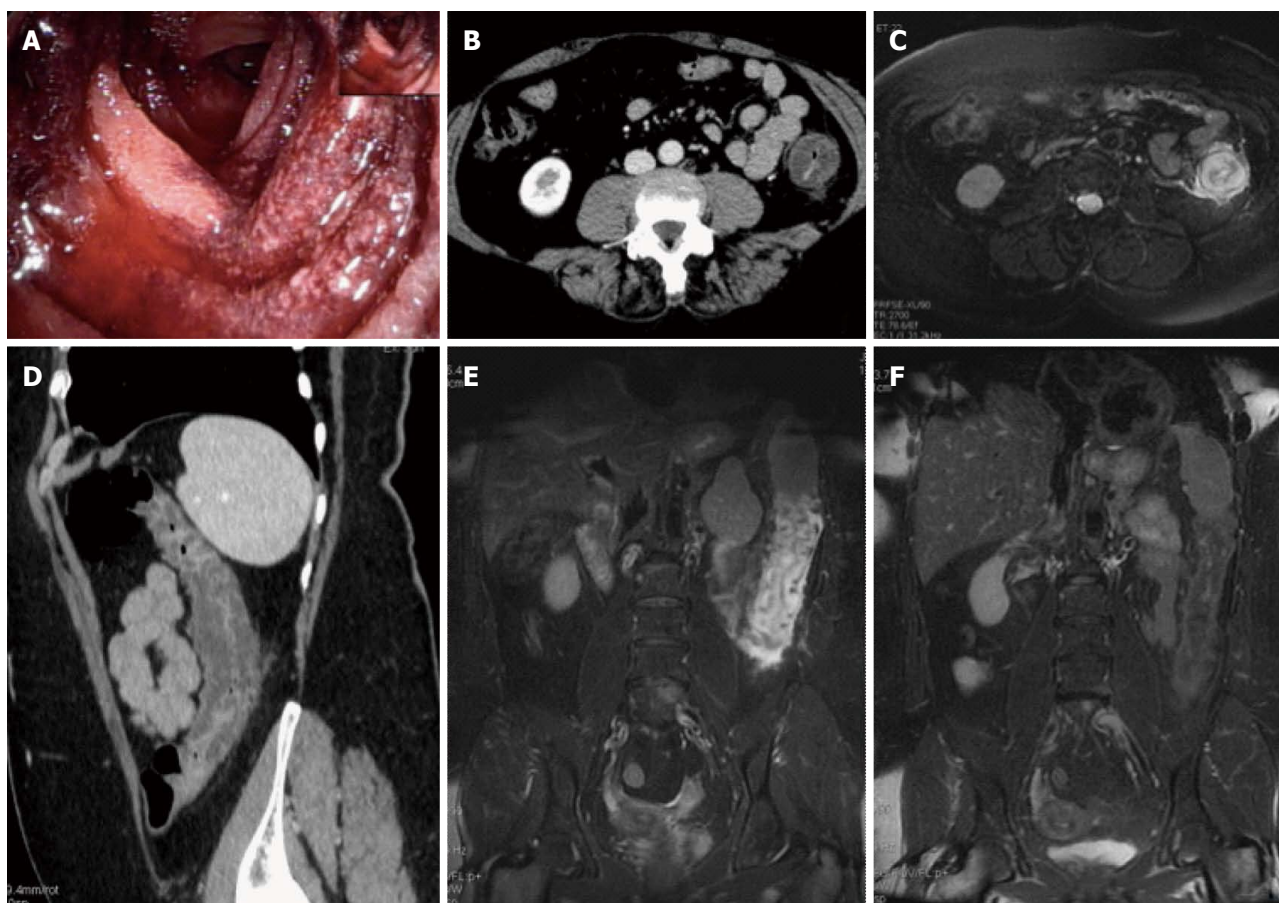
The gross appearance of the affected colonic wall at CT, divided into three morphologic groups, according to Balthazar *et al*<sup>[14]</sup>, showed that Type I CT was present in 5 out of 7 patients (71.4%) and Type II CT in 2 out of 7 patients (28.6%). The diagnostic CT examinations in 5 patients with Type I CT appearance was performed within a mean of 36 h (ranging from 2 and 48 h) after the clinical onset of symptoms, showing an acute form of



**Table 1** Patients demographic characteristic

Patient	Sex	Age (yr)	Pre-existing disease	Number of CT/MR	Diagnostic proof	Status of discharge
1	F	54	Multiple myeloma relapsed treated with Lenalinomide	1/2	E	L
2	F	89	Ischemic heart disease, hypertension, rheumatoid arthritis	1/1	E	L
3	F	80	Hypertension, thrombotic disease	1/2	E + B	L
4	F	97	Diverticulitis, pancreatitis, hypertension in treatment	1/1	E	L
5	M	67	Crohn's disease	1/1	B	D
6	F	57	Hypertension in treatment	1/2	E	L
7	M	62	Heart failure	1/3	E	L

CT: Computed tomography; MR: Magnetic resonance; F: Female; M: Male; B: Biopsy; E: Endoscopy; D: Died; L: Living.



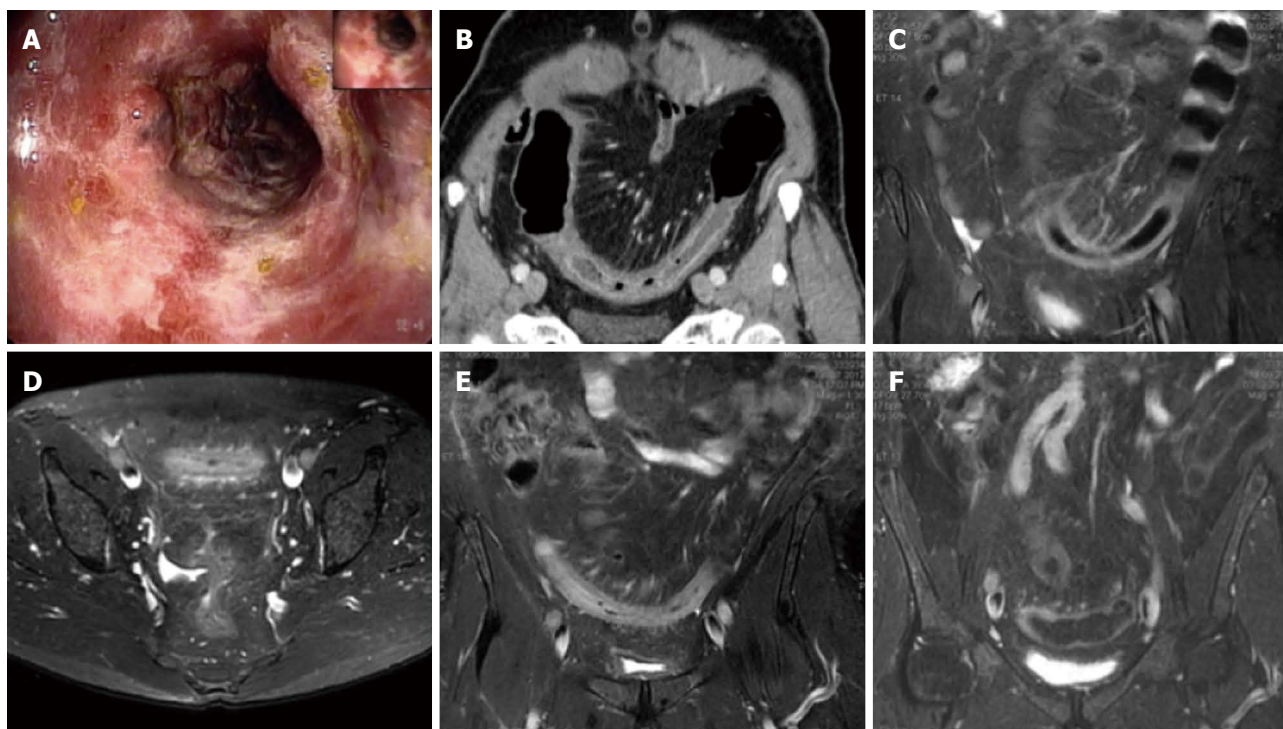
**Figure 1** Magnetic resonance imaging follow-up of a patients with ischemic colitis resolved promptly. Ischemic colitis (IC) of left side colon in a 57 year old woman with a recent history of acute hypertensive crisis, who presented with left lower quadrant pain and massive rectal bleeding. A: Endoscopic procedure showed multiple necrotic area; B and C: Contrast-enhanced computed tomography (CT) and axial T2 fast-recovery fast-spin echo sequence (FRFSE) magnetic resonance imaging (MRI), after 32 h from CT, showed acute IC (Type I CT and MRI) with wall thickening, three layer sandwich sign and a mild amount of free fluid in the paracolic gutter; D and E: 2D coronal reformat CT and coronal T2 FRFSE MRI, at the same time, showed the entire involved tract; F: Ischemia resolved without complications with conservative therapy as shown in the follow-up MRI.

IC.

As the morphologic CT groups, the gross appearance of the affected colonic wall upon MRI could be divided into 3 groups: (1) Type I MRI, in the first group, wall thickening with a 3 layer sandwich sign showing high signal intensity of the intermediate layer and the low signal intensity of the inner and outer layers, were present in 5 MR examinations (4 patients, 1 patient had 2 MR examinations with an interval of 48 h due to worsening of clinical symptoms). The MR examinations with

Type I appearance were performed within a mean of 36 h (ranging from 1 to 54 h) after the CT examination and the same patients with Type I MRI showed the Type I appearance at CT, according to an acute form of IC (Figures 1 and 2); (2) Type II MRI, in the second group, which consisted of 2 patients, there was a wall thickening with a 2 layer sign (high signal intensity of the inner layer and low signal intensity of the outer layer). The MR examinations with Type II appearance were performed within a mean of 420.5 h (ranging from





**Figure 2** Magnetic resonance imaging follow-up of a patient with ischemic colitis and worsening of clinical symptoms. Ischemic colitis (IC) of sigmoid colon in a 62-year-old man with left lower quadrant pain and elevate lactate dehydrogenase levels, who presented with melena and a recent history of stenting procedures for ischemic cardiopathy. A: Endoscopic procedure showed multiple necrotic area; B: 2D (two dimensional) coronal reformat contrast-enhanced computed tomography (CT) showed acute IC (Type I CT); C-E: The patient had 2 magnetic resonance examinations (C and D-E) with an interval of 48 h due to worsening of clinical symptoms, with an increase of the length and thickness of the involved tract (D-E); F: The ischemic process resolved without complication after parenteral nutrition, as showed in the follow-up magnetic resonance imaging, performed after 384 h from the date of CT examination.

121 to 720 h) after the CT examination, according to a subacute form of IC; and (3) Type III MRI, in the third group, which consisted of 2 patients, the MR appearance showed a wall thickening with a inverted 2 layer sign (high signal intensity of the outer layer and low signal intensity of the inner layer). The MR examinations with Type III appearance were performed within a mean of 420.5 h (ranging from 384 to 457 h) after the CT examination, according to a subacute form of IC.

In the remaining 3 MRI examinations, performed within a mean of 410 h (ranging from 99.5 to 720 h) the colonic wall appeared normal.

No patients had circumferential intramural air consistent with pneumatosis coli.

Mild to moderate amounts of free abdomen fluid were present in all patients (100%) on the CT examination, and in 4 out of 4 of the first MR examinations (performed within 48 h from the date of CT examination) and in 1 out of 5 in the second MRI examinations (performed between 48 h and 15 d from the date of CT examination).

The free fluid was mainly located in the paracolic gutters and in the Douglas. No high-attenuation fluid consistent with blood was present. No reduction in caliber of mesenteric vessels was found.

On the basis of the result of the initial CT examination, endoscopic findings and clinical evaluation, no surgery was performed on any of the patients. Surgery was

performed 3 wk after the initial episode because of the development of a sealed-off perforation of the transverse colon in one of the patients.

The site of the affected tract and its length (mm), gross appearance on CT (type according Balthazar *et al.*<sup>[14]</sup>, thickness of the affected wall and presence or absence of peritoneal fluid) and on MRI (type, thickness of the affected colonic wall and presence or absence of peritoneal fluid), in relation to the date of each examination were reported in Table 2.

## DISCUSSION

The diagnosis of IC largely depends on clinical suspicion, especially since many other conditions (*e.g.*, infectious colitis, inflammatory bowel disease, diverticulitis, colon cancer) are presented with abdominal pain, diarrhoea and hematochezia<sup>[3]</sup>. Endoscopy has become the diagnostic test of choice in establishing the diagnosis of IC, although, it can be limited because it could be performed without bowel preparation to prevent hypoperfusion caused by dehydrating cathartics; in addition a minimal air insufflation should be used to prevent perforation<sup>[7,8]</sup>.

From a radiological point of view, a CT examination is actually considered the main technique for the noninvasive diagnosis of mesenteric ischemia and also in cases of acute abdomen from different and various origins, because it can suggest IC when it is unsuspected, can diag-

**Table 2** Gross appearance on computed tomography and magnetic resonance imaging

Patient	Involved tract/length (mm)	CT I range 2-168 h <sup>1</sup>	MRI I within 48 h <sup>2</sup>	MRI II > 48 h < 15 d <sup>2</sup>	MRI III > 15 d <sup>2</sup>
1	Left side colon and splenic flexure/314	Type I /12 mm; free fluid	Type I /10 mm; free fluid		Type III /6 mm; no free fluid
2	Left side colon, splenic flexure and transverse colon/609	Type I /12 mm; free fluid		Type II /7 mm; no free fluid	
3	Left side colon and splenic flexure/380	Type II /9.5 mm; free fluid		Type II /7 mm; no free fluid	normal appearance; no free fluid
4	Transverse colon/219	Type II /6 mm; free fluid		normal appearance; no free fluid	
5	Entire colon/1000	Type I /8.5 mm; free fluid	Type I /9.5 mm; free fluid		
6	Left side colon/218	Type I /17.5 mm; free fluid	Type I /15 mm; free fluid	Normal appearance; no free fluid	
7	Sigmoid colon/145.5	Type I /8.2 mm; free fluid	Type I /7 mm; free fluid	Type I /8 mm; free fluid	Type III /5 mm; no free fluid

<sup>1</sup> After the clinical onset; <sup>2</sup> After the date of the CT examination. MRI: Magnetic resonance imaging; CT: Computed tomography.

nose complications and exclude other illnesses. However it requires the use of ionizing radiation and an iodinated contrast agent, limiting the possibility to use this technique in a short term follow-up<sup>[9-14]</sup>.

Recently Iacobellis *et al.*<sup>[15]</sup> has reported that MRI can play a relevant role in the diagnostic management of acute IC and may be substituted for other invasive endoscopic procedures in the diagnosis and grading of IC when an ischemic injury is suggested. Prior publications have described the feasibility of using MRI to evaluate a full range of colonic disease processes, including only one case of IC but without pathological confirmation and using precontrast and postcontrast imaging<sup>[16]</sup>. Our study is based on the assumption that a parallel between the experimental colonic ischemic damage in the animal model and humans is reasonable<sup>[15]</sup>. Then our aim has been to validate the utility of MRI in the clinical diagnosis and follow-up of IC, using combined Fast Imaging Employing Steady State Acquisition on a coronal plane and T2-weighted fast-recovery fast-spin echo sequences, both on an axial and coronal plane. About 71.4% of patients that underwent a CT examination within 48 h showed Type I of gross appearance of an involved colonic wall (wall thickening with heterogeneous enhancement and zones of low attenuation compatible with severe colonic edema and enhancement of the mucosa consistent with an acute process, a shaggy contour, a loss of colonic haustra, with varying degrees of pericolic streakiness). The same patients with Type I CT showed Type I appearance at MRI, according to an acute form of IC. The reason why the gross appearance of the involved colonic wall had a thickened and edematous appearance is related to the fact that usually IC is a form of non-occlusive ischemic disease and in most cases, however, there is no evidence of obstruction of a major artery or vein. Then a decrease in blood flow to 20% of the normal flow, associated with small-vessel disease (hypoxia), and reperfusion injury when the blood flow is reestablished are the responsible factors<sup>[17,18]</sup>. Consequently, any part of the colon can be involved, with no

correlation established between the length and site of the involvement and distribution of the superior mesenteric or inferior mesenteric artery or vein<sup>[17,19,20]</sup>. Segments commonly affected by IC are the splenic flexure (Griffith point) because the marginal artery of Drummond (a system of arcades connecting the major arteries) is occasionally tenuous here and is absent in up to 5% of patients, and the anastomotic plexus between the inferior mesenteric artery distribution and the hypogastric vascular supply (point of Sudeck) at the rectosigmoid junction, because it is distal to the last collateral connection with proximal arteries<sup>[7,21]</sup>. In our case population a segmental distribution was apparent in 85.71% (6 patients).

The striking differences in the gross morphology of ischemic segment as detected at MRI is probably related to the timing of the examination and to the pathophysiology of the developing anoxic process. In the initial phases of anoxia, mucosal damage occurs first; with more severe and prolonged forms of anoxia, submucosa hemorrhage, edema, and pericolic congestive and edematous changes developing later due to the reperfusion event. Indeed, in the subacute phase (MRI examination performed between 3 and 30 d) the gross morphology has been changed (MRI Type II or III) with a reduction of the thickness of the involved wall (mean 7.2 mm, range 5-8 mm) and a double ring appearance (high signal intensity of the inner layer and low signal intensity of the outer layer for II type MRI and high signal intensity of the outer layer and low signal intensity of the inner layer for Type III MRI), probably for the reduction of edematous phenomena like the CT Type II according to Balthazar.

Although up to 85% of cases of IC managed conservatively improve within 1 or 2 d and resolve completely within 1 or 2 wk, close to one-fifth of patients develop peritonitis or deteriorate clinically and require surgery. Surgical resection is required when an irreversible ischemic injury and chronic colitis develop as both can lead to bacteremia and sepsis, colonic stricture, persistent abdominal pain and bloody diarrhoea, and protein-losing

enteropathy<sup>[8]</sup>. The advantage of the use of MRI for clinical management of IC, is the possibility to perform a short term follow-up without the employment of ionizing radiation or intravenous contrast material. As demonstrated in our study MRI could be used, instead of CT to suggest the diagnosis of the IC in the proper clinical setting, particular when a segmental distribution is evident, in the depiction of other abnormal conditions that may be seen in patients suspected of having IC, and in a short term follow-up, when a clinical worsening occurs, to adequately manage the patient.

Some limitations of the present study should be outlined. Firstly, our patient selection process does not allow for an evaluation of the sensitivity or specificity of MRI for the diagnosis or detection of IC. The other major limitation of both CT and MR imaging in the diagnosis of colonic ischemia is the lack of specificity. The gross morphologic features overlap with those of inflammatory colitis, although the segmental distribution is more often seen in ischemia<sup>[22]</sup>. In spite of the limited number of subjects who were examined, we are convinced that MRI can provide a valid imaging for the identification of pathological findings of acute IC. Moreover MRI in combination with clinical suspicion, endoscopic and histological findings, can play a key role in the diagnosis and management of IC. In particular MRI can discriminate patients with urgent operative intervention from patients in which a follow-up can be proposed as an alternative to surgery. All these allow for an earlier detection and effective follow-up of IC with a possible earlier adequate treatment.

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## COMMENTS

### Background

The incidence of ischemic colitis (IC) is underestimated because it often has a mild transient nature, clinical presentation can be nonspecific and highly variable, therefore, the diagnosis largely depends on clinical suspicion. In this context the role of imaging techniques remains controversial. Standard radiology yields non-specific and late findings, while computed tomography (CT), the main technique for the noninvasive diagnosis of mesenteric ischemia, is well suited to confirm the clinical suspicion of IC, to suggest IC when it is unsuspected and to diagnose complications, however it requires the use of radiation and an iodinated contrast agent, limiting the possibility to use this technique in a short term follow-up.

### Research frontiers

The role of magnetic resonance imaging (MRI) in the diagnostic management of acute IC is still controversial, and nothing is known about the *in vivo* magnetic resonance findings of IC or about the relationship between MR findings and the onset of clinical symptoms.

### Innovations and breakthroughs

To be known, this is the first study using MRI for the evaluation of IC and for the comparison between MRI and CT findings in this pathological condition.

### Applications

MRI in combination with clinical suspicion, endoscopic and histological findings,

can play a key role in the diagnosis and management of IC. In particular MRI can discriminate patients with urgent operative intervention from patients in which a follow-up can be proposed as an alternative to surgery. In particular the advantage of the use of MRI for clinical management of IC, is the possibility to perform a short term follow-up without the employment of ionizing radiation or intravenous contrast material.

### Terminology

Fast imaging employing steady state acquisition sequence (FIESTA): provides images of fluid filled structures with very short acquisition times. The FIESTA sequence uses the T2 steady state contrast mechanism to provide high signal noise ratio images with strong signal from fluid tissues while suppressing background tissue for contrast and anatomic detail of small structures; IC: inflammation of the colon due to colonic ischemia resulting from alterations in systemic circulation or local vasculature.

### Peer review

This paper is well written, is original and has good information. It merits to be admitted to publish without substantial changes.

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## Ensure preparation and capsule endoscopy: A two-center prospective study

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### Abstract

**AIM:** To compare small bowel (SB) cleanliness and capsule endoscopy (CE) image quality following Ensure®, polyethylene glycol (PEG) and standard preparations.

**METHODS:** A preparation protocol for CE that is both efficacious and acceptable to patients remains elusive. Considering the physiological function of the SB as a site for the digestion and absorption of food and not as a stool reservoir, preparation consisting of a liquid, fiber-free formula ingested one day before a CE study might have an advantage over other kinds of preparations. We conducted a prospective, blind-to-preparation, two-center study that compared four types of preparations. The participants' demographic and clinical data were collected. Gastric and SB transit times were calculated. The presence of bile in the duodenum was scored by a single, blinded-to-preparation gastroenterologist expert in CE, as was cleanliness within the proximal, middle and distal part of the SB. A four-point scale was used (grade 1 = no bile or residue, grade 4  $\geq$  90% of lumen full of bile or residual material).

**RESULTS:** The 198 consecutive patients who were referred to CE studies due to routine medical reasons were divided into four groups. They all observed a 12-h overnight fast before undergoing CE. Throughout the 24 h preceding the fast, control group 1 ( $n = 45$  patients) ate light unrestricted meals, control group 2 ( $n = 81$ ) also ate light meals but free of fruits and vegetables, the PEG group ( $n = 50$ ) ate unrestricted light meals and ingested the PEG preparation, and the Ensure group ( $n = 22$ ) ingested only the Ensure formula. Preparation with Ensure improved the visualization of duodenal mucosa (a score of 1.76) by decreasing the bile content compared to preparation with PEG (a score of 2.9) ( $P = 0.053$ ). Overall, as expected, there was less residue and stool in the proximal part of the SB than in the middle and distal parts in all groups. The total score of cleanliness throughout the length of the SB showed some benefit for Ensure (a score of 1.8) over control group 2 (a score of 2) ( $P = 0.06$ ). The cleanliness grading of the proximal and distal parts of the SB was similar in all four groups ( $P = 0.6$  for both). The cleanliness in the middle part of the SB in the PEG (a score of 1.8) and Ensure groups (a score of 1.7) was equally better than that of control group 2 (a score of 2.1) ( $P = 0.057$  and  $P = 0.07$ , respectively). All 50 PEG patients had diarrhea as an anticipated side effect, compared with only one patient in the Ensure group.

**CONCLUSION:** Preparation with Ensure, a liquid, fiber-free formula has advantages over standard and PEG preparations, with significantly fewer side effects than PEG.

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**Key words:** Capsule endoscopy; Preparation; Polyethylene glycol; Ensure; Fiber-free diet; Image quality

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## INTRODUCTION

Capsule endoscopy (CE) has become a commonly applied technology for examining the small bowel (SB) mucosa<sup>[1-8]</sup>. A review of the literature revealed that there is no universally accepted protocol for bowel preparation before a CE study<sup>[9-23]</sup>. A proper preparation is one that decreases the presence of debris, food remnants, bile and stool content in the intestinal lumen and significantly increases diagnostic accuracy. Preparation by a 12-h fast and light meals one day before the procedure, as suggested by the manufacturer of the capsule, is inadequate. Investigators have prepared the bowels of their patients with different doses of purgatives [polyethylene glycol (PEG) or sodium phosphate solution] or prokinetics (erythromycin, metoclopramide, domperidone and tegaserod) and/or anti-bubble agents (simethicone)<sup>[9-23]</sup>. The main disadvantage of purgatives is that they are not well tolerated by patients. Moreover, the efficacy of both purgatives and prokinetics is controversial.

The physiologic function of the small intestine is very different from that of the colon. While the colon functions as a stool reservoir and is full of stool even during a fast, the small intestine is free of stool or any content after a 24- to 48-h fast. Since instructing patients to observe such prolonged fasting before undergoing CE is unreasonable, ingestion of a liquid, fiber-free formula instead of food during the 24 h preceding a CE examination would appear to be a more practical option for ensuring sufficient cleanliness of the small intestine as well as good patient compliance. This idea has not been investigated before.

The aim of this two-center prospective study was to compare the SB cleanliness and the quality of CE images of patients who were prepared by ingestion of Ensure (a fiber-free liquid formula) with those of patients who were assigned the PEG and two control groups.

## MATERIALS AND METHODS

This prospective, blind-to-preparation investigation was conducted in two Israeli medical centers, the Tel-Aviv Sourasky Medical Center (TASMC) and the Hillel-Yaffe Medical Center (HYMC). The protocol of the study was approved by the local Helsinki committees of both centers. Consecutive patients who were referred to CE studies were asked to sign informed consent forms to participate in the study. The usual medical indications for CE referral were: (1) suspected Crohn's disease (CD); (2) gastrointestinal (GI) bleeding of unknown origin (normal gastroscopy and colonoscopy); (3) iron deficiency anemia of unknown origin (normal gastroscopy and colonoscopy, negative celiac serology); (4) suspected malabsorption

disease; (5) unexplained abdominal pain; (6) unexplained chronic diarrhea; and (7) abnormal SB findings on abdominal computerized tomogram (CT).

The exclusion criteria were: age < 18 and > 70 year, pregnancy, previous surgery involving the GI tract, previous intestinal obstruction, and refusal to participate in the study. Because of its sucrose content, diabetes mellitus was an exclusion criterion for the Ensure group only. The relevant demographic and medical data were collected, including: age, gender, the indication for a CE study, chronic diseases, medications, previous surgeries, and previous GI endoscopic examinations.

### Study group assignment

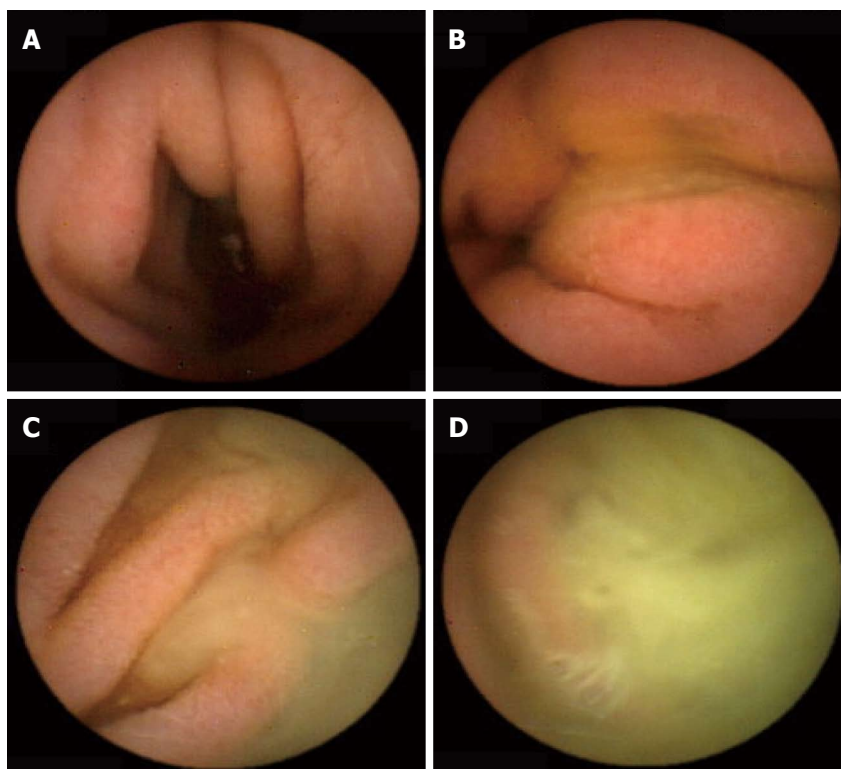
The patients were divided into four groups and they all observed a 12-h overnight fast before undergoing CE, whatever preparation protocol they followed.

The patients who were evaluated at HYMC were randomly divided into two groups. Those assigned to the PEG group were instructed to eat light meals and to ingest two liters of PEG one day before undergoing CE. Those assigned to the Ensure group were instructed to drink up to four cans of the Ensure Plus<sup>®</sup> formula ad libitum throughout 24 h, and to stop all other food/beverage intake except for water. Ensure Plus<sup>®</sup> (Abbott Laboratories) is a popular, fiber-free, polymeric-balanced formula with a caloric content of 355 kcal/can, 13 g protein, 50.1 g carbohydrates, 11.4 g fat, minerals, trace elements and vitamins.

The patients who underwent the CE study due to medical indications in TASMC during the same period of time comprised the two control groups: the control group 1 patients were instructed to eat light meals during the day before the CE, and the control group 2 patients followed a low-fiber diet (without fruits, vegetables and fiber supplements) for 2 d before CE and ate light meals one day before undergoing CE.

The CE procedure was standard in both centers. All patients ingested a PillCam<sup>™</sup> SB 2 wireless video capsule (Given<sup>®</sup> Diagnostic Imaging System, Yokneam, Israel) with a small amount of water. An array of 8 sensors was attached to the abdominal wall, and a recorder with a battery was fastened around their waists with a belt. The patients were allowed to drink liquids two hours after ingesting the capsule and to eat a light meal four hours later. Eight hours after capsule ingestion, the recorder was disconnected and the sensors were removed. The recorded digital information was downloaded onto the computer and the images were analyzed using RAPID<sup>®</sup> software. The CE films were examined by gastroenterologists with CE expertise in both medical centers. Afterwards, a single independent expert who was unaware of the provenance of the films reviewed all films for the presence of bile and degree of cleanliness.

The following data were collected and analyzed from the CE studies: (1) Indications for the CE study grouped into 3 categories (suspected CD, obscure GI bleeding, others); (2) Gastric transit time as calculated from the



**Figure 1** Grading of the presence of bile in the duodenum. A: Grade 1; B: Grade 2; C: Grade 3; D: Grade 4.

first view of the gastric mucosa to the first image of the duodenum, expressed in minutes; (3) SB transit time as calculated from the first view of the duodenum up to the first cecal image, expressed in minutes; (4) Final diagnosis grouped into six categories (established CD, angiodysplasia, polyps, nonspecific findings, normal examination, others); (5) Cecum arrival: a CE study was defined as having been completed if the capsule reached the cecum. An abdominal X ray was performed to exclude capsule retention in cases of non-arrival; (6) The bile presence score: the presence of bile in the duodenum lumen was evaluated using a scale of 1 to 4, with 1 representing none and 4 indicating more than 90% of the lumen being full of bile (Figure 1); (7) The cleanliness score: SB cleanliness was evaluated by a 4-point scale, with 1 indicating no residual material in the lumen and 4 indicating more than 90% of the lumen having residual material (Figure 2). The SB section of the CE study was divided into three parts: proximal, middle and distal. Each part received a separate cleanliness score; (8) Extra-SB findings, *i.e.*, the number of recorded findings in the esophagus, stomach and cecum; and (9) Follow-up, including the number of Ensure cans that were consumed, possible side effects of Ensure and PEG, level of satisfaction from preparation in PEG and Ensure groups, patient compliance with the preparation's protocol.

### Statistical analysis

Descriptive statistics are given as mean and standard deviation for continuous variables and frequency distribution for categorical variables. Comparisons between patients

with different protocols of preparation with regard to demographics (age, gender), clinical parameters (indications for CE study) and CE data (final diagnosis, gastric and SB transit times, cecal arrival rate, duodenal bile score and cleanliness score) were performed using the Chi-square, Fisher's Exact and unpaired *t*-tests. The non-parametric Mann-Whitney test was also applied if the continuous parameters did not follow a normal distribution. Pairwise comparisons were performed when the results of the overall tests were significant. The False Discovery Rate method for adjustment of significance level was used. Comparison of continuous variables between groups was performed by one-way analysis of variance (ANOVA) and the non-parametric ANOVA Kruskal-Wallis test.

Ryan-Einot-Gabriel-Welsch pair-wise comparisons between groups were employed when the ANOVA indicated significant results. Additionally, post-hoc tests using Mann-Whitney tests with Bonferroni adjustment were performed when Kruskal-Wallis test revealed significant relations between groups and other clinical parameters. The relationships between transit time and other continuous parameters were examined by the Spearman's correlation coefficients. All statistical analyses were performed using SAS for Windows 9.2. The statistical tests were defined as having a confidence interval of 95%.  $P < 0.05$  was considered statistically significant.

## RESULTS

A total of 139 patients were screened to participate in the PEG and Ensure groups. Sixty-seven of them were



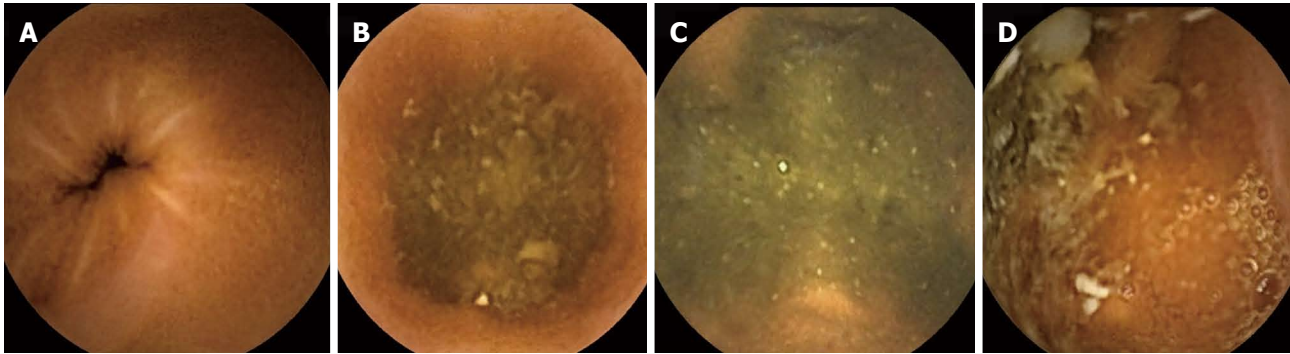


Figure 2 Grading of small bowel cleanliness. A: Grade 1; B: Grade 2; C: Grade 3; D: Grade 4.

Table 1 Demographics of the entire study population *n* (%)

	Group 1 Overnight fast	Group 2 Low fiber diet and overnight fast	Group 3 PEG and overnight fast	Group 4 Ensure and overnight fast	<i>P</i> value
Number of patients	45	81	50	22	
Male/female	21/24	42/39	27/23	8/14	
Average age (yr)	55.3	53	48.5	41.1	0.0138 (groups 1 and 2 <i>vs</i> 4)
Indication for CE					
Suspected CD	11 (24.4)	20 (24.7)	18 (36)	13 (59)	0.0151 (groups 1 and 2 <i>vs</i> 4)
Obscure GI bleeding	29 (64.4)	50 (61.7)	24 (48)	6 (27.3)	0.0124 (groups 1 and 2 <i>vs</i> 4)
Others	5 (11.2)	11 (13.6)	8 (16)	3 (13.7)	0.9265
Number of incomplete cases <sup>1</sup>	3 (6.7)	5 (6.2)	7 (14)	3 (13.6)	0.14
		+1 gastroparesis		+2 gastroparesis	

<sup>1</sup>Incomplete cases = non-arrival to the cecum. CE: Capsule endoscopy; CD: Crohn's disease; PEG: Polyethylene glycol; GI: Gastrointestinal.

excluded from the study due to the following reasons: About 13 patients had diabetes mellitus, 18 patients were younger than 18 years, 11 patients had previous intestinal surgeries, 8 patients had an urgent CE study, and 17 patients refused to participate in the study. The remaining 72 patients were divided into either the PEG (*n* = 50) or Ensure (*n* = 22) groups. Control group 1 consisted of 45 patients and control group 2 consisted of 81 patients.

Table 1 presents the demographic data of these four groups of patients. The PEG and Ensure groups were younger than both control groups. About 24%-25% of all patients in the control groups were referred to the CE study in order to rule out CD, about 60%-65% to identify a reason for obscure GI bleeding and about 11%-14% for other indications. The percentage of cases with suspected CD was higher in the PEG and Ensure groups than in the control groups. The reason for these age and referral differences between the study and control groups was related to the refusal of more of the older patients to drink PEG or Ensure. The rate of cases with non-arrival of the capsule to the cecum was not significantly different between the groups (*P* = 0.14). Capsule retention was excluded in all cases of incomplete studies by abdominal X-ray. One control patient and two Ensure patients had an extremely prolonged gastric transit time (> 4 h) without any anatomic reason on gastroscopy. These three patients were considered as having gastroparesis and were not included in the evaluation of the rate of non-arrival to the cecum. These patients with a gastroparesis were

25, 33 and 56 years old with no previous intestinal surgery and no diabetes mellitus. They were referred to CE study to exclude CD and celiac disease and to identify a reason for iron deficiency anemia.

In order to provide a reliable analysis of image quality of CE studies, all cases of incomplete studies (non-arrival to cecum and gastroparesis) and those with incomplete data were excluded from the analyses in Table 2. All four resultant groups were very similar to the original groups with respect to age and indications for CE study. Overall, 107 of those 157 cases had a normal study or non-specific findings (68%). The rate of positive findings by CE was 32%. Twelve of 53 patients with suspected CD were diagnosed as having definite CD, with a yield of 23% (7.6% of all CE studies). There was an 18% rate of various extra-SB findings. Both gastric transit time and SB transit time for the four groups showed no statistical difference (*P* = 0.29 and *P* = 0.7, respectively), ranging between 29.1-40.1 min and 220.4-228 min for gastric and SB transit times, respectively.

The presence of duodenal bile was similar in both control groups (Table 3). Much more bile interfered with the visualization of duodenal mucosa in the PEG group (a score of 2.9) than in the Ensure group (a score of 1.76), and the difference almost reached a level of significance (*P* = 0.053). Overall, as expected, there was less residue and stool in the proximal part of the SB than in the middle and distal parts in all groups. The cleanliness grading of the proximal and distal parts of the SB was similar in



**Table 2** Parameters of study population with complete data *n* (%) (mean  $\pm$  SD)

	Group 1 Overnight fast	Group 2 Low fiber diet and overnight fast	Group 3 PEG and overnight fast	Group 4 Ensure and overnight fast	<i>P</i> value
Number of patients	36	61	43	17	
Male/female	15/21	34/27	24/19	5/12	
Average age (yr)	55.9	53	46.9	41.9	
Indication for CE					
Suspected CD	8 (22.2)	17 (27.8)	17 (39.5)	10 (58.9)	
Obscure GI bleeding	23 (63.9)	37 (60.7)	18 (41.9)	4 (23.5)	
Others	5 (13.9)	7 (22.5)	8 (18.6)	3 (17.6)	
Final SB diagnosis					
Established CD	2 (5.56)	6 (9.8)	2 (4.6)	2 (11.8)	
Angiectasia	4 (11.1)	11 (18)	4 (2.3)	1 (5.9)	
SB Polyp	1 (2.8)	0	0	2 (11.8)	
Celiac disease	0	3 (4.9)	0	0	
Nonspecific findings	2 (5.56)	7 (11.5)	5 (11.6)	3 (17.6)	
Normal study	25 (69.4)	29 (47.5)	29 (67.4)	7 (42.2)	
Others	2 (5.56)	5 (8.2)	3 (7)	2 (11.8)	
Extra-SB findings	5 (13.9)	16 (26.2)	6 (14)	4 (23.5)	
Mean gastric transit time (min)	29.1 $\pm$ 35.5	34.9 $\pm$ 31.3	35.8 $\pm$ 29.8	40.1 $\pm$ 41	General = 0.29; 0.075 (group 1 vs group 2); 0.08 (group 1 vs group 3)
Mean SB transit time(min)	228 $\pm$ 95.4	220.4 $\pm$ 81.4	239.6 $\pm$ 84	222.4 $\pm$ 100.4	General = 0.7

CE: Capsule endoscopy; SB: Small bowel; CD: Crohn's disease; GI: Gastrointestinal.

**Table 3** Scoring of bile presence and cleanliness of small bowel (mean  $\pm$  SD)

	Group 1 Overnight fast	Group 2 Low fiber diet and overnight fast	Group 3 PEG and overnight fast	Group 4 Ensure and overnight fast	<i>P</i> value
Number of patients	36	61	43	17	
Duodenal bile score	1.96 $\pm$ 0.7	1.9 $\pm$ 0.6	2.9 $\pm$ 0.6	1.76 $\pm$ 0.6	General = 0.15; 0.053 (group 3 vs group 4)
Cleanliness of proximal SB	1.8 $\pm$ 0.6	1.8 $\pm$ 0.4	1.7 $\pm$ 0.4	1.6 $\pm$ 0.34	General = 0.6
Cleanliness of middle SB	1.9 $\pm$ 0.7	2.1 $\pm$ 0.6	1.8 $\pm$ 0.6	1.7 $\pm$ 0.5	General = 0.16; 0.057 (group 2 vs group 3); 0.07 (group 2 vs group 4)
Cleanliness of distal SB	2.2 $\pm$ 0.7	2.2 $\pm$ 0.6	2.3 $\pm$ 0.8	2.1 $\pm$ 0.6	General = 0.6
Total score of clearness	1.97 $\pm$ 0.7	2 $\pm$ 0.6	1.9 $\pm$ 0.6	1.8 $\pm$ 0.5	General = 0.4; 0.06 (group 2 vs group 4)

SB: Small bowel; PEG: Polyethylene glycol.

all four groups ( $P = 0.6$  and  $P = 0.6$ , respectively). The scores of the middle part of the SB in the PEG and Ensure groups were lower than that of the control group 2 ( $P = 0.057$  and  $P = 0.07$ , respectively). The total cleanliness score for the entire length of the SB showed some difference between that same control group and the Ensure group ( $P = 0.06$ ), with a benefit for the latter.

Patient compliance among the PEG and Ensure groups was similar (1.25 and 1.2, respectively,  $P = 0.7$ ), as was subjective feeling during the preparation for CE ( $P = 0.163$ ). The patients succeeded in drinking an average of two cans of Ensure, ranging from 0.25 to 4 cans per day. All 50 PEG patients had diarrhea during the day they drank it, compared to only one of the 22 patients in the Ensure group.

## DISCUSSION

Our prospective, blind-to-preparation, two-center study

demonstrated that a preparation by a liquid, fiber-free formula (Ensure) before a CE study has several advantages over other types of preparation. First, it improved the visualization in the duodenum by slightly decreasing the amount of bile compared with the control groups and by decreasing the amount of bile to a level that almost reached statistical significance compared with the PEG group ( $P = 0.053$ ). Secondly, the total score of cleanliness throughout the length of the SB showed some benefit for the Ensure preparation over one of the control groups ( $P = 0.06$ ). Finally, the cleanliness in the middle part of the SB of the patients in both the PEG and Ensure groups was better than that of one of control groups ( $P = 0.057$  and  $P = 0.07$ , respectively). However, all patients in the PEG group had diarrhea as a side effect, compared to only one patient in the Ensure group.

Many studies have dealt with the issue of preparation before a CE study by means of different kinds of

prokinetics and purgatives<sup>[9-23]</sup>. In contrast, our current study approached this problem by considering the physiological function of the SB as a place for the digestion and absorption of food and not as a reservoir for stools. As such, we expected that preparation with a liquid, fiber-free formula for one day before the CE study (followed by a 12-h overnight fast) should ensure SB cleanliness by keeping the amount of residue in the SB to a minimum. The results of our study bore out these expectations by demonstrating a positive impact of Ensure preparation on the bile score in the duodenum compared with the score for the PEG group and a total cleanliness score compared with one of the control groups. The Ensure preparation proved itself as good as PEG in terms of cleanliness in the middle part of the SB, with much less reported diarrhea. The statistical differences that showed strong trends but that rarely decreased below 0.05 to indicate significance could probably be related to the relatively small number of patients in the Ensure preparation group.

Our choice of Ensure Plus to serve as a very low-residue diet was arbitrary. We could have used any other commercially available free-fiber liquid formula and even a milkshake or soy-milkshake.

The efficacy of the PEG preparation is controversial. Some studies showed its benefit over a 12-h fast<sup>[17,18,20]</sup>, while others reported no advantage<sup>[16,23]</sup>. It is obvious that preparation with 2 liters of PEG is as good as 4 liters<sup>[20]</sup>. More bile in the images of the PEG group led to poorer image quality in the duodenum than that of the Ensure group. The cleanliness in the middle part of the SB was similar to that of the Ensure group and better than that of the control groups, without there being any effect on the cleanliness of the proximal and distal parts. However, diarrhea as a common side effect was a significant disadvantage of PEG compared with Ensure and the control groups.

This study has a number of limitations. Although we succeeded in recruiting enough patients in the PEG and control groups for conducting capsule studies, the Ensure group was small. Therefore, some of our results could not reach a level of statistical significance. In addition, there were some basic differences between the groups: the PEG and ENSURE groups included younger populations with a higher percentage of CD patients. One important advantage of this study was the fact that all the scoring was done by a single blind-to-preparation expert in CE studies.

In summary, this prospective, two-center study raised, for the first time, the option of preparation for CE study with a liquid, fiber-free formula, and showed that it has some advantage over standard preparation or/and PEG, with significantly fewer side effects than PEG.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Capsule endoscopy (CE) has become a commonly used technology for examining the small bowel (SB) mucosa. A proper preparation decreases the amount of debris, food remnants, bile and stool present in the intestinal lumen and significantly increases diagnostic accuracy. However, there is no universally accepted protocol for SB preparation before a CE study. A standard preparation by 12-h fast and light meals one day before the procedure is inadequate. Preparations with purgatives are not well tolerated by patients, and their efficacy is controversial.

### Research frontiers

Considering the physiological function of the SB as a site for the digestion and absorption of food and not as a stool reservoir, a preparation consisting of a liquid, fiber-free formula (like Ensure®) ingested one day before a CE study might have an advantage over other kinds of preparations. The aim of this two-center prospective, blind-to-preparation study was to compare the SB cleanliness and the quality of CE images of patients who were prepared by ingestion of Ensure® with those of patients who were assigned to the polyethylene glycol (PEG) group and two control groups that received standard preparation.

### Innovations and breakthroughs

Preparation with Ensure® improved the visualization of duodenal mucosa by decreasing the bile content compared to preparation with PEG. The total score of cleanliness throughout the length of the SB showed some benefit for Ensure® over one of control groups. The cleanliness in the middle part of the SB in the PEG and Ensure® groups was equally better over one of the control groups. As expected, all 50 PEG patients had diarrhea as a side effect, compared with only one patient in the Ensure group. This study presents for the first time a novel idea that preparation for undergoing CE of the SB should be completely different from the preparation for endoscopic examination of a colon and should be based on fasting and a liquid, fiber-free formula and not on purgatives.

### Applications

This study results suggest that the preparation with Ensure®, a liquid, fiber-free formula has advantages over standard and PEG preparations, with significantly fewer side effects than PEG.

### Terminology

Capsule endoscopy: CE is a technology that uses a swallowed wireless video capsule for photographing the inside of the esophagus, stomach, and small intestine. The images are transmitted to a belt-held recorder. It is a well-accepted tool which provides a direct and noninvasive approach to view the entire small bowel mucosa. This technology has enriched the knowledge on small bowel pathology and revolutionized the overall management of SB diseases.

### Peer review

The manuscript is well-written and refers to an important issue in light of the increasing utility of the CE modality. The authors present a novel approach to a common "real life" issue and provide a new opportunity for an easy and convenient preparation.

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## Effect of alternative antibiotics in treatment of cefotaxime resistant spontaneous bacterial peritonitis

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### Abstract

**AIM:** To evaluate effective alternative antibiotics in treatment of cefotaxime-resistant spontaneous bacterial peritonitis.

**METHODS:** One hundred cirrhotic patients with spontaneous bacterial peritonitis [ascitic fluid polymorphonuclear cell count (PMNLs)  $\geq 250$  cells/mm<sup>3</sup> at admission] were empirically treated with cefotaxime sodium 2 g/12 h and volume expansion by intravenous human albumin. All patients were subjected to history taking, complete examination, laboratory tests (including a complete blood cell count, prothrombin time, biochemical tests of liver and kidney function, and fresh urine sediment), chest X-ray, a diagnostic abdominal paracentesis, and the sample subjected to total and differential cell count, chemical examination, aerobic and anaerobic cultures. Patients were divided after 2 d by a second ascitic PMNL count into group I;

patients sensitive to cefotaxime ( $n = 81$ ), group II ( $n = 19$ ); cases resistant to cefotaxime (less than 25% decrease in ascitic PMNL count). Patients of group II were randomly assigned into meropenem ( $n = 11$ ) or levofloxacin ( $n = 8$ ) subgroups. All patients performed an end of treatment ascitic PMNL count. Patients were considered improved when: PMNLs decreased to  $< 250$  cells/mm<sup>3</sup>, no growth in previously positive culture cases, and improved clinical manifestations with at least 5 d of antibiotic therapy.

**RESULTS:** Age, sex, and Child classes showed no significant difference between group I and group II. Fever and abdominal pain were the most frequent manifestations and were reported in 82.7% and 80.2% of patients in group I and in 94.7% and 84.2% of patients in group II, respectively. Patients in group II had a more severe ascitic inflammatory response than group I and this was demonstrated by more ascitic lactate dehydrogenase (LDH) [median: 540 IU/L (range: 150-1200 IU/L) *vs* median: 240 IU/L (range: 180-500 IU/L),  $P = 0.000$ ] and PMNL [median: 15 000 cell/mm<sup>3</sup> (range: 957-23 822 cell/mm<sup>3</sup>) *vs* 3400 cell/mm<sup>3</sup> (range: 695-26 400 cell/mm<sup>3</sup>),  $P = 0.000$ ] counts. Ascitic fluid culture was positive in 32% of cases. Cefotaxime failed in 19% of patients; of these patients, 11 (100%) responded to meropenem and 6 (75%) responded to levofloxacin. Two patients with failed levofloxacin therapy were treated according to the *in vitro* culture and sensitivity (one case was treated with vancomycin and one case was treated with ampicillin/sulbactam). In group II the meropenem subgroup had higher LDH (range: 108-860 IU/L *vs* 120-491 IU/L,  $P = 0.042$ ) and PMNL counts (range: 957-23 822 cell/mm<sup>3</sup> *vs* 957-15 222 cell/mm<sup>3</sup>,  $P = 0.000$ ) at initiation of the alternative antibiotic therapy; there was no significant difference in the studied parameters between patients responsive to meropenem and patients responsive to levofloxacin at the end of therapy (mean  $\pm$  SD: 316.01  $\pm$  104.03



PMNLs/mm<sup>3</sup> vs 265.63 ± 69.61 PMNLs/mm<sup>3</sup>,  $P = 0.307$ ). The isolated organisms found in group II were; *enterococci*, *acinetobacter*, expanded-spectrum  $\beta$ -lactamase producing *Escherichia coli*,  $\beta$ -lactamase producing *Enterobacter* and *Staphylococcus aureus*.

**CONCLUSION:** Empirical treatment with cefotaxime is effective in 81% of cases; meropenem is effective in cefotaxime-resistant cases.

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**Key words:** Spontaneous bacterial peritonitis; Cefotaxime; Ascitic polymorphonuclear count; Cirrhosis; Meropenem; Levofloxacin

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## INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is infection of ascitic fluid in cirrhotic patients. In 1985, results of a randomized trial demonstrated that cefotaxime, as a third-generation cephalosporin, achieved cure in SBP episodes for 85% of patients, compared to 56% of patients who received ampicillin plus tobramycin<sup>[1]</sup>. Since then, cefotaxime has become the empiric antibiotic of choice for the treatment of SBP. Mortality rates reduced from 90% when it was first described to around 20%-40%<sup>[2]</sup>. The widespread use of diagnostic paracentesis, as well as the prompt initiation of empiric antibiotic therapy, based on the results of ascitic polymorphonuclear (PMNL) cell count, has contributed in the improvement of survival in these patients<sup>[3]</sup>. Recent reports showed that resistance to third generation cephalosporins (including cefotaxime) is increasing<sup>[4,5]</sup> and this may increase the need to develop alternative antibiotics. The primary aim of this study was to evaluate effective alternative antibiotics in treatment of cefotaxime-resistant spontaneous bacterial peritonitis.

## MATERIALS AND METHODS

### Study design

This prospective study was conducted at the Tropical Medicine Department, Zagazig University Hospitals, Zagazig, Sharkia Governorate, Egypt during the period from October 2010 to October 2011. This study was approved by the Ethical committee of our institution. Written informed consents were obtained from all patients. For patients with any grade of hepatic encephalopathy, the written informed consents were obtained from the first degree relatives.

### Patient management

All patients were inpatients and underwent diagnostic abdominal paracentesis with a differential leucocyte count, microbiological culture, and biochemical analysis at admission. Cefotaxime sodium 2 g/12 h (*iv*) was used as the initial empirical therapy because it is supported by the current guidelines as the first line of therapy for SBP<sup>[2]</sup>. It was subsequently maintained or replaced depending on the clinical course, a second abdominal paracentesis with PMNL count, and the *in vitro* susceptibility of the isolated microorganisms, up to a planned period of 5-7 d in a hospitalized basis.

After 48 h, a second abdominal paracentesis was done; cases with < 25% reduction in PMNL count were considered resistant to cefotaxime and were then randomly assigned to receive either meropenem 1 g/8 h (*iv*) or levofloxacin 1 g/12 h (*iv* drip). They were randomized by matching the sample (resistant group) and were classified into matched pairs; one from each pair was randomly enrolled into either the meropenem subgroup or the levofloxacin subgroup. One patient who was initially assigned to the levofloxacin subgroup was moved to the meropenem subgroup due to drug availability at the time of therapy. The final number was 11 patients in the meropenem subgroup and 8 in the levofloxacin subgroup.

The choice of these antibiotics was not only a matter of availability but also mainly due to previous reports of activity against bacterial species causing SBP<sup>[6,7]</sup> both *in vitro*<sup>[8]</sup> and *in vivo*<sup>[9]</sup> at national<sup>[10]</sup> and international<sup>[11]</sup> levels.

Five days after initiation of alternative antibiotic therapy an end of treatment diagnostic abdominal paracentesis was performed; cases with < 25% reduction in PMNL count from the second puncture counts were treated according to the results of *in vitro* culture and sensitivity that were available. All cases with initially positive ascitic fluid culture were re-cultured. Intravenous expansion was performed with human albumin 20 g *iv* infusion at the time of diagnosis and after 48 h.

All patients underwent a final diagnostic paracentesis with a differential leucocyte count at the end of therapy. The antibiotic dosage was adjusted to renal function throughout the treatment period. Diuretics were routinely discontinued at the time of diagnosis of SBP and therapeutic paracentesis was not allowed during the study.

### Clinical and laboratory assessment

All patients were subjected to history taking, complete clinical examination, laboratory tests (including a complete blood cell count, prothrombin time, biochemical tests of liver and kidney function, and fresh urine sediment), chest x-ray film, a diagnostic abdominal paracentesis, and the sample was subjected to total and differential cell count, chemical examination, aerobic and anaerobic culture. These were performed in all the cirrhotic patients with ascites on the day of admission and

**Table 1** Demographic and clinical features of all patients *n* (%)

Variable	Group I ( <i>n</i> = 81)	Group II ( <i>n</i> = 19)	$\chi^2$	<i>P</i> value
Gender				
Male	48 (59.3)	11 (57.9)	0.01	0.91
Female	33 (40.7)	8 (42.1)		
Age (yr)	49.4 ± 7.74	51.5 ± 8.08	1.04	0.29
mean ± SD				
Abdominal pain				
Yes	65 (80.2)	16 (84.2)	0.01	0.9
No	16 (19.8)	3 (15.8)		
Fever				
Yes	67 (82.7)	18	0.93	0.33
No	14 (17.3)	1 (5.3)		
Child Class				
B	17 (21)	1 (5.3)	1.62	0.2
C	64 (79)	18 (94.7)		
Abdominal tenderness				
Yes	43 (53.1)	12 (63.2)	0.11	0.427
No	38 (46.9)	7 (36.8)		
Upper GI bleeding				
Yes	8 (9.9)	4 (21.1)	1.820	0.177
No	73 (90.1)	15 (78.9)		
Encephalopathy				
Yes	25 (30.9)	12 (63.2)	6.885	0.009
No	56 (69.1)	7 (36.8)		
Asymptomatic				
Yes	15 (18.5)	0 (0.00)	4.08	0.04
No	66 (81.5)	19 (100)		

GI: Gastrointestinal.

whenever they developed symptoms and signs suspicious for SBP (*i.e.*, fever, change in mental status, abdominal pain, development of renal failure, hypotension, *etc.*) during the hospitalization period.

The ascitic fluid samples were collected under aseptic conditions in tubes containing ethylenediamine tetraacetic acid anticoagulant and then tested to determine white blood cell and PMNL counts by automated cell blood counter; chemical analysis for glucose, lactate dehydrogenase (LDH) and total protein were also done. All the specimens were analyzed within one hour. Moreover, 10 mL of ascitic fluid was inoculated directly at the patient's bedside into aerobic and anaerobic blood culture bottles for bacteriological examination.

### Microbiological analysis

Ascitic fluid cultures were performed at the time of the initial paracentesis in the blood culture bottles (Remaux, France) at the bedside. All were incubated aerobically and anaerobically at 37 °C for 2 d before a subculture was done. Cases were considered negative after 7 d. All initially positive cases were re-cultured and cases were considered resolved from infection when PMNLs count had dropped to < 250/mm<sup>3</sup> and all culture cases were negative.

### Definitions

**Cirrhosis:** Cirrhosis diagnosed based on clinical, biochemical, histological and/or radiological findings and

Child class was assessed for each patient.

**SBP:** SBP was defined as the presence of  $\geq 250$  PMNLs/mm<sup>3</sup> in the ascitic fluid<sup>[2]</sup>.

**Resolution of SBP:** Resolution of SBP was defined as reduction of the elevated ascitic fluid PMNL count to < 250/mm<sup>3</sup>, negative culture in previously positive cases with resolution of the clinical manifestations and normalization of the impaired renal function.

**Resistance:** Resistance to empiric therapy was considered when < 25% reduction in PMNL count from the base line was achieved after 48 h<sup>[2]</sup>.

**Exclusion criteria:** (1) Non-cirrhotic ascites (including malignant ascites); (2) Cases with secondary bacterial peritonitis; and (3) Bacterascites (*i.e.*, positive ascitic fluid culture with < 250 neutrophils/mm<sup>3</sup>).

### Statistical analysis

Data were checked, entered and analyzed using SPSS Version 15. Data were expressed as mean ± SD for quantitative variables, number and percentage for qualitative ones.  $\chi^2$  or Fisher exact, *t* test and paired *t* test were used when appropriate. *P* < 0.05 was considered significant.

## RESULTS

### General characteristics and clinical presentation

Baseline characteristics of patients are presented in Table 1. Age and gender were comparable in group I and group II. In group I, the numbers in Child class B and C were 21% and 79% while in group II they were 5.3% and 94.7%, respectively. This means that most cefotaxime resistance cases were associated with advanced liver disease.

Fever and abdominal pain were the most frequent manifestations and were reported in 82.7% and 94.7% of patients in group I and in 80.2% and 84.2% of patients in group II, respectively. Asymptomatic patients were found in 18.5% of the cefotaxime sensitive group, but all patients of the resistant group presented with symptoms and signs. Regarding the laboratory parameters (Table 2), patients in group II had a greater peripheral blood leucocyte response than group I.

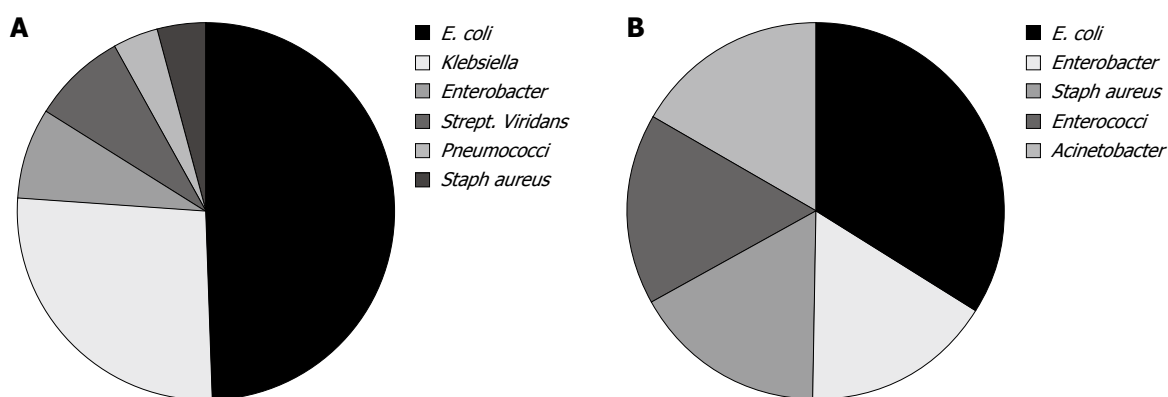
### Microorganisms

Ascitic fluid culture was positive in only 32% of cases. There were 26 ascitic fluid culture positive cases within group I (out of 81); the isolated organisms were *Escherichia coli* (*E. coli*) in 13 (50%), *Klebsiella* in 7 (27%), *Enterobacter*, and *Strep. viridans* each in 2 (8%), *Pneumococci* and *S. aureus* each in 1 (4%) (Figure 1A). There were 6 ascitic fluid culture positive cases within group II (out of 19); the isolated organisms were *E. coli* in 2 (33%), *Enterobac-*

**Table 2** Laboratory parameters of all patients at admission

Variable/mean $\pm$ SD	Group I ( <i>n</i> = 81)	Group II ( <i>n</i> = 19)	<i>t</i>	<i>P</i> value
Laboratory parameters				
INR	1.87 $\pm$ 0.48	1.85 $\pm$ 0.57	0.091	0.927
Albumin (g/dL)	2.16 $\pm$ 0.32	2.23 $\pm$ 0.22	0.852	0.396
Total bilirubin (mg/dL)	5.45 $\pm$ 4.50	4.23 $\pm$ 2.60	1.133	0.260
Direct bilirubin (mg/dL)	3.44 $\pm$ 3.25	2.50 $\pm$ 1.95	1.202	0.232
Creatinine (mg/dL)	1.62 $\pm$ 0.85	1.80 $\pm$ 0.59	0.882	0.38
Urea (mg/dL)	83.87 $\pm$ 37.16	88.33 $\pm$ 42.99	0.457	0.649
Potassium (mmol/L)	3.76 $\pm$ 0.65	3.65 $\pm$ 0.79	0.586	0.559
Sodium (mmol/L)	130.36 $\pm$ 9.26	129.93 $\pm$ 10.53	0.178	0.859
Platelets ( $\times 10^3$ /mL)	53.2 $\pm$ 20.1	54.6 $\pm$ 31.5	0.235	0.815
Ascitic fluid parameters				
Glucose (mg/dL)	121.05 $\pm$ 41.62	101.52 $\pm$ 53.032	1.744	0.084
Protein (mg/dL)	1389.95 $\pm$ 840.65	1553.63 $\pm$ 566.38	0.805	0.423
LDH (IU/L)	240 (180-500)	540 (108-1200)	4.406	0.000
PMNLs (/mm <sup>3</sup> )	3400 (695-264 00)	15 000 (957-23 822)	3.852	0.000

Data are presented by mean  $\pm$  SD or median (range). INR: International normalized ratio; WBC: White blood cell; LDH: Lactate dehydrogenase; PMNLs: Polymorph nuclear leucocytes.

**Figure 1** Isolated organisms in groups. A: Group I ; B: Group II . *E. coli*: *Escherichia coli*.

*ter*, *S. aureus*, *Enterococci* and *Acinetobacter* each in one (16%) (Figure 1B).

### Ascitic fluid analysis

Data for all patients are presented in Tables 2 and 3. Patients in group II had a more severe ascitic inflammatory response than group I and this was demonstrated by the more ascitic LDH and PMNL counts. In group I, apart from the significant reduction in PMNL count, none of the biochemical parameters showed significant changes at the end of therapy. In group II the meropenem subgroup had higher LDH and PMNL count at initiation of the alternative antibiotic therapy; there was no significant difference in the studied parameters between patients responsive to meropenem and levofloxacin at the end of therapy.

### Treatment outcomes

Cases were treated for a median of 5-12 d. Cases in group I (*n* = 81) were treated by cefotaxime for 5-7 d, while patients in group II were treated as follows: the me-

roopenem subgroup was treated for 5 d, the levofloxacin subgroup was treated for 5 d, 2 cases who were resistant to levofloxacin were treated with vancomycin in one case and ampicillin/sulbactam in one case, according to the *in vitro* culture and sensitivity results, for further 5 d and were recultured and examined by an end of treatment ascitic PMNL count before being considered cured.

The clinical and laboratory parameters of group II patients showed no major improvement with the initial empirical therapy; whereas noticeable improvements in many of the parameters were achieved when the alternative antibiotic therapy was given. This was obvious in hepatic encephalopathy (HE) which was encountered in 30.9% of patients in group I and 63.2% of patients in group II (grade II-III HE), where an improvement of consciousness level was achieved after antibiotic treatment of SBP. All patients of group II, in spite of starting treatment of HE, showed no major improvement with cefotaxime, but improvement of consciousness level occurred after the resolution of SBP by the alternative antibiotic therapy.

**Table 3** Ascitic fluid parameters in group II at the beginning and end of alternative therapy

Variable	Meropenem subgroup (n = 11)	Levofloxacin subgroup (n = 8)	t	P value
At initiation of alternative therapy				
Glucose (mg/dL)	100.45 ± 80.33	113.50 ± 9.58	0.390	0.702
Protein (mg/dL)	1850.00 ± 529.26	1360.83 ± 572.08	1.771	0.097
LDH (IU/L)	450 (108-1200)	237 (120-831)	2.223	0.042
PMNLs/mm <sup>3</sup>	18 061 (957-23822)	3540 (957-15222)	3.622	0.000
At end of therapy				
Glucose (mg/dL)	102.81 ± 39.35	124.00 ± 29.17	1.150	0.268
Protein (mg/dL)	1660.00 ± 418.85	1163.33 ± 516.78	2.156	0.052
PMNLs/mm <sup>3</sup>	316.01 ± 104.03	265.63 ± 69.61	1.056	0.307

Data are presented by mean ± SD or median (range). LDH: Lactate dehydrogenase; PMNLs: Polymorph nuclear leucocytes.

## DISCUSSION

Cefotaxime as a standard treatment of SBP has been tried for many years with many reports of evolving resistance to cefotaxime and other third-generation cephalosporins in SBP<sup>[2,5]</sup>. Resistance to cefotaxime in this work is 19%, close to 21.5% recently reported by Ariza *et al.*<sup>[5]</sup>.

In this study cefotaxime resistance is not related to age or sex of patients, and the male predominance in our study is directly related to the higher rates of cirrhosis and risk of exposure and is in agreement with previous studies from other parts of the world<sup>[12,13]</sup>.

Clinical manifestations of SBP are nonspecific; the most frequently encountered symptoms and signs are fever (69%) and abdominal pain (59%),<sup>[14]</sup>. This was the position in our study where fever and abdominal pain were the most frequent clinical manifestations.

However approximately 10% of patients with SBP are asymptomatic<sup>[14]</sup>; in our study asymptomatic patients made up 18.5% of the cefotaxime sensitive group, but all patients in the resistant group had clinical manifestations. This notion, together with the low yield of ascitic fluid culture, raised the value of PMNL count  $\geq 250$  cell/mm<sup>3</sup> as the most important parameter for the diagnosis of SBP.

It is well known that infection in cirrhotic patients, especially SBP, precipitates HE and has been related to variceal bleeding both in terms of pathogenesis of portal pressure increase and severity of bleeding episodes<sup>[15]</sup> hence the related mortality was reduced by prompt antibiotic therapy<sup>[16]</sup> and this is confirmed by our study.

SBP occurs due to bacterial translocation, mainly in patients with advanced cirrhosis and severe liver functional damage<sup>[13,14,17]</sup>; this is confirmed in this study as SBP developed only in patients with Child classes B and C.

In this study, resistance to cefotaxime is associated with more advanced liver disease, because 94.7% and 5.3% of cases were Child C and B respectively. This notion needs further evaluation.

The low proportion of positive ascitic fluid cultures is probably due to the relatively low concentration of

bacteria in the ascitic fluid as compared with the infections in other organic fluids (*e.g.*, urine)<sup>[18]</sup>. For the same reason, a therapy based on the isolation of the responsible bacteria is seldom achievable and antibiotic treatment cannot be delayed to the moment when microbiological results are available<sup>[19]</sup>. That is why cefotaxime as an empirical therapy should be begun without delay.

In the cefotaxime sensitive group gram negative bacteria were the most frequent (22/26; 85%) and *E. coli* was the most predominant; however Gram positive bacteria were isolated in 4 patients. In the resistant group, bacteria were isolated in 6 patients; these results are closely similar to Taşkıran *et al.*<sup>[20]</sup> study which revealed positive ascitic fluid culture in 29.4% of cases with predominantly Gram negative bacteria. Angeloni *et al.*<sup>[12]</sup> revealed positive cultures in 30% of episodes with predominantly Gram negative bacteria; in these episodes the percentage of treatment failure of the initial therapy with cefotaxime was 32% and this result is similar to our findings.

In our patients, cefotaxime failed because the isolated organisms were intrinsically resistant to cefotaxime as *Enterococci* (one patient) and *Acinetobacter* spp (one patient) or were capable of degrading the expanded-spectrum cephalosporins as expanded-spectrum  $\beta$ -lactamase producing (ESBL) *E. coli* (two patients) or  $\beta$ -lactamase producing *Enterobacter* species (one patient) or bacteria with inherent insufficient susceptibility to cefotaxime, such as *S. aureus* (one patient). Our study supports previous reports<sup>[21]</sup> about the introduction of new organisms in the development of SBP.

The efficacy of empiric antibiotic treatment can rarely be based on the amelioration of symptoms or on microbiological results. Therefore, a reduction of PMNL count below 250 cell/mm<sup>3</sup> or a 25% reduction of the initial value has been suggested as the main criterion for establishing the efficacy of antibiotics and the need for switching therapy. Our study confirms the validity of such an approach. Based on PMNL count, we identified the failure of the initial therapy on time.

Meropenem use was successful in 100% of resistant cases. Levofloxacin was successful in 75% of resistant cases, a result lower than reported for *in vivo*<sup>[9,22]</sup> and *in vitro* isolates where all aerobic isolates of SBP were sensitive to levofloxacin<sup>[23]</sup>. Other studies recommended meropenem use, not only in severe cases but also when ESBL producing *E. coli* is seen in the cultures and points to a relation between quinolone resistance and ESBL production<sup>[7,24]</sup>. Types of the isolated bacteria in group II of our study explain the efficacy of meropenem over levofloxacin.

Albumin use was suggested in SBP management<sup>[25]</sup>, although it was not included in the treatment protocols. However the guidelines for the prevention and treatment of hepato-renal syndrome<sup>[26]</sup> suggested that albumin administration may reduce the incidence of renal failure and mortality in patients with SBP; consequently, all



our patients received albumin. Some patients recorded a temporary increase in serum creatinine. All these patients showed improvement of kidney function with the use of proper antibiotics and resolution of SBP.

In conclusion, our study suggests that use of cefotaxime as the first line of treatment is considered valid, since a switch to another antibiotic was necessary only in 19% of our cases and we suggest meropenem as an effective alternative in resistant cases. This study also confirmed the value of a second abdominal paracentesis 48 h after initiation of empiric therapy to detect resistant cases early.

## COMMENTS

### Background

Spontaneous bacterial peritonitis is infection of ascitic fluid in cirrhotic patients with high mortality rates. Cefotaxime has become the empiric antibiotic of choice for the treatment since 1985. Mortality rates reduced from 90% when cefotaxime was first described to around 20%-40%. Recent reports showed that resistance to third generation cephalosporins (including cefotaxime) is increasing and this may increase the need to develop alternative antibiotics.

### Research frontiers

Eradication of ascitic fluid infection is the goal of antibiotic therapy. This study confirmed the validity of cefotaxime as an empiric first line therapy for spontaneous bacterial peritonitis and identified meropenem as an effective alternative in cases of cefotaxime resistance.

### Innovations and breakthroughs

Cefotaxime as the empiric first line therapy for spontaneous bacterial peritonitis is effective in 81% of cases. Meropenem is effective in 100% of cefotaxime-resistant cases, while levofloxacin is effective in 75% of cefotaxime-resistant cases.

### Applications

This article emphasises the value of meropenem and levofloxacin as effective alternatives to cefotaxime in resistant cases.

### Terminology

Spontaneous bacterial peritonitis is infection of ascitic fluid in patients with cirrhosis; Cefotaxime (a third generation cephalosporin) is utilized primarily for its activity against Gram-negative aerobic organisms. The drug exhibits moderate activity against Gram-positive bacteria. Meropenem is an ultra-broad spectrum injectable antibiotic used to treat a variety of infections. It is a beta lactam antibiotic and belongs to the subgroup of carbapenems; Levofloxacin is a synthetic chemotherapeutic antibiotic which belongs to fluoroquinolone drug class and is used to treat severe or life-threatening bacterial infections or bacterial infections that have failed to respond to other antibiotic classes.

### Peer review

The clinical study focused on the effect of cefotaxime as the empiric first line therapy for spontaneous bacterial peritonitis and effectiveness of meropenem and levofloxacin as alternatives in case of cefotaxime resistance. This will be useful for future planning of therapy and clinical application.

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## Radiofrequency ablation during continuous saline infusion can extend ablation margins

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### Abstract

**AIM:** To determine whether fluid injection during radiofrequency ablation (RFA) can increase the coagulation area.

**METHODS:** Bovine liver (1-2 kg) was placed on an aluminum tray with a return electrode affixed to the base, and the liver was punctured by an expandable electrode. During RFA, 5% glucose; 50% glucose; or saline fluid was infused continuously at a rate of 1.0 mL/min through the infusion line connected to the infusion port. The area and volume of the thermocoagulated region of bovine liver were determined after RFA. The Joule heat generated was determined from the temporal change in output during the RFA experiment.

**RESULTS:** No liquid infusion was  $17.3 \pm 1.6$  mL, similar to the volume of a 3-cm diameter sphere ( $14.1$  mL). Mean thermocoagulated volume was significantly larger with continuous infusion of saline ( $29.3 \pm 3.3$  mL) than with 5% glucose ( $21.4 \pm 2.2$  mL), 50%

glucose ( $16.5 \pm 0.9$  mL) or no liquid infusion ( $17.3 \pm 1.6$  mL). The ablated volume for RFA with saline was approximately 1.7-times greater than for RFA with no liquid infusion, representing a significant difference between these two conditions. Total Joule heat generated during RFA was highest with saline, and lowest with 50% glucose.

**CONCLUSION:** RFA with continuous saline infusion achieves a large ablation zone, and may help inhibit local recurrence by obtaining sufficient ablation margins. RFA during continuous saline infusion can extend ablation margins, and may be prevent local recurrence.

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**Key words:** Radiofrequency ablation; *Ex vivo* bovine liver; Large ablation zone; Saline; Glucose; Joule heat

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DOI: <http://dx.doi.org/10.3748/wjg.v19.i8.1278>

### INTRODUCTION

Radiofrequency ablation (RFA) is a technique by which deposition of electromagnetic energy is used to thermally ablate hepatic tumor tissue<sup>[1]</sup> and is now the standard method for local treatment of hepatic malignancy<sup>[2-9]</sup>.

Several types of device are currently used for such therapy, such as the Cool-tip system (single electrode) made by Covidien, and the RFA systems made by Boston Scientific and AngioDynamics (the Starburst XL electrode)<sup>[10]</sup>. The former uses a single-electrode puncture needle, whereas the latter both employ a needle elec-

trode with an expandable array of fine needles extending from the tip. The expandable array of the StarBurst XL electrode is also fitted with an infusion port. To date, various techniques have been devised to secure adequate ablation margins<sup>[11]</sup>. We investigated whether ablation margins could be extended by fluid infusion from the infusion port of an expandable electrode.

We conducted an *ex vivo* experiment on bovine liver to investigate whether ablation margins could be extended by fluid infusion from a StarBurst XL electrode in order to avoid the influence of the cooling effect *in vivo* experiment.

## MATERIALS AND METHODS

### RFA setting and ablation protocol

A StarBurst XL electrode (AngioDynamics; Queensbury, NY) and RITA1500X generator (AngioDynamics) were used to provide RFA. One to two kilograms of bovine liver from an abattoir was used which had been exposed to room temperature for several hours until the baseline mean liver temperature ranged between 15 °C and 21 °C, placed on an aluminum tray with a return electrode affixed to the base and the liver was punctured by the StarBurst XL electrode, with a maximum array expansion of 5 cm in diameter, under ultrasonographic guidance by convex probe using a Logiq 7 ultrasound device (GE Healthcare, Tokyo, Japan). Stepped ablation was performed, with the electrode first expanded to 2 cm after reaching the set temperature of 105 °C, then expanded by 3 cm after reaching the set temperature of 105 °C, with single ablation maintained at each diameter for 7 min. An infusion line was connected to the infusion port on the electrode, and the other end of the infusion line was connected to a syringe pump. Continuous liquid infusion was initiated at the same time as radiofrequency output. Three infusion liquids were used with the continuous infusion rate was set at 1.0 mL/min: 5% glucose; 50% glucose; and saline<sup>[12]</sup>.

After stopping radiofrequency output, specimens were sectioned along the longitudinal and transverse axes of each lesion. Measurement of the coagulation diameter perpendicular to the electrode axis was based on consensus of two observers (Ishikawa T, Kubota T). Macroscopic changes in specimens have been demonstrated to correlate well with coagulation necrosis at histological examination<sup>[13]</sup>. Furthermore, the central white area of the RF-induced ablation zone was found to correspond to the necrotic zone<sup>[14]</sup>.

The liver blocks containing lesions were dissected along the axis along which the electrode was inserted, and the central white area of the RF-induced ablation zone was found to correspond to the necrotic zone<sup>[15]</sup>. For macroscopic examination, two observers used calipers to measure, in the central white area of coagulation necrosis in each pathologic specimen, the overlapping width, the longest diameter of each ablation sphere along the electrode, and the shortest diameter at the

midpoint between the two electrodes<sup>[16]</sup>.

The region of discoloration indicating ablation of the bovine liver was measured, and the volume of ablation was calculated using the formula for calculating the volume of an ellipsoid ( $V = 4/3abc\pi$ ).

An intergroup comparison of calculated thermocoagulated volumes was performed using Student's *t*-test with Bonferroni correction.

In addition, the generated Joule heat was calculated from the data for output changes over time during the RFA experiment.

### Statistical analysis

In the intergroup comparison of calculated thermocoagulated volumes, an unpaired Student's *t*-test was used and *P* values were calculated with a correction of the  $\alpha$  significance level by Bonferroni's method based on the number of test repetitions. For all statistical analyses, values of *P* < 0.05 were considered statistically significant.

## RESULTS

Figure 1 shows cross-sectional photographs of ablated regions of liver and graphs of changes over time in temperature, output and impedance during RFA for each continuous infusion condition. Changes in impedance over time after array expansion to 3 cm tended to gradually increase in RFA without infusion and with glucose, but the steady state of impedance was seen when RFA was used with saline (Figure 1). The ablation zone also widened and presented a circular shape when saline was used.

Experimental result shows mean  $\pm$  SD values for dimensions of ablation, calculated volume, mean output during RFA, and calculated Joule heat. Mean thermocoagulated volume was significantly larger with continuous infusion of saline ( $29.3 \pm 3.3$  mL) than with 5% glucose ( $21.4 \pm 2.2$  mL), 50% glucose ( $16.5 \pm 0.9$  mL) or no liquid infusion ( $17.3 \pm 1.6$  mL) (*P* < 0.01). The ablated volume for RFA with saline was approximately 1.7-times greater than for RFA with no liquid infusion, representing a significant difference between these two conditions.

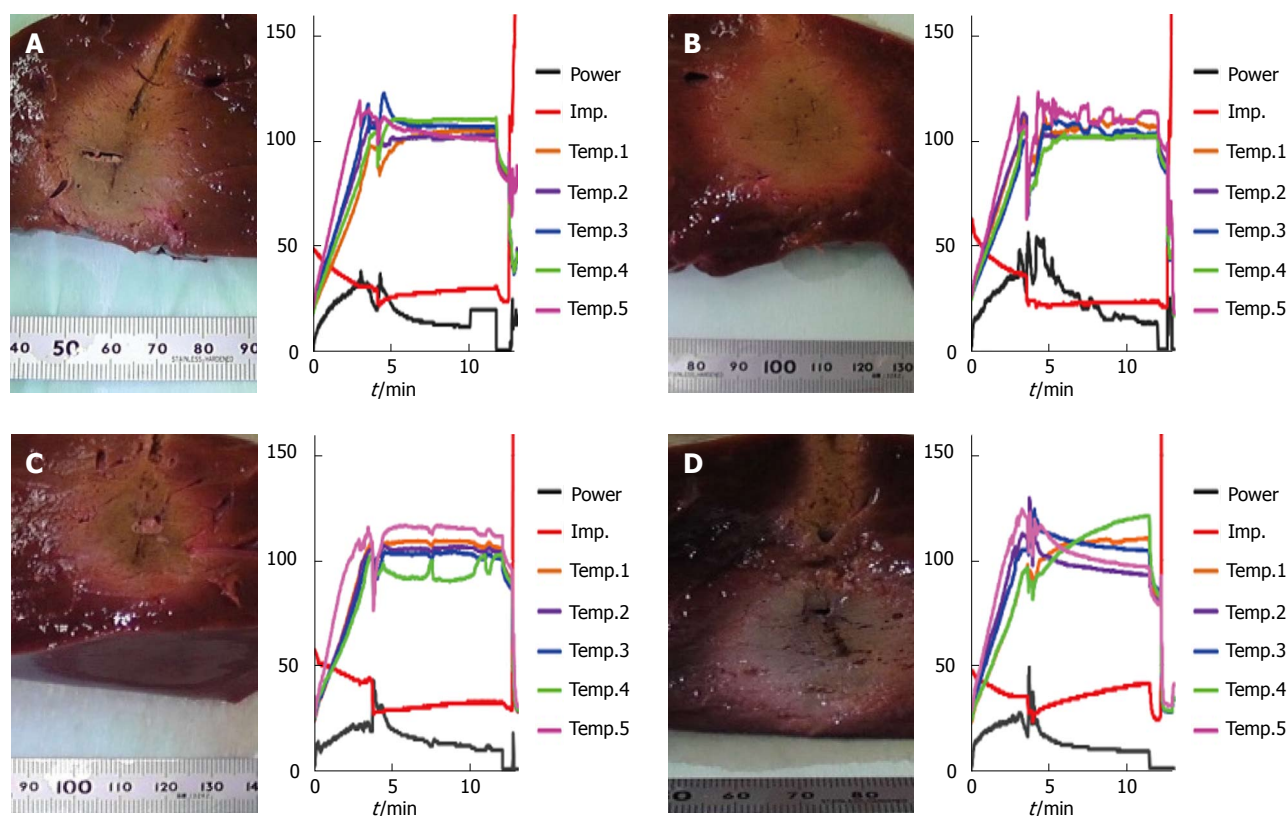
An increasing trend in ablation volume was also seen in RFA with 5% glucose, although not to the same extent as with saline. In RFA with 50% glucose, the ablated volume was very similar to RFA with no liquid infusion, and differed significantly from RFA with saline.

Mean radiofrequency output tended to be higher in RFA with saline than with no liquid infusion, but tended to be low in RFA with glucose. The mean total Joule heat generated during RFA was highest with saline, and low with glucose.

## DISCUSSION

RFA is a therapeutic technique that utilizes the principle of conductive heating, in which heat is generated by friction from moving electrons when current, is passed through a conductor<sup>[17]</sup>. Specifically, by passing electricity





**Figure 1** Transection of liver specimen with radiofrequency ablation lesion (on the left side) and graphic depiction of changes in temperature (middle) with tissue impedance (red line) and power (black line) during radiofrequency ablation (on the right side) in the four groups. A: No continuous liquid infusion; B: Saline; C: 5% glucose; D: 50% glucose.

through a needle electrode inserted into the human body, coagulation and necrosis of a malignant neoplasm or other lesion tissue is induced<sup>[18,19]</sup>. The basic principle of RFA as treatment for hepatic malignancy is the generation of Joule heat by high-frequency current in living tissue between the needle electrode and a return electrode. Although the heat generated by this high-frequency current arises in all the conducting tissue pathways, high temperatures only develop in tissues near the electrode, where the current density is high. Barely any heat arises in tissues further from the electrode because of the fall in current density and the cooling (or radiator) effect of blood flow.

Although this principle brings about ablation in tissue near the electrode, the vapor microbubbles generated in the ablation region are removed by blood flow, lowering the electrical conductivity of tissue and consequently sometimes preventing adequate ablation of the target zone<sup>[2]</sup>. Efficient generation of conductive heating is therefore necessary to achieve adequate ablation margins<sup>[20]</sup>. Temperature-controlled systems for facilitating adequate ablation of the target zone by preventing excessive heating of tissue have been commercialized, but our experiments aimed to test the effect of RFA used in combination with continuous infusion of a liquid to the target tissue. Specifically, we conducted *ex vivo* experiments on bovine liver to investigate whether infusion of a liquid from the infusion port of a StarBurst XL electrode could

enhance the efficiency of conductive heating.

The mean ablated volume when RFA was performed by StarBurst XL electrode with no liquid infusion was  $17.3 \pm 1.6$  mL, similar to the volume of a 3-cm diameter sphere (14.1 mL). On the other hand, when RFA was used with a continuous infusion of liquid, the mean ablation volume was  $29.3 \pm 3.3$  mL with saline,  $21.4 \pm 2.2$  mL with 5% glucose, and  $16.5 \pm 0.9$  mL with 50% glucose ( $P < 0.01$ ). This shows that the ablated region expanded significantly when saline was used. In RFA with continuous liquid infusion, the ablated volume was significantly higher than in RFA without liquid infusion, and when saline in particular was used, the mean point estimate volume increased approximately 1.7-fold. In order to maintain the steady state of impedance for RFA during saline continuous injection, current density between electrodes should therefore be decreased to within the range within which current causes an increase in tissue temperatures, without boiling. We speculate that changing the electrical conductance of the tissue by infusing saline could be a good solution. Furthermore, we propose the following three reasons for these results.

First, in RFA without liquid infusion, water in tissues near the electrode forms microbubbles, some of which move to the area around the tissue. The moisture percentage near the electrode thus decreases and electrical conductivity of the tissue falls. This is supported by the finding that impedance gradually increased during RF

output. In contrast, when RFA is used with continuous liquid infusion, the moisture percentage in tissue near the electrode remains stable or is increased, maintaining or increasing electrical conductivity and leading to expansion of the ablated region. Furthermore, saline was likely more effective in extending the ablated region than glucose when used with RFA because saline is a conductive solution.

Second, ablation of tissue near the electrode results from the thermal conversion effect of Joule heat due to a high current density. The thermal conversion effect of Joule heat alone is insufficient to induce ablation of the surrounding tissue, and we speculate that such ablation is the result of synergistic effects involving, for example, heat conduction from tissue near the electrode to surrounding tissue, or transfer of heated water or high-temperature microbubbles generated near the electrode to the surrounding tissue. Infusion of liquid would promote this transfer of heated water or high-temperature microbubbles to the surrounding tissue, with the ultimate effect of extending the ablated region.

Third, the type of infusion liquid was relevant, with a significant difference in ablated volume seen between the saline and 50% glucose solutions. This is probably because although a highly concentrated glucose solution would be expected to extend the ablation region through the effects described in the two points above, the non-ionic nature of the solution may lower the electrical conductivity of the target tissue. Considering that ablated volumes were similar in RFA with 50% glucose infusion and without any liquid infusion, the negative effects of this highly concentrated but non-ionic solution cancelled out the effects described in the first two points above, resulting in no marked extension of the ablated region.

Shorter treatment time (RFA time) may be another effect of RFA with continuous liquid infusion. This could be due to an increase in thermocoagulation efficiency at the target tissue arising from the transfer of high-temperature microbubbles or heated water to the surrounding tissue, as suggested in the second point above.

Furthermore, RFA with continuous liquid infusion tended to produce a larger thermocoagulated region with a lower total Joule heat compared to RFA without liquid infusion. Saline infusion in particular demonstrated high ablation efficiency at the target tissue, and could therefore prove highly useful<sup>[21]</sup>.

This high ratio reveals the strong ablative action on the target tissue, and suggests that RFA with continuous saline infusion can achieve efficient ablation of tissue near the electrode. While this *ex-vivo* procedure cannot be directly adopted for use in clinical settings, RFA during continuous saline infusion can achieve a large thermocoagulation region in a short time and may be able to inhibit local recurrence by securing ablation margins.

There are several limitations to this study. The most important limitation was the absence of blood flow. *In vivo*, the volume of coagulation will be smaller and the amount of applied energy will be higher due to the

removal of heat by the presence of blood flow<sup>[22]</sup>. This *ex-vivo* procedure cannot be directly adopted for use in a clinical setting. Using the same RF device, the size of the ablation zone *in vivo* was smaller than when *ex vivo*<sup>[23]</sup>. However, RFA during saline injection has potentially broad clinical merit and can assist in the planning of a methodological strategy. The relationship between parameters was calculated for a particular RF system, therefore, a different multipolar RF system would change the mathematical modeling. Moreover, the physiologic variability of thermal and electrical tissue properties is supposed to lead to a variable efficiency of applied energy under *in vivo* conditions. Consequently, the accuracy of mathematical modeling derived from *ex vivo* data will be restricted in the clinical application of multipolar RF ablation. Therefore, mathematical modeling has to be adjusted for *in vivo* conditions and due to the physiologic variability<sup>[24]</sup> of parameters the accuracy of predicted volumes is supposed to be reduced.

Finally, the findings of this study reflect certain model-specific characteristics, such as its hypovascularity, outcomes in other more clinically relevant tumor model may differ. Therefore, extrapolation to other types of tumors should be performed with caution.

In conclusion, we confirmed the effect of continuous liquid infusion in expanding the RFA thermocoagulated region in *ex vivo* experiments using a Starburst XL electrode system. Our results indicate that RFA with continuous saline infusion can significantly expand the volume of thermocoagulation in temperature-controlled RFA systems. RFA with continuous saline infusion can achieve a large thermocoagulation region in a short time and may be able to inhibit local recurrence by securing ablation margins<sup>[25,26]</sup>.

## COMMENTS

### Background

Radiofrequency ablation (RFA) is now the standard method for local treatment of hepatic malignancy. To date, various techniques have been devised to secure adequate ablation margins. This study was investigated whether ablation margins could be extended by fluid infusion from the infusion port of an expandable electrode.

### Research frontiers

In this study, authors confirmed that ablation margins by RFA could be extended by saline infusion from the infusion port of an expandable electrode.

### Innovations and breakthroughs

While this *ex-vivo* procedure cannot be directly adopted for use in clinical settings, RFA during continuous saline infusion can achieve a large thermocoagulation region in a short time and may be able to inhibit local recurrence by securing ablation margins.

### Applications

In this study, RFA with continuous saline infusion can achieve a large thermocoagulation region in a short time and may be able to inhibit local recurrence by securing ablation margins.

### Peer review

This is a very novel study that Starburst RFA device and infusion saline and dextrose to study differences in burn volume with RFA. The results are interesting and suggest that RFA during continuous saline infusion can extend ablation margins.

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## Ethnicity association of *Helicobacter pylori* virulence genotype and metronidazole susceptibility

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### Abstract

**AIM:** To characterise the *cag* pathogenicity island in *Helicobacter pylori* (*H. pylori*) isolates by analysing the strains' *vacA* alleles and metronidazole susceptibilities in light of patient ethnicity and clinical outcome.

**METHODS:** Ninety-five *H. pylori* clinical isolates obtained from patients with dyspepsia living in Malaysia were analysed in this study. Six genes in the *cagPAI* region (*cagE*, *cagM*, *cagT*, *cag13*, *cag10* and *cag67*) and *vacA* alleles of the *H. pylori* isolates were identified by polymerase chain reaction. The isolates' metronidazole susceptibility was also determined using the *E*-test

method, and the resistant gene was characterised by sequencing.

**RESULTS:** More than 90% of the tested isolates had at least one gene in the *cagPAI* region, and *cag67* was predominantly detected in the strains isolated from the Chinese patients, compared with the Malay and Indian patients ( $P < 0.0001$ ). The majority of the isolates (88%) exhibited partial deletion (rearrangement) in the *cagPAI* region, with nineteen different patterns observed. Strains with intact or deleted *cagPAI* regions were detected in 3.2% and 8.4% of isolates, respectively. The prevalence of *vacA* s1m1 was significantly higher in the Malay and Indian isolates, whereas the isolates from the Chinese patients were predominantly genotyped as *vacA* s1m2 ( $P = 0.018$ ). Additionally, the isolates from the Chinese patients were more sensitive to metronidazole than the isolates from the Malay and Indian patients ( $P = 0.047$ ). Although we attempted to relate the *cagPAI* genotypes, *vacA* alleles and metronidazole susceptibilities to disease outcome, no association was observed. The *vacA* alleles were distributed evenly among the strains with intact, partially deleted or deleted *cagPAI* regions. Interestingly, the strains exhibiting an intact *cagPAI* region were sensitive to metronidazole, whereas the strains with a deleted *cagPAI* were more resistant.

**CONCLUSION:** Successful colonisation by different *H. pylori* genotypes is dependent on the host's genetic makeup and may play an important role in the clinical outcome.

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**Key words:** *Helicobacter pylori*; *cag* pathogenicity island; *vacA* alleles; Metronidazole susceptibility

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## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) demonstrates great genetic diversity, as evidenced by the apparently unlimited number of unique strains differing in genome size, gene order, gene content and allelic profile<sup>[1]</sup>. The high levels of genetic diversity in clinical isolates and the presence of certain virulence genotypes may confer a selective advantage to certain strains in different hosts. Therefore, the virulence genotype of *H. pylori* is a potentially useful predictor of the clinical outcome of gastric mucosal colonisation.

*H. pylori* possess a number of virulence determinants that modulate the organism's interaction with a host<sup>[2,3]</sup>. These virulence factors include the secreted, vacuolating cytotoxin VacA and the gene products of the pathogenicity island (*cagPAI*). The presence of these genetic loci correlates with the more severe *H. pylori*-associated pathologies, peptic ulcers and gastric cancer. Screening for the *cagPAI* genes is frequently performed due to the genes' involvement in virulence characteristics. An infection with *cagPAI*-positive *H. pylori* strains significantly increases the risk of developing severe gastric mucosal inflammation, duodenal ulceration and gastric cancer<sup>[4-6]</sup>. It has been observed that only one-half to two-thirds of isolates from Western patients express the *cagPAI* region, whereas almost all of the East Asian strains express this region. As there is limited information on the structure of the *cagPAI* in Malaysian patients, the role of *cagPAI* rearrangement in *H. pylori* isolates from various ethnic populations within this country should be investigated.

The *vacA* gene is another *H. pylori* virulence factor that exhibits great polymorphism and diverse allelic combination. The variable structure, resulting in different allelic arrangements in the gene, is related to differences in cytotoxin production and to the distinct clinical outcomes of *H. pylori* infection<sup>[7-9]</sup>. For example, a recent study performed at our institute has demonstrated a correlation between the presence of genetic polymorphisms in the *vacA* gene and the severity of gastritis<sup>[10]</sup>.

*H. pylori* infection can be effectively cured by antibiotics. More specifically, metronidazole is commonly used in conjunction with either amoxicillin or clarithromycin and a proton-pump inhibitor to eradicate *H. pylori* infection<sup>[11,12]</sup>. Metronidazole is also used in the treatment of other diseases, such as gynaecological infections, and has contributed to the increased antibiotic resistance of *H. pylori*<sup>[13]</sup>. In Malaysia, the prevalence of metronidazole-resistant strains is 37%, which may be one of the factors contributing to the failed eradication of *H. pylori* infection in the country<sup>[14]</sup>.

The population of Malaysia consists of three major

ethnic groups (Malay, Chinese and Indian) that are historically associated with differences in *H. pylori* infection<sup>[15-17]</sup>. *H. pylori*-associated gastroduodenal diseases typically do not reflect the prevalence of *H. pylori* infection<sup>[15]</sup>. Furthermore, geographical and population differences in *H. pylori* virulence factors and disease severity underscore the importance of investigating the relationship between the genotypes of the causative strains and the clinical outcome. Therefore, to further understand the dynamics of the genetic traits of the *H. pylori cagPAI* regions, *vacA* alleles and metronidazole susceptibilities in Malaysia's multi-ethnic population, we investigated the possible association between these *H. pylori* virulence factors and infection in patients of different ethnicities and clinical outcomes.

## MATERIALS AND METHODS

### Patients

Patients with dyspepsia who had undergone an upper gastrointestinal scope (OGDS) at Universiti Kebangsaan Malaysia Medical Centre (Kuala Lumpur, Malaysia) between May 2004 and December 2007 were recruited. Any patients who had received antibiotics 4 wk prior to OGDS were excluded. Written informed consent was obtained from each patient, and the protocol was approved by the Research Ethics Committee (protocol number FF-075-2003) of the University.

### *H. pylori* culture

Biopsies were subcultured for *H. pylori* on Columbia agar base (Oxoid, Basingstoke, England) containing Dent's supplement (Oxoid, Basingstoke, England) and 7% defibrinated sheep blood. The plates were incubated at 37 °C for 5 d under microaerophilic conditions.

### DNA extraction

*H. pylori* genomic DNA was prepared using a High Pure polymerase chain reaction (PCR) Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

### Detection of metronidazole susceptibility and resistance genotype

Metronidazole susceptibility was determined using the E-test method (AB Biodisk, Solna, Sweden). First, an *H. pylori* culture suspension with a McFarland turbidity of 3.0 was used to inoculate Columbia agar supplemented with 10% defibrinated sheep blood. The plates were then incubated at 35 ± 2 °C for 3-5 d under microaerophilic conditions. The isolates were classified as resistant to metronidazole when the MIC value was > 8 µg/mL<sup>[18]</sup>. The *rdxA* genes were amplified by PCR using specific primers, as previously described<sup>[19]</sup>, and then subjected to sequencing.

### Detection of *cagPAI* genes

Two loci were selected in the *cag I* region (*cagE* and

*cagM*), and four loci were chosen from the *cagII* region (*cagT*, *cag13*, *cag10* and *cag67*). Additionally, the insertion sequence IS605 was selected. The presence of these selected genes was determined by PCR using specific primers, as previously described<sup>[20]</sup>. PCR was conducted in 25 µL volumes containing 1 × PCR buffer, 0.2 mmol/L dNTP mix, 10 pmol of each primer, 1 U Taq polymerase (BioTherm, GeneCraft, Germany) and 1 µL of the DNA sample. The PCR amplification conditions consisted of 35 cycles at 94 °C for 1 min, 50–55 °C for 30 s, 72 °C for 1 min, and one cycle for the final extension at 72 °C for 10 min. The amplified product was electrophoresed on a 1% agarose gel.

### Detection of *vacA* alleles

The *vacA* genotyping was performed by multiplex PCR using specific primers, as previously described<sup>[10,21]</sup>. PCR was conducted in 25-µL volumes containing 1 × PCR buffer, 0.2 mmol/L dNTP mix, 6 pmol of each primer, 0.75 U Taq polymerase (BioTherm, GeneCraft, Germany) and 1 µL of the DNA sample. The PCR amplification conditions consisted of an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and one cycle for the final extension at 72 °C for 7 min. The amplified products were electrophoresed on a 1.5% agarose gel.

### Statistical analysis

A statistical analysis was performed using the  $\chi^2$  test. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### *H. pylori* clinical isolates

A total of 95 clinical *H. pylori* isolates were obtained from 40 male and 55 female patients with a mean age of  $55.47 \pm 16.87$  years (age range of 17–89 years) and from three different ethnic groups (12 Malays, 59 Chinese and 24 Indians). The endoscopic findings were as follows: nonulcer dyspepsia ( $n = 76$ ), gastric ulcer ( $n = 9$ ), duodenal ulcer ( $n = 6$ ) and both gastric and duodenal ulcers ( $n = 2$ ). The OGDS findings were not available for two of the patients. Nonulcer dyspepsia was further classified as endoscopically normal stomach ( $n = 12$ ), antrum-predominant gastritis ( $n = 43$ ), corpus-predominant gastritis ( $n = 2$ ) and pangastritis ( $n = 19$ ). No significant association was found between patient ethnicity (Chinese versus non-Chinese) and the endoscopic findings (non-ulcer dyspepsia versus peptic ulcer).

### *cagPAI* genotype

Of the 95 *H. pylori* isolates, 87 (91.6%) were *cagPAI*-positive, and the remaining 8 (8.4%) lacked all selected *cagPAI* genes. For the *cagPAI*-positive strains, intact *cagPAI* genes were detected only in 3 isolates, whereas other isolates exhibited partially deleted (rearranged) *cagPAI*

**Table 1** Distribution of *Helicobacter pylori cagPAI* genes isolated from patients of different ethnicities  $n$  (%)

<i>cagPAI</i> genes	Ethnic groups			<i>P</i> value
	Malay	Chinese	Indian	
<i>cagI</i> region				
<i>cagE</i>	7 (58.3)	41 (69.5)	14 (58.3)	0.541
<i>cagM</i>	9 (75.0)	45 (76.3)	19 (79.2)	0.948
<i>cagII</i> region				
<i>cagT</i>	11 (91.7)	49 (83.1)	18 (75.0)	
<i>cag13</i>	0	4 (6.8)	0	
<i>cag10</i>	6 (50.0)	29 (49.2)	16 (66.7)	0.336
<i>cag67</i>	6 (50.0)	47 (79.7)	5 (20.8)	< 0.0001

genes. Additionally, the *IS605* gene was detected in 4 (4.2%) isolates. Further analyses indicated that the positivity of the *H. pylori* isolates was 61.1% (58/95), 53.7% (51/95), 4.2% (4/95), 82.1% (78/95), 76.8% (73/95) and 65.3% (62/95) for *cag67*, *cag10*, *cag13*, *cagT*, *cagM* and *cagE*, respectively.

As shown in Table 1, the presence of *cagE* and *cagM* in the *cagI* region was distributed evenly among the isolates from patients of different ethnicities, with no significant differences between the ethnicities. The *cagT* gene was frequently detected in isolates from the Malay patients, whereas *cag13* was only detected in the isolates from the Chinese patients. The presence of *cag67* was significantly higher in the isolates from the Chinese patients than in the isolates from the Malay patients and was lowest in the isolates from the Indian patients. The *cagPAI* genes were detected in greater than 50% of the isolates from the nonulcer and ulcer patients, except for *cag13*, which was only detected in the patients with a normal stomach or gastritis. Thus, different *cagPAI* genes were present in the majority of the isolates examined, irrespective of disease state.

Of the 95 clinical isolates, only 3 (3.2%) contained an intact *cagPAI*, whereas 84 (88.4%) exhibited partial deletions within the *cagPAI* region, and 8 (8.4%) lacked the entire selected gene in the *cagPAI*. As shown in Table 2, thirteen isolates appeared to have a diverged *cagPAI*, in which the two selected genes for the *cagI* region had been deleted, although the genes in the *cagII* region were conserved, with at least one gene present. In contrast, ten strains had only one gene in either the *cagI* or the *cagII* region. The single gene detected was *cagM*, *cagT* and *cag67* in 3, 6 and 1 isolate, respectively. The most commonly partially deleted *cagPAI* genotypes were detected in 26 (27.4%), 16 (16.8%) and 13 (13.7%) isolates, with *cagPAI* regions of types C, I and N, respectively. These isolates exhibited at least four genes in the *cagPAI* region, and the genes in the *cagI* region were conserved in these isolates. Additionally, the *IS605* gene was detected in the isolates from the Chinese ( $n = 2$ ) and Indian ( $n = 2$ ) patients. Three of these isolates contained all of the selected genes for *cagPAI*, except *cag13*, and one isolate lacked the *cag13* and *cag10* genes.

As shown in Table 3, three isolates with an intact *cag*-

**Table 2** *cagPAI* genotypes of *Helicobacter pylori* clinical isolates *n* (%)

<i>cagPAI</i> type	<i>cag</i> II region				<i>cag</i> I region		Total
	<i>cag67</i>	<i>cag10</i>	<i>cag13</i>	<i>cagT</i>	<i>cagM</i>	<i>cagE</i>	
A	+	+	+	+	+	+	3 (3.2)
B	+	-	+	-	-	-	1 (1.1)
C	+	+	-	+	+	+	26 (27.4)
D	+	+	-	+	+	-	2 (2.1)
E	+	+	-	-	+	+	1 (1.1)
F	+	+	-	-	+	-	1 (1.1)
G	+	+	-	-	-	-	1 (1.1)
H	+	-	-	-	-	-	1 (1.1)
I	+	-	-	+	+	+	16 (16.8)
J	+	-	-	+	+	-	2 (2.1)
K	+	-	-	+	-	+	1 (1.1)
L	+	-	-	-	+	+	1 (1.1)
M	+	-	-	+	-	-	2 (2.1)
N	-	+	-	+	+	+	13 (13.7)
O	-	+	-	+	+	-	2 (2.1)
P	-	+	-	+	-	-	2 (2.1)
Q	-	-	-	+	+	+	1 (1.1)
R	-	-	-	+	+	-	2 (2.1)
S	-	-	-	+	-	-	6 (6.3)
T	-	-	-	-	+	-	3 (3.2)
U	-	-	-	-	-	-	8 (8.4)

PAI were obtained from the Chinese patients. Nineteen of the 59 (32.2%) isolates from the Chinese patients possessed partially deleted *cagPAI* regions of type C, compared with 25% and 16.7% of the isolates from the Malay and Indian patients, respectively. Interestingly, the isolates from the Indian patients showed a high proportion of partially deleted *cagPAI* regions of type N (37.5%) and deleted *cagPAI* regions (16.7%). An analysis of the disease state, with reference to strain genotype, revealed a relationship between the *cagPAI* genotype and disease severity. The isolates from those patients with peptic ulcer disease (PUD) primarily exhibited *cagPAI* regions of type C, which contained more genes in the rearranged *cagPAI* genotypes. In contrast, a larger number of isolates from the patients with pangastritis or antrum-predominant gastritis possessed *cagPAI* regions of type I or N, respectively, compared with the isolates from the patients with peptic ulcers.

### *vacA* genotype

The *vacA* s1m1, s1m2 and s2m2 genotypes were identified in 51.6% (49/95), 42.1% (40/95) and 4.2% (4/94) of the isolates, respectively. Additionally, two (2.1%) isolates possessed the *vacA* s1m1m2 genotype. The *vacA* s1m1 genotype was further classified as *vacA* s1am1 (63.3%, 31/49), s1cm1 (22.4%, 11/49) or s1as1cm1 (14.3%, 7/49), and the *vacA* s1m2 genotype was further classified as *vacA* s1am2 (65%, 26/40), s1cm2 (22.5%, 9/40) or s1as1cm2 (12.5%, 5/40). An analysis of the distribution of the *vacA* alleles with respect to patient ethnicity and disease state is shown in Table 4. We found a significant distribution of different *vacA* alleles according to patient ethnicity. The *vacA* s1m1 genotype

was significantly more prevalent in the isolates from the Malay and Indian patients, whereas the *vacA* s1m2 genotype was predominantly detected in the isolates from the Chinese patients ( $P = 0.018$ ). The *vacA* s2m2 genotype was only isolated from the Malay and Indian patients, and a mixed genotype of the m region was identified in the isolates from the Chinese patients. Further analyses of the *vacA* subgenotypes with respect to patient ethnicity revealed that the highest proportion of isolates harbouring the *vacA* s1am1 subgenotype were from Indian patients (93.8%, 15/16). The prevalence of the *vacA* s1cm1 genotype was similar in proportion in the isolates from the Malay (37.5%, 3/8) and Chinese (32%, 8/25) patients, although this subgenotype was not detected in the isolates from the Indian patients. In contrast, the *vacA* s1am2 subgenotype was detected in all of the ethnic groups, whereas the *vacA* s1cm2 subgenotype was only identified in the isolates from the Chinese patients. No significant difference was observed in the distribution of the *vacA* s1m1 and s1m2 strains with respect to the disease state ( $P = 0.686$ ).

### Metronidazole susceptibility

In this study, metronidazole resistance was observed in 45.3% (43/95) of the isolates. The resistant strains exhibited MIC values ranging from 8 to > 256 mg/L, with an MIC value > 256 mg/L for the majority of the strains (62.8%, 27/43). As shown in Table 4, a strong association was observed between metronidazole susceptibility and patient ethnicity. The Chinese patients were more prone to infection with metronidazole-sensitive *H. pylori* strains, whereas the Malay and Indian patients were more likely to be infected with the antibiotic-resistant strains. The strains isolated from the patients with PUD were more sensitive to metronidazole than the strains from the patients with pangastritis, although this difference was not statistically significant. DNA sequence analyses of the *ndxA* gene revealed the presence of missense, frameshift and nonsense mutations in 30, 3 and 4 isolates, respectively. All of the tested metronidazole-sensitive strains harboured only missense mutations, whereas all three types of mutations were identified in the antibiotic-resistant strains.

### Distribution of *H. pylori* *cagPAI* genotypes, *vacA* alleles and metronidazole susceptibilities

As shown in Table 5, the isolates with intact *cagPAI* regions carrying the *vacA* s1m2 allele were sensitive to metronidazole. Of the isolates with a rearranged *cagPAI* region, the type C strains predominantly harboured the *vacA* s1m2 allele, whereas a high proportion of the types I and N *cagPAI* strains harboured the *vacA* s1m1 allele. However, these strains were found to be more sensitive to metronidazole. In contrast, the isolates exhibiting a deleted *cagPAI* region predominantly carried the *vacA* s1m2 allele and were more resistant to metronidazole.

**Table 3** Distribution of strains with intact, partially deleted (rearranged) or deleted *cagPAI* regions among patients of different ethnicities and disease states *n* (%)

<i>cagPAI</i> status	Ethnic group			Disease state			
	Malay ( <i>n</i> = 12)	Chinese ( <i>n</i> = 59)	Indian ( <i>n</i> = 24)	Normal ( <i>n</i> = 12)	Gastritis (antral) ( <i>n</i> = 43)	Pangastritis ( <i>n</i> = 19)	PUD ( <i>n</i> = 17)
Intact							
Type A	0	3 (5.1)	0	1 (8.3)	2 (4.7)	0	0
Partially deleted							
Type C	3 (25.0)	19 (32.2)	4 (16.7)	3 (25.0)	9 (20.9)	7 (36.8)	7 (41.2)
Type I	2 (16.7)	14 (23.7)	0	2 (16.7)	5 (11.6)	5 (26.3)	3 (17.6)
Type N	2 (16.7)	2 (3.4)	9 (37.5)	1 (8.3)	7 (16.3)	2 (10.5)	2 (11.8)
Deleted							
Type U	1 (8.3)	3 (5.1)	4 (16.7)	1 (8.3)	3 (7.0)	1 (5.3)	2 (11.8)

PUD: Peptic-ulcer disease.

**Table 4** Distribution of *vacA* alleles and metronidazole susceptibilities among *Helicobacter pylori* clinical isolates from patients of different ethnicities and disease states *n* (%)

<i>cagPAI</i> status	Ethnic group			Disease state			
	Malay ( <i>n</i> = 12)	Chinese ( <i>n</i> = 59)	Indian ( <i>n</i> = 24)	Normal ( <i>n</i> = 12)	Gastritis (antral) ( <i>n</i> = 43)	Pangastritis ( <i>n</i> = 19)	PUD ( <i>n</i> = 17)
Intact							
Type A	0	3 (5.1)	0	1 (8.3)	2 (4.7)	0	0
Partially deleted							
Type C	3 (25.0)	19 (32.2)	4 (16.7)	3 (25.0)	9 (20.9)	7 (36.8)	7 (41.2)
Type I	2 (16.7)	14 (23.7)	0	2 (16.7)	5 (11.6)	5 (26.3)	3 (17.6)
Type N	2 (16.7)	2 (3.4)	9 (37.5)	1 (8.3)	7 (16.3)	2 (10.5)	2 (11.8)
Deleted							
Type U	1 (8.3)	3 (5.1)	4 (16.7)	1 (8.3)	3 (7.0)	1 (5.3)	2 (11.8)

PUD: Peptic-ulcer disease.

## DISCUSSION

The *cagPAI* and *vacA* genotypes are widely used to characterise the virulence of *H. pylori* and the relationship of such virulence to disease severity, although direct associations with peptic ulcers and gastric cancer have not been established<sup>[22,23]</sup>. Rather, the development of severe histological changes in the gastric mucosa may depend on the synergistic effect of bacterial and host factors<sup>[24]</sup>.

The genotypic characteristics of the *H. pylori* *cagPAI* genes show great variability worldwide. The rearrangement of the *cagPAI* varies between different populations and geographical regions, with variations of 50%–65% in areas of the Indian subcontinent<sup>[25,26]</sup>, 32% in France<sup>[27]</sup>, 9% in Sweden<sup>[28]</sup> and the United States<sup>[29]</sup> and 1% in Japan<sup>[30]</sup>. In the present study, more than 90% of the clinical isolates were positive for at least one of the selected *cagPAI* genes. The *cagPAI* genes tested in this study did not include the *cagA* gene, as we previously demonstrated<sup>[31]</sup> that the majority of the *H. pylori* strains circulating in our study population were *cagA*-positive. In the six genetic loci studied, the most frequently deleted gene was *cagI3*, and the least frequently deleted gene was *cagT*. The IS605 insertion sequence was present in less than 5% of the *H. pylori* isolates examined, and the majority of the isolates harboured a rearranged *cagPAI* gene. Analyses of

the individual *cagPAI* genes showed that *cag67* was more conserved in the isolates from the Chinese patients, whereas the rearrangement of *cagE* occurred at a higher frequency in the isolates from the Malay and Indian patients, although this difference was not significant.

We also observed that the majority of the *H. pylori* clinical isolates expressed partially deleted *cagPAI* regions and that only few isolates exhibited either intact or deleted *cagPAI* regions. Previous studies have reported the occurrence of different proportions of intact *cagPAI* in their isolates. Ali *et al.*<sup>[32]</sup> found that 37.4% of isolates exhibited intact *cagPAI* regions, whereas other researchers<sup>[30,33]</sup> reported that more than 96% of isolates contained intact *cagPAI*. In the present study, the isolates expressing intermediate genotypes (deletions within the *cagPAI* region) were more commonly encountered than those isolates with intact or deleted *cagPAI* regions. Moreover, a large proportion of the isolates exhibiting *cagPAI* rearrangement lacked an IS605 sequence, suggesting that this insertion sequence did not play a role in *cagPAI* disruption in the clinical isolates.

Additionally, we found that no single gene in the *cagPAI* region can be used as a genetic marker for an intact *cagPAI* because a large proportion of the clinical isolates exhibited gene rearrangement in the *cagPAI* region. Due



**Table 5** Distribution of *Helicobacter pylori* *cagPAI* genotypes, *vacA* alleles and metronidazole susceptibilities *n* (%)

<i>cagPAI</i> status	<i>vacA</i>		Metronidazole susceptibility		<i>vacA</i> /metronidazole susceptibility			
	s1m1	s1m2	Sensitive	Resistant	s1m1/sensitive	s1m1/resistant	s1m2/sensitive	s1m2/resistant
Intact ( <i>n</i> = 2) <sup>1</sup>	0	2 (100)	2 (100)	0	0	0	2 (100)	0
Partially deleted								
Type C ( <i>n</i> = 26)	10 (38.5)	16 (61.5)	14 (53.8)	12 (46.2)	5 (19.2)	5 (19.2)	9 (34.6)	7 (26.9)
Type I ( <i>n</i> = 16)	12 (75.0)	4 (25.0)	10 (62.5)	6 (37.5)	7 (43.8)	5 (31.2)	3 (18.8)	1 (6.2)
Type N ( <i>n</i> = 13)	11 (84.6)	2 (15.4)	8 (61.5)	5 (38.5)	7 (53.8)	4 (50.0)	1 (12.5)	1 (12.5)
Deleted ( <i>n</i> = 6) <sup>2</sup>	1 (16.7)	5 (83.3)	3 (37.5)	5 (62.5)	0	1 (16.7)	2 (33.3)	3 (50.0)

<sup>1</sup>One strain with the *vacA* s1m1m2 genotype was not included; <sup>2</sup>Two strains with the *vacA* s2m2 genotype were not included.

to the region's large size (approximately 40 kbp), the absence of these genes in certain clinical isolates did not necessarily indicate the complete absence of the *cagPAI*, as indicated in 9 isolates positive for a single gene. Regarding *cagPAI* rearrangement, 19 different genotypes (types B to T) have been identified. The three most common *cagPAI* genotypes identified in the clinical strains revealed that the genes at the left end of the *cagPAI* region were more unstable and prone to rearrangement than the genes in the middle and at the right end of the *cagPAI* region. This finding was supported by analyses of the individual genes, which demonstrated more gene rearrangement at the left end of the *cagPAI* region (*cag13*, *cag10* and *cag67*). Therefore, the genes in the middle and at the right end of the *cagPAI* region may play an important role in the pathogenesis of *H. pylori* infection in the study population.

In the current study, specific *cagPAI* genotypes were isolated from patients of different ethnicities. All of the isolates with intact *cagPAI* regions and less *cagPAI* rearrangement were from the Chinese patients, whereas the Indian patients were infected with strains exhibiting more rearranged or deleted *cagPAI* regions, and the Malay patients tended to be infected with various *cagPAI* genotypes. Thus, the presence of different *cagPAI* genotypes in different ethnicities may be related to the genetic characteristics of both the colonising *H. pylori* strain and the host. Although the relationship between the *cagPAI* genotype and disease state was unclear, we observed that certain types of infecting strains exhibited different *cagPAI* rearrangements in nonulcer and ulcer patients.

A significant difference was observed in the prevalence of *vacA* genotypes among patients of distinct ethnicities. As the Chinese patients were more likely to be infected with the *vacA* s1m2 strains, these strains may be regarded as more pathogenic, consistent with previous reports showing a higher frequency of peptic ulcers and gastric cancer in the Chinese patients in a similar study population<sup>[15,34]</sup>. In our study population, the smaller proportion of patients with PUD than NUD may have contributed to the lack of a significant association between the endoscopic findings and patient ethnicity, as analysis was conducted on the samples with positive cultures. Additionally, we demonstrated that the *vacA* s1m2 genotype was significantly associated with enhanced gas-

tric inflammation<sup>[10]</sup>. In contrast, the *vacA* s2m2 strains were isolated only from the Malay and Indian patients, suggesting that these strains are less virulent. Consistent with this finding, Chinese ethnicity has been associated with infection by strains lacking the *vacA* s2m2 allele<sup>[35,36]</sup>. As Miernyk *et al.*<sup>[37]</sup> recently reported a high proportion of *vacA* s2m2 strains isolated from Alaskan natives, it would be interesting to examine whether the disease outcome in Alaskan natives is similar to the outcome in the Malay and Indian patients in our study population. The current study also revealed the proportions of different *vacA* subgenotypes in each ethnic population. More specifically, the *vacA* s1cm1 genotype was not detected in the isolates from the Indian patients, whereas the *vacA* s1cm2 genotype was only detected in the isolates from the Chinese patients.

A previous report demonstrated a higher prevalence of resistance to metronidazole than to other antibiotics in *H. pylori* isolates from Malaysia<sup>[14]</sup>. In the current study, we attempted to further relate the metronidazole susceptibility of *H. pylori* to patient ethnicity and to correlate this susceptibility with the *cagPAI* and *vacA* genotypes. We noted a significant association between the metronidazole-sensitive *H. pylori* strains and Chinese ethnicity, whereas the Malay and Indian patients were more likely to be infected with the metronidazole-resistant strains. This finding may reflect differences in metronidazole use between different ethnic populations. The pattern of metronidazole susceptibility in different ethnicities paralleled the specific genotypes of the infecting strains. For example, the strains of the specific *cagPAI* genotype and *vacA* allele isolated from the Chinese patients were more sensitive to metronidazole.

The presence of frameshift and nonsense mutations in the antibiotic-resistant *H. pylori* strains suggested that these mutations confer resistance to metronidazole. However, more than half of the resistant strains exhibited missense mutations that were also detected in the antibiotic-sensitive strains. This finding implied that *rdxA* is not the only gene involved in the metronidazole-resistant phenotype and thus is not a reliable epidemiological marker. Rather, other genes or mechanisms may be implicated in the generation of resistance<sup>[38-40]</sup>.

We observed no association between the specific *H. pylori* genotypes and the strains' antibiotic susceptibilities

in severe disease. These results may be complicated by the fact that most of the patients in our study population had gastritis. Although the *cagPAI* and *vacA* alleles are important virulence factors in infection, the development of disease is likely to involve a highly complex interplay of many bacterial and/or host factors.

The distribution of the *vacA* s1m2 genotype was broad, as this genotype was detected in the majority of the strains with intact *cagPAI* regions or *cagPAI* rearrangement of type C (positive for more *cagPAI* genes than other types) and the metronidazole-resistant strains. This finding may indicate that the *vacA* s1m2 strains have variable pathogenic properties when combined with other genotypic characteristics. The isolates with the deleted *cagPAI* regions were also primarily linked to metronidazole resistance. As these isolates may induce less inflammation in the host gastric epithelia, their genotypic characteristics may reduce antibiotic delivery and thus hinder the eradication of *H. pylori*. In contrast, the *vacA* s1m2 genotype could not contribute to metronidazole resistance, as this genotype was distributed evenly between the antibiotic-sensitive and -resistant strains<sup>[41,42]</sup>.

In conclusion, we report a large proportion of *H. pylori* isolates harbouring *cagPAI* rearrangement and demonstrate that metronidazole susceptibility varies with patient ethnicity. The distinct distribution of the *H. pylori* *cagPAI* genotypes, *vacA* alleles and metronidazole susceptibilities in the different ethnicities of Malaysia may contribute to varying risk of gastroduodenal diseases. These distinct, patient ethnicity-associated *H. pylori* genotypes may have important clinical and epidemiological implications. Finally, the present study of *H. pylori*-specific genotypes from different host genetic backgrounds enhances our understanding of bacterium-host interactions and bacterial ecology in various niches. Further information on the characteristics of *H. pylori* will allow a more precise identification of virulent strains and a better definition of risk factors in susceptible hosts.

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## COMMENTS

### Background

The prevalence of *Helicobacter pylori* (*H. pylori*) infection in Malaysia consistently revealed ethnic differences, with Indians exhibiting higher infection rates than the Chinese and Malays. In contrast, peptic-ulcer disease and gastric carcinoma are known to be more prevalent in the Chinese and lowest in the Indians. The great genetic diversity of *H. pylori* may play an important role in the consequences of infection in different hosts. The *cagPAI* and *vacA* genes are well-established *H. pylori* virulence factors that interact with the host cells and disrupt downstream signalling pathways. The *cagPAI* structures, *vacA* alleles

and metronidazole susceptibilities of different strains in hosts of varying genetic makeup may enhance the knowledge of bacterium-host interactions in *H. pylori* infections in multiethnic populations.

### Research frontiers

The specific genotypes stemming from the *cagPAI* and *vacA* alleles were identified in *H. pylori* strains isolated from different ethnic groups. The strains isolated from the Chinese and peptic-ulcer disease (PUD) patients were more sensitive to antibiotics, indicating a selective advantage that occurred during early infection and persisted in chronic infection. In the present study, however, no association between the specific *H. pylori* genotypes and PUD could be determined due to the small number of PUD cases. Consistent with this observation, previous epidemiological studies have reported that less than 20% of patients infected with *H. pylori* are diagnosed as PUD.

### Innovations and breakthroughs

Past reports have highlighted the importance of *H. pylori* genetic diversity in chronic infection. The current study emphasises and adds to findings from the same institution, demonstrating that the genetic background of the *H. pylori* strains may play an important role in the risk of gastroduodenal disease in different ethnic groups.

### Applications

The results of this study provide insight into the effects of the genomic diversity of *H. pylori* on hosts of different genetic backgrounds. Therefore, these findings can be used in the development of advanced screening tools for diagnosing and determining the prognosis of *H. pylori* infection.

### Terminology

The term *cagPAI* is defined as the *cag* pathogenicity island, a common gene sequence believed to be responsible for pathogenesis. This sequence contains approximately 40 kbp of nucleotides encoding over 40 genes. The pathogenicity island is typically absent from the *H. pylori* strains isolated from human carriers of *H. pylori* who remain asymptomatic.

### Peer review

The authors investigated the diversity of the *H. pylori* virulence factors (the *cagPAI* and *vacA* alleles) and metronidazole susceptibilities of strains isolated from patients of different ethnicities. The association between specific *H. pylori* genotypes and patient ethnicity provides insight into the pathogenesis of *H. pylori* infection in different hosts and possibly the different risk factors in *H. pylori* infection.

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## Abnormal splenic artery diameter/hepatic artery diameter ratio in cirrhosis-induced portal hypertension

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**RESULTS:** The receiver operating characteristic analysis showed that the cutoff points for abnormal splenic artery internal diameter and S/P ratio were  $> 5.19$  mm and  $> 1.40$ , respectively. The sensitivity, specificity, positive predictive value, and negative predictive value were 74.2%, 45.2%, 97.1%, and 6.6%, respectively. The prevalence of an abnormal S/P ratio in the patients with cirrhosis and portal hypertension was 83.4%. Patients with a higher S/P ratio had a lower risk of developing ascites [odds ratio (OR) = 0.708, 95%CI: 0.508-0.986,  $P = 0.041$ ] and a higher risk of developing esophageal and gastric varices (OR = 1.483, 95%CI: 1.010-2.175,  $P = 0.044$ ) and forming collateral circulation (OR = 1.518, 95%CI: 1.033-2.230,  $P = 0.034$ ). After splenectomy, the portal venous pressure and maximum and mean portal venous flow velocities were reduced, while the flow rate and maximum and minimum flow velocities of the hepatic artery were increased ( $P < 0.05$ ).

**CONCLUSION:** The prevalence of an abnormal S/P ratio is high in patients with cirrhosis and portal hypertension, and it can be used as an important marker of splanchnic hemodynamic disturbances.

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### Abstract

**AIM:** To determine an optimal cutoff value for abnormal splenic artery diameter/proper hepatic artery diameter (S/P) ratio in cirrhosis-induced portal hypertension.

**METHODS:** Patients with cirrhosis and portal hypertension ( $n = 770$ ) and healthy volunteers ( $n = 31$ ) underwent volumetric computed tomography three-dimensional vascular reconstruction to measure the internal diameters of the splenic artery and proper hepatic artery to calculate the S/P ratio. The cutoff value for abnormal S/P ratio was determined using receiver operating characteristic curve analysis, and the prevalence of abnormal S/P ratio and associations between abnormal S/P ratio and major complications of portal hypertension were studied using logistic regression.

**Key words:** Portal hypertension; Cirrhosis; Splenic artery internal diameter; Proper hepatic artery internal diameter; Complications; Splenectomy

Zeng DB, Dai CZ, Lu SC, He N, Wang W, Li HJ. Abnormal splenic artery diameter/hepatic artery diameter ratio in cirrhosis-induced portal hypertension. *World J Gastroenterol* 2013; 19(8): 1292-1298 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i8/1292.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i8.1292>

### INTRODUCTION

Splanchnic and systemic hemodynamic disorders are

common in patients with cirrhosis<sup>[1,2]</sup>. The systemic hyperdynamic circulation manifests as increased cardiac output and heart rate, and decreased arterial blood pressure and systemic vascular resistance<sup>[3,4]</sup>. The splanchnic hemodynamic disorder mainly manifests as increased vascular resistance and comparative visceral ischemia, which are associated with hepatic encephalopathy, hepatorenal syndrome, and portal hypertension<sup>[5]</sup>. Currently, portal hypertension has been found to be attributed to abnormally high portal venous inflow and high resistance in the portal system<sup>[6]</sup>.

The splanchnic hemodynamic disorder in portal hypertension has been studied in a few studies, and some results support the significance of splenic and hepatic artery changes in portal hypertension. As early as in 1970, Yamauchi *et al*<sup>[7]</sup> observed an increase in the radius of the splenic artery in patients with portal hypertension, and such increase was not present in splenomegaly without portal hypertension. They found an association between the radius of the splenic artery and portal pressure and the degree of reduction in the portal pressure after splenectomy in patients with portal hypertension, and the dilatation of the splenic artery was accompanied by an increase in the diameter of the proper hepatic artery in those patients<sup>[7]</sup>. Subsequent studies confirmed the increases in the diameters of the splenic and hepatic arteries in patients with liver cirrhosis as compared with healthy controls<sup>[8,9]</sup>, and further found that changes in the splenic artery occur prior to the onset of clinically evident portal hypertension<sup>[8]</sup>.

Splenic and hepatic arterial blood flow distribution abnormalities are also very common in patients who have received liver transplantation. A study by Quintini *et al*<sup>[10]</sup> confirmed that hepatic artery hypoperfusion in liver transplant recipients can be induced by increased portal venous blood flow because over-perfusion of the portal vein cleared adenosine at the terminal end of the hepatic artery, resulting in hepatic artery contraction, *i.e.*, “hepatic arterial buffer response”. Persistence of this response may cause further deterioration of splenic and hepatic arterial blood flow abnormalities, and result in a series of related complications known as splenic artery steal syndrome (SASS) or splenic artery syndrome that includes liver dysfunction, ischemic bile duct injury, and graft dysfunction or loss. Kirbas *et al*<sup>[11]</sup> performed multi-detector computed tomography (CT) in post-liver transplanted patients, and the authors proposed that a splenic artery diameter > 4 mm or a splenic artery diameter/hepatic artery diameter ratio > 1.5 indicates presence of SASS, thus establishing the importance of splenic artery diameter and the splenic artery diameter/hepatic artery diameter ratio in the clinical diagnosis of splenic and hepatic arterial blood flow distribution abnormalities.

Small-for-size syndrome (SFSS) is a common clinical complication that occurs after segmental liver transplantation<sup>[12,13]</sup>. Although the pathogenesis of the SFSS is still controversial, it is assumed that portal hypoperfusion with high intravascular shear stress is a causative factor<sup>[14-16]</sup>. In 2003, a group of researchers reported that

portal flow should be modulated perioperatively in adult living-donor liver transplantation if a suboptimal graft-to-recipient body weight ratio is accompanied by high recipient portal inflow<sup>[17]</sup>. The authors found that splenic artery ligation could decrease recipient portal inflow, increase recipient hepatic arterial flow, and resolve refractory ascites, and neither SFSS nor vascular complications occurred in recipients undergoing splenic artery ligation<sup>[17]</sup>. Grutadauria *et al*<sup>[18]</sup> obtained similar findings when they applied splenic artery embolization (SAE) for the treatment of SFSS after living-related donor liver transplantation. Their results showed that clinical conditions in patients with SFSS rapidly improved after SAE, and they thought that early SAE can protect the segmental graft against the harmful effects of the portal overflow<sup>[18]</sup>. Other animal and clinical studies of patients with liver cirrhosis and idiopathic portal hypertension showed that splenectomy can reduce portal venous blood flow and portal venous pressure, and increase hepatic arterial blood flow<sup>[19,20]</sup>.

In this study, we examined whether splenic artery diameter and the splenic artery internal diameter/proper hepatic artery internal diameter (S/P) ratio are of clinical significance in patients with cirrhosis and portal hypertension. We determined the optimal cutoff values for abnormal splenic artery diameter and S/P ratio, observed the prevalence of an abnormal S/P ratio, and studied the associations between the S/P ratio and major complications of portal hypertension in patients with cirrhosis. The preoperative and postoperative portal vein hemodynamic indexes were further compared in patients undergoing splenectomy to explore the significance of splenectomy in the treatment of portal hypertension and its complications.

## MATERIALS AND METHODS

### Participants

A total of 1000 patients with liver cirrhosis and portal hypertension who sought medical treatment at our hospital from January 2011 to May 2011 were recruited. All patients had a pathological diagnosis of liver cirrhosis. Clinical diagnosis of portal hypertension was made in the setting of end-stage liver disease, and in the presence of ascites and/or varices. Of the 1000 patients, 230 were excluded: 109 with hepatic artery variations, 66 with post-splenectomy, 51 with partial splenic artery embolization, and 4 with transjugular intrahepatic portosystemic stent shunting. Thus, a total of 770 patients with liver cirrhosis and portal hypertension were enrolled (554 males and 216 females), of whom 558 had biochemical examination results. Thirty-one healthy volunteers who participated in the donor screening for the living-related liver transplantation were also enrolled. This study was approved by the Institutional Review Boards of our hospital, and all the participants provided written informed consent.

### Laboratory tests

All the patients received routine blood, coagulation, and liver function tests in a fasting state. The routine blood

tests were performed with an SYSMES-2100 automatic blood cell analyzer and reagents (SYSMES Co., Japan). Coagulation tests were performed with a CA-7000 automatic coagulation instrument and ancillary reagents (SYSMES Co., Japan). Liver function tests were performed with an AU5400 automatic biochemical analyzer and related reagents (Mishima Olympus Co., Japan).

### **Measurement of splenic and proper hepatic artery internal diameter**

All the patients underwent abdominal enhanced computed tomography (CT) scanning with a General Electric (GE) Lightspeed VCT 64-slice scanner (GE, Milwaukee, WI, United States). For scanning, the collimator width was 0.625 mm, the tube current was 380 mA, the tube voltage was 120 kV, the slice thickness was 5 mm, the slice interval was 5 mm, and the reconstruction slice thickness was 0.625 mm. A high-pressure syringe was used to inject 100 mL contrast agent iopromide (370 mg/mL) via the antecubital vein at a flow rate of 2.5-3.5 mL/s, and the scan phases were the arterial phase (20-25 s), venous phase (65-70 s), and delayed phase (180 s). The data were input into a GE ADW 4.3 image workstation, and multiplanar reconstruction, maximum intensity projection, and volume rendering post-processing were performed. The splenic and proper hepatic artery internal diameters were measured in the arterial phase. The internal diameter of the proper hepatic artery was measured within 1 cm from its origin. The internal diameter of the splenic artery was measured at 3 locations: within 1 cm from its origin, at the upper edge of the pancreas, and within 1 cm from the point where the splenic artery branches originate. The mean value was calculated and recorded as the internal diameter of the splenic artery. The S/P ratio was then calculated.

### **Measurement of hemodynamic indexes of portal vein and hepatic artery before and after splenectomy**

The free portal venous pressure was measured by catheterization via the right gastroepiploic vein before and after splenectomy. Doppler ultrasound (Esaote, Italy) was used to measure the hemodynamic indexes of the portal vein and hepatic artery within 1 wk prior to splenectomy and within 2 wk after splenectomy. Indexes measured were the portal venous flow rate and the maximum and mean portal venous flow velocities, the flow rate and maximum and minimum flow velocities of the hepatic artery, and the resistance index (RI) of the hepatic artery.

### **Statistical analysis**

Demographic characteristics were presented as number (%) for sex, causes of cirrhosis, and level of liver function, mean  $\pm$  SD for age, and median [interquartile range (IQR): Q1-Q3] for the internal diameters of the splenic artery and proper hepatic artery and the S/P ratio because data were not normally distributed. Data of patients with cirrhosis and healthy controls were compared using Pearson  $\chi^2$  test for sex, two-sample *t* test for age, and Mann-Whitney *U* test for splenic artery diameter,

proper hepatic artery diameter, and the S/P ratio, respectively. Receiver operating characteristic (ROC) curve analysis through binary logistic regression was carried out to identify the optimal cutoff values of abnormal internal diameter of the splenic artery and S/P ratio. The prevalence of an abnormal S/P ratio in patients with cirrhosis and portal hypertension was calculated based on the identified cutoff value. Associations between the S/P ratio and complications of cirrhosis and portal hypertension were analyzed using univariate logistic regression. The preoperative and postoperative portal vein hemodynamic indexes and routine blood test data of patients undergoing splenectomy were presented as mean  $\pm$  SD or median (IQR: Q1-Q3) if data were not normally distributed. The preoperative and postoperative data were compared using paired *t* test or Wilcoxon sign-rank test if data were not normally distributed. All statistical assessments were two-tailed and the significance level  $\alpha$  was set at 0.05. All statistical analyses were performed using SPSS 17.0 statistics software (SPSS Inc, Chicago, IL, United States).

## **RESULTS**

### **Demographic characteristics of participants**

A total of 770 patients with cirrhosis and portal hypertension (554 males and 216 females) and 31 healthy controls (16 males and 15 females) were enrolled. Their demographic characteristics are summarized in Table 1. The mean age of patients with cirrhosis and portal hypertension was  $53.3 \pm 11.3$  years, and the mean age of healthy controls was  $46.0 \pm 12.0$  years. Among the patients with cirrhosis and portal hypertension, 622 had cirrhosis due to hepatitis B, 61 had alcoholic cirrhosis, 39 had cirrhosis due to hepatitis C, 24 had autoimmune liver disease, 7 had drug-induced liver damage, 4 had Budd-Chiari syndrome, 4 had cirrhosis caused by hepatitis B and C, 3 had alcoholic cirrhosis with hepatitis B virus infection, 2 had idiopathic portal hypertension, 1 had hepatolenticular degeneration, 1 had cryptogenic cirrhosis, 1 had nonalcoholic fatty liver cirrhosis, and 1 had cirrhosis due to unknown cause.

### **Internal diameter of splenic artery and S/P ratio**

Patients with cirrhosis and portal hypertension had a higher internal diameter of the splenic artery and S/P ratio than healthy controls (both,  $P < 0.05$ ). The median internal diameter of the splenic artery was 5.35 mm (IQR: 4.67-6.18 mm) in patients with cirrhosis and portal hypertension and 4.60 mm (IQR: 4.32-5.32 mm) in healthy controls. The median S/P ratio was 1.48 (IQR: 1.19-1.82) in patients with cirrhosis and portal hypertension and 1.36 (1.13-1.48) in healthy controls (Table 1). The internal diameter of the proper hepatic artery was not significantly different between patients with cirrhosis and portal hypertension and healthy controls.

ROC curve analysis showed that the optimal cutoff values of abnormal internal diameter of the splenic artery and S/P ratio were  $> 5.19$  mm and  $> 1.40$ , respectively (Figure 1). The sensitivity, specificity, positive pre-

**Table 1** Demographic characteristics of participants *n* (%)

Variables	Patients with cirrhosis and portal hypertension ( <i>n</i> = 770)	Healthy controls ( <i>n</i> = 31)	<i>P</i> value
Sex			0.025 <sup>1</sup>
Male	554 (71.9)	16 (51.6)	
Female	216 (28.1)	15 (48.4)	
Age (yr)	53.3 ± 11.3	46.0 ± 12.0	< 0.001 <sup>1</sup>
Causes of cirrhosis			NA
Hepatitis B	622 (80.8)	-	
Hepatitis C	39 (5.1)	-	
Alcoholic cirrhosis	61 (7.9)	-	
Autoimmune liver disease	24 (3.1)	-	
Others	24 (3.1)	-	
Level of liver function			NA
I	230 (29.9)	-	
II	209 (27.1)	-	
III	119 (15.5)	-	
Undefined	212 (27.5)	-	
Splenic artery internal diameter (mm)	5.35 (4.67-6.18)	4.60 (4.32-5.32)	0.002 <sup>1</sup>
Proper hepatic artery internal diameter (mm)	3.63 (2.97-4.43)	3.72 (3.14-4.19)	0.830
S/P ratio	1.48 (1.19-1.82)	1.36 (1.13-1.48)	0.012 <sup>1</sup>

<sup>1</sup>Indicates significant difference between patients with cirrhosis and portal hypertension and healthy controls. Data were presented as number (%) for sex, cause of cirrhosis, and level of liver function; mean ± SD for age; and median (interquartile range: Q1-Q3) for the internal diameters of the splenic artery and proper hepatic artery and the splenic artery internal diameter/proper hepatic artery internal diameter (S/P) ratio because data were not normally distributed. Data of patients with cirrhosis and healthy controls were compared using Pearson  $\chi^2$  for sex, two-sample *t* test for age, and Mann-Whitney *U* test for S/P ratio, respectively. NA: Not assessed.

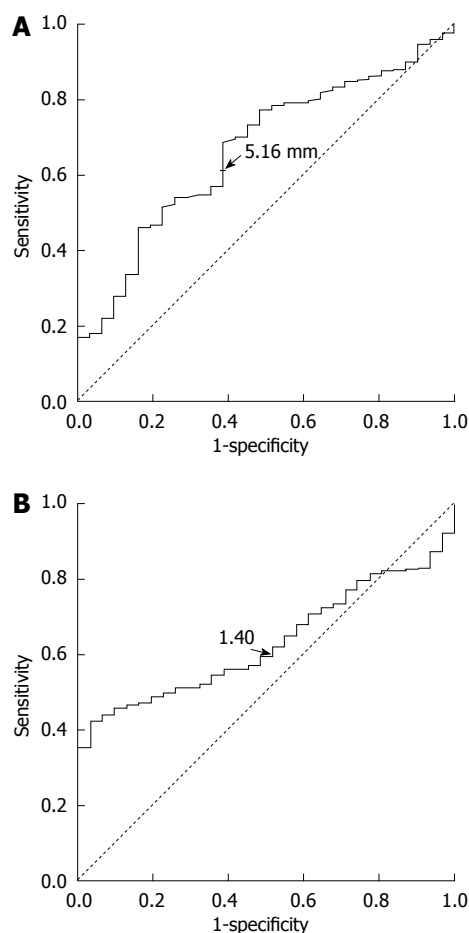
dictive value, and negative predictive value were 74.2%, 45.2%, 97.1%, and 6.6%, respectively. Using the cutoff value of 1.40, 640 patients (83.4%) with cirrhosis and portal hypertension had an abnormal S/P ratio, and 28 healthy controls (90.3%) had a normal S/P ratio.

### Associations between S/P ratio and complications in patients with cirrhosis and portal hypertension

Results of univariate logistic regression are presented in Table 2. The results showed that patients with a higher S/P ratio had a lower risk of developing ascites [odds ratio (OR) = 0.708, 95%CI: 0.508-0.986, *P* = 0.041]. However, patients with a higher S/P ratio had a higher risk of developing esophageal and gastric varices (OR = 1.483, 95%CI: 1.010-2.175, *P* = 0.044) and forming collateral circulation (OR = 1.518, 95%CI: 1.033-2.230, *P* = 0.034).

### Portal vein hemodynamic indexes and routine blood test results in patients with cirrhosis and portal hypertension before and after splenectomy

A total of 17 patients with cirrhosis and portal hypertension underwent splenectomy. Table 3 presents the comparison of the preoperative and postoperative portal vein hemodynamic indexes in these patients. After splenectomy, the portal venous pressure and maximum and mean portal venous flow velocities were significantly reduced



**Figure 1** Receiver operating characteristic curve analysis for identifying the optimal cutoff values. A: Abnormal internal diameter of the splenic artery; B: Abnormal splenic artery internal diameter/proper hepatic artery internal diameter (S/P) ratio for patients with cirrhosis and portal hypertension. Binary logistic regression showed that the areas under the receiver operating characteristic curve were 0.667 (95%CI: 0.581-0.753) and 0.633 (95%CI: 0.572-0.694) for the internal diameter of the splenic artery and S/P ratio, respectively (both, *P* < 0.05). The optimal cutoff values were identified to be > 5.19 mm for abnormal internal diameter of the splenic artery and > 1.40 for abnormal S/P ratio.

compared with the preoperative values (all, *P* < 0.05). The postoperative flow rate, maximum flow velocity, and minimum flow velocity of the hepatic artery were significantly higher than the preoperative values (all, *P* < 0.05). The RI of the hepatic artery was significantly reduced after splenectomy (*P* < 0.001).

Table 4 presents the comparison of the results of the preoperative and postoperative routine blood tests in patients with cirrhosis and portal hypertension who underwent splenectomy. After splenectomy, total bilirubin, direct bilirubin, white blood cell (WBC) count, and platelet count were significantly increased (all, *P* < 0.05), and creatinine levels were significantly decreased (*P* < 0.05).

## DISCUSSION

The results of this study showed that patients with cirrhosis and portal hypertension had a significantly greater internal diameter of the splenic artery and higher S/P ratio than healthy controls, but not a significantly differ-



**Table 2** Univariate logistic regression analyses of associations between splenic artery internal diameter/proper hepatic artery internal diameter ratio and complications in patients with cirrhosis and portal hypertension *n* (%)

Complication	Total ( <i>n</i> = 770)	Patients with normal S/P ratio ( <i>n</i> = 130)	Patients with abnormal S/P ratio ( <i>n</i> = 640)	OR (95%CI)	<i>P</i> value
Ascites					
Yes	426 (55.7)	304 (53.5)	122 (61.9)	0.708 (0.508-0.986)	0.041 <sup>1</sup>
No	339 (44.3)	264 (46.5)	75 (38.1)	Reference	
Hepatic encephalopathy					
Yes	77 (10.1)	58 (10.2)	19 (9.6)	1.065 (0.617-1.838)	0.820
No	688 (89.9)	510 (89.8)	178 (90.4)	Reference	
Esophageal and gastric varices					
Yes	209 (27.4)	166 (29.3)	43 (21.8)	1.483 (1.010-2.175)	0.044 <sup>1</sup>
No	555 (72.6)	401 (70.7)	154 (78.2)	Reference	
Upper gastrointestinal bleeding					
Yes	111 (14.5)	90 (15.9)	21 (10.7)	1.581 (0.954-2.621)	0.076
No	653 (85.5)	477 (84.1)	176 (89.3)	Reference	
Collateral circulation formation					
Yes	611 (79.9)	464 (81.7)	147 (74.6)	1.518 (1.033-2.230)	0.034 <sup>1</sup>
No	154 (20.1)	104 (18.3)	50 (25.4)	Reference	

<sup>1</sup>Indicates significant association. The incidences of ascites, hepatic encephalopathy, esophageal and gastric varices, Upper gastrointestinal bleeding, and collateral circulation formation were not available in 5, 5, 6, 6, and 5 patients, respectively. OR: Odds ratio.

**Table 3** Comparisons of preoperative and postoperative portal vein hemodynamic indexes in patients with cirrhosis and portal hypertension who received splenectomy (*n* = 17)

Variables	Before splenectomy	After splenectomy	<i>P</i> value
Portal vein			
Pressure (cm H <sub>2</sub> O)	39.19 ± 5.87	27.04 ± 3.03	< 0.001 <sup>1</sup>
Flow rate (mL/min)	1005.50 (833.00-1811.25)	868.00 (539.50-1215.75)	0.116
Maximum flow velocity (cm/s)	23.85 (18.53-31.20)	15.85 (12.68-20.58)	0.009 <sup>1</sup>
Mean flow velocity (cm/s)	20.85 (15.90-25.95)	12.25 (10.48-16.40)	0.011 <sup>1</sup>
Hepatic artery			
Flow rate (mL/min)	153.50 (128.75-188.77)	365.00 (190.00-511.00)	0.048 <sup>1</sup>
Minimum flow velocity (cm/s)	16.25 ± 15.23	32.60 ± 15.23	0.001 <sup>1</sup>
Resistance index	0.74 ± 0.05	0.64 ± 0.07	< 0.001 <sup>1</sup>

<sup>1</sup>Indicates significant difference between preoperative and postoperative values. Data were presented as mean ± SD, or median (interquartile range: Q1-Q3) if not normally distributed. Preoperative and postoperative data were compared using paired *t*-test or Wilcoxon sign-rank test if data were not normally distributed.

**Table 4** Comparisons of preoperative and postoperative routine blood test results in patients with cirrhosis and portal hypertension who received splenectomy (*n* = 17)

Variable	Before splenectomy	After splenectomy	<i>P</i> value
ALT (U/L)	29.39 ± 16.86	36.47 ± 28.9	0.439
AST (U/L)	33 (21.9-45.4)	37 (26.65-48.45)	0.653
Total bilirubin (μmol/L)	16.8 (12.95-24.9)	21.4 (16.2-39.55)	0.005 <sup>1</sup>
Direct bilirubin (μmol/L)	4.74 ± 2.13	7.56 ± 4.31	0.003 <sup>1</sup>
Albumin (g/L)	37.67 ± 3.73	36.74 ± 3.28	0.439
Creatinine (μmol/L)	70.44 ± 22.28	58.69 ± 14.78	0.036 <sup>1</sup>
WBC count (E + 09/L)	1.73 (1.26-2.24)	9.05 (6.43-12.84)	< 0.001 <sup>1</sup>
RBC count (E + 12/L)	3.45 ± 0.73	3.42 ± 0.47	0.87
Platelet count (E + 09/L)	47.41 ± 24.48	307 ± 132.4	< 0.001 <sup>1</sup>

<sup>1</sup>Indicates significant difference between preoperative and postoperative values. Data were presented as mean ± SD, or median (interquartile range: Q1-Q3) if not normally distributed. Preoperative and postoperative data were compared using paired *t* test or Wilcoxon sign-rank test if data were not normally distributed. ALT: Alanine amino transferase; AST: Aspartate amino transferase; WBC: White blood cell; RBC: Red blood cell.

ent internal diameter of the proper hepatic artery. The finding of an increased diameter of the splenic artery in patients with cirrhosis and portal hypertension is con-

sistent with the findings of prior studies<sup>[7-9]</sup>. The results indicate that excessive perfusion of the splenic artery increases the return blood flow in the splenic vein, thereby

increasing the blood flow in the portal venous system resulting in portal hypertension. However, an increase in the diameter of the hepatic artery was observed in patients with portal hypertension in some early studies<sup>[7-9]</sup>, a finding not observed in our study. A possible reason for this discrepancy is that different imaging methods were used to measure the arterial internal diameter and current enhanced CT scanning is more accurate than the imaging methods used in earlier studies.

The optimal cutoff values for the abnormal internal diameter of the splenic artery and S/P ratio were identified to be  $> 5.19$  mm and  $> 1.40$ , respectively, by the ROC curve analysis. When the abnormal S/P ratio  $> 1.40$  was used, 83.4% of patients with cirrhosis and portal hypertension had an abnormal S/P ratio. The prevalence of an abnormal S/P ratio is very high in patients with cirrhosis and portal hypertension, indicating that an abnormal S/P ratio can be used as a marker of splanchnic hemodynamic disturbances in these patients. Machálek *et al*<sup>[21]</sup> summarized measurements of the splenic artery internal diameter of 30 normal spleens, and found that splenic artery thickening was indicated by an internal diameter of the splenic artery greater than  $5.6 \pm 1.3$  mm. In our study, we found that an internal diameter of the splenic artery greater than 5.19 mm is the best cutoff value to predict cirrhosis and portal hypertension, which is consistent with the finding of Machálek *et al*<sup>[21]</sup>. Kirbas *et al*<sup>[11]</sup> proposed that a splenic artery diameter  $> 4$  mm or a splenic artery diameter/hepatic artery diameter ratio  $> 1.5$  indicates presence of SASS after liver transplantation, which is similar to the S/P ratio cutoff point of 1.40 for patients with cirrhosis and portal hypertension determined in our study. However, Kirbas *et al*<sup>[11]</sup> reported a much lower abnormal value for the internal diameter of the splenic artery than the value reported by Machálek *et al*<sup>[21]</sup> and our study, which may be due to the a different measurement method used by them.

Our results indicated associations between the S/P ratio and major complications in patients with portal hypertension. Patients with a higher S/P ratio had a lower risk of developing ascites (OR = 0.708, 95%CI: 0.508-0.986,  $P = 0.041$ ) and a higher risk of developing *esophageal* and *gastric* varices (OR = 1.483, 95%CI: 1.010-2.175,  $P = 0.044$ ) and forming collateral circulation (OR = 1.518, 95%CI: 1.033-2.230,  $P = 0.034$ ). The results indicate that the S/P ratio has a great clinical significance for predicting major complications of portal hypertension in patients with cirrhosis.

Studies have also shown that splenectomy can reduce portal venous blood flow and portal venous pressure, increase hepatic arterial blood flow, and improve liver function, thereby decreasing major complications of portal hypertension<sup>[22,23]</sup>. Our study showed that splenectomy significantly reduced portal venous pressure and *maximum* and mean portal venous flow velocities, and increased the flow rate, maximum flow velocity, and minimum flow velocity of the hepatic artery. The study of SASS after liver transplantation by Mogl *et al*<sup>[24]</sup> found a significant elevation of the RI of the hepatic artery ( $0.79 \pm 0.14$ ) in

patients with SASS, which was significantly reduced ( $0.65 \pm 0.09$ ) after splenic artery embolization. In our study, the preoperative and postoperative RIs of the hepatic artery were  $0.74 \pm 0.05$  and  $0.64 \pm 0.07$ , respectively, which is consistent with the findings of Mogl *et al*<sup>[24]</sup>. Our results also showed that postoperative WBC count and platelet levels were significantly increased compared with the preoperative values, indicating that these indexes can be restored to normal values. These results indicate that the major complications of cirrhosis and portal hypertension and splanchnic hemodynamic disturbances can be improved by splenectomy.

The hepatic-venous pressure gradient (HVPG) is the gold standard for the evaluation of the presence and severity of portal hypertension<sup>[25]</sup>. A limitation of this study is that portal hypertension was diagnosed using clinical criteria, but not based on HVPG results. In addition, the mean age of our healthy controls was younger than that of patients with cirrhosis and portal hypertension. However, the diameter of the splenic artery was not significantly affected by age or gender in adults.

In conclusion, the prevalence of an abnormal S/P ratio is high in patients with cirrhosis and portal hypertension, and it can be used as an important marker of splanchnic hemodynamic disturbances in these patients. The S/P ratio is closely related to major complications of portal hypertension including ascites, esophageal and gastric varices, and collateral circulation formation. Splenectomy is effective for decreasing portal venous pressure and delaying the progress of liver cirrhosis and portal hypertension.

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## COMMENTS

### Background

The splanchnic hemodynamic disorder in portal hypertension has been studied, and the significance of splenic and hepatic artery changes in portal hypertension has been noted. In patients who have received liver transplantation, the importance of the splenic artery diameter and the splenic artery diameter/hepatic artery diameter ratio in the clinical diagnosis of splenic and hepatic arterial blood flow distribution abnormalities has been established.

### Research frontiers

Splenic and hepatic arterial blood flow abnormalities are common in patients with cirrhosis and portal hypertension, but the clinical significance of the splenic artery diameter and splenic artery internal diameter/proper hepatic artery internal diameter (S/P) ratio in those patients has not been clearly elucidated.

### Innovations and breakthroughs

For the first time, the authors determined the optimal cutoff values of abnormal internal diameter of the splenic artery and S/P ratio in patients with cirrhosis and portal hypertension, which were  $> 5.19$  mm and  $> 1.40$ , respectively. A high prevalence of an abnormal S/P ratio and close associations between the S/P ratio and the major complications of portal hypertension including ascites, esophageal and gastric varices, and collateral circulation formation were found in patients with cirrhosis and portal hypertension.

### Applications

The study results suggest that an abnormal internal diameter of the splenic artery and S/P ratio are of clinical significance in patients with cirrhosis and

portal hypertension, and an abnormal S/P can be used as an important marker of splanchnic hemodynamic disturbances in these patients.

### Terminology

In this study, the splenic and proper hepatic artery internal diameters refer to the values measured in the arterial phase of abdominal enhanced computed tomographic (CT) scanning. The internal diameter of the proper hepatic artery was measured within 1 cm from its origin. The internal diameter of the splenic artery was measured at 3 places: within 1 cm from its origin, at the upper edge of the pancreas, and within 1 cm from the point that the splenic artery branches originate. The mean value was calculated and recorded as the internal diameter of the splenic artery. The S/P ratio was then calculated.

### Peer review

It is a nice study which sought and found correlations between splenic artery internal diameter/proper hepatic artery internal diameter ratio, calculated by a non-invasive tool (3D CT scan) and clinical complications of portal hypertension. The study is original and the manuscript is well written.

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## Ileal pouch anal anastomosis with modified double-stapled mucosectomy-the experience in China

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### Abstract

**AIM:** To investigate the feasibility and long-term functional outcome of ileal pouch-anal anastomosis with modified double-stapled mucosectomy.

**METHODS:** From January 2002 to March 2011, forty-five patients underwent ileal pouch anal anastomosis with modified double-stapled mucosectomy technique and the clinical data obtained for these patients were reviewed.

**RESULTS:** Patients with ulcerative colitis ( $n = 29$ ) and familial adenomatous polyposis ( $n = 16$ ) underwent ileal pouch-anal anastomosis with modified double-stapled mucosectomy. Twenty-eight patients underwent one-stage restorative proctocolectomy, ileal pouch anal

anastomosis, protective ileostomy and the ileostomy was closed 4-12 mo postoperatively. Two-stage procedures were performed in seventeen urgent patients, proctectomy and ileal pouch anal anastomosis were completed after previous colectomy with ileostomy. Morbidity within the first 30 d of surgery occurred in 10 (22.2%) patients, all of them could be treated conservatively. During the median follow-up of 65 mo, mild to moderate anastomotic narrowing was occurred in 4 patients, one patient developed persistent anastomotic stricture and need surgical intervention. Thirty-five percent of patients developed at least 1 episode of pouchitis. There was no incontinence in our patients, the median functional Oresland score was 6, 3 and 2 after 1 year, 2.5 years and 5 years respectively. Nearly half patients (44.4%) reported "moderate functioning", 37.7% reported "good functioning", whereas in 17.7% of patients "poor functioning" was observed after 1 year. Five years later, 79.2% of patients with good function, 16.7% with moderate function, only 4.2% of patients with poor function.

**CONCLUSION:** The results of ileal pouch anal anastomosis with modified double-stapled mucosectomy technique are promising, with a low complication rate and good long-term functional results.

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**Key words:** Ileal pouch anal anastomosis; Stapled mucosectomy; Ulcerative colitis; Familial adenomatous polyposis; Surgical technique

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## INTRODUCTION

Ileal pouch anal anastomosis (IPAA) is the operative approach for patients requiring restorative proctocolectomy (RPC) due to familial adenomatous polyposis (FAP) and ulcerative colitis (UC). There are two types of IPAA have been performed: a handsewn technique with mucosectomy<sup>[1]</sup> and a stapling technique with the retention of the mucosa of the rectal stump<sup>[2]</sup>. Both approaches have advantages and disadvantages. For handsewn technique with mucosectomy, all colorectal mucosa is removed which is thought freeing the patients from the risk of further inflammatory disease, dysplasia or cancer in UC and reducing the risk of polyposis or cancer in FAP, but the manipulation of the anal canal and the excision of the anal transition zone (ATZ) is associated with significant impairment in anal sensation, therefore results in the risk of postoperative problems with continence<sup>[3]</sup>. For stapling technique with the retention of mucosa which is favoured as it is simpler and provides better functional results, the retention of the potentially diseased rectal mucosa exposes patients not only to disease recurrence but also to malignant degeneration<sup>[4,5]</sup>.

We modify double-stapled techniques by sparing 5 cm short rectal muscular cuff and retaining the mucosa only in ATZ. The aim of this study is to evaluate whether ileal pouch-anal anastomosis with modified double-stapled mucosectomy is a safe and feasible procedure and its long-term functional outcomes.

## MATERIALS AND METHODS

Between January 2002 and March 2011, 16 FAP patients and 29 UC patients received IPAA in our hospital. There were 22 males and 23 females with a mean age of 42.4 years (range: 17-67 years). The study is approved by the institutional ethics committee. The main symptoms included diarrhea, mucus or blood in the stool, abdominal pain, and weight loss. Colonoscopic examinations were performed in all patients, and biopsy results were consistent with adenomatous polyposis and ulcerative colitis. Malignant adenomatous polyps were found in three patients diagnosed with FAP.

### *Surgical procedure and technique*

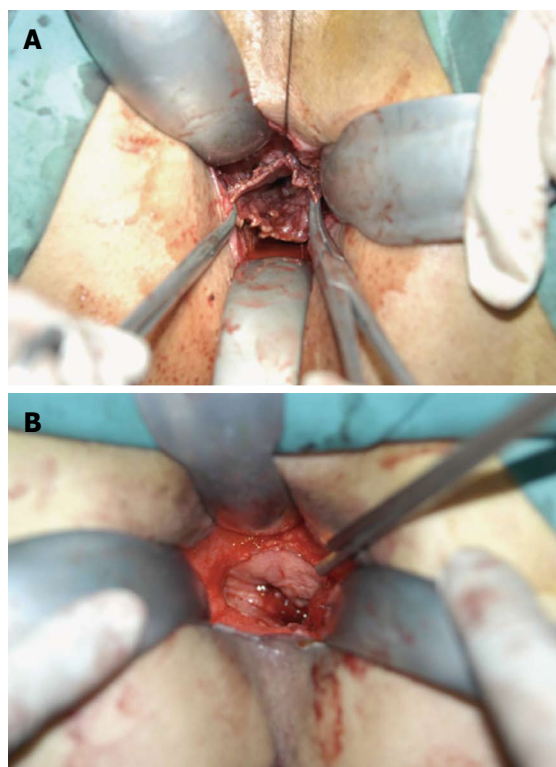
All patients underwent 2-stage procedures. For patients underwent elective surgery, we performed restorative proctocolectomy, ileal J-pouch anal anastomosis and protective ileostomy. Ileostomy was closed 4-12 mo postoperatively.

For patients underwent urgent surgery, we performed colectomy and ileostomy in first stage. Six month later, the proctectomy and ileal J-pouch anal anastomosis were completed. No protective stoma was made.

The operative technique was performed as previously described<sup>[6]</sup> and is modified. After completing mobilization of the colon, the rectum and the ileocecal region,



**Figure 1** Resected specimen.



**Figure 2** Surgical procedure. A: The mucosal and submucosal tube was incised; B: The end-to-end anastomosis was created by circular stapler.

the end of the ileum (5 cm from the ileocecal region for patient with FAP and more than 15 cm for patient with UC) was cut and closed, a J-shaped pouch with a length of 15 cm was made using a linear stapler (Ethicon, PROXIMATE® Linear Cutter 100). Then, the rectum was resected at the level above the seminal vesicle and the specimen was removed (Figure 1). The mesoileum was trimmed to mobilize the pouch and eliminate any tension on it. A 2 cm incision at the end of pouch was made, the anvil was inserted into it after creating a purse-string suture. The anus was effaced using Lone-Star Retractor, 1:100 000 adrenaline normal saline is injected under the rectal mucosa to separate it from the muscular

**Table 1** Demographic and perioperative data

Age at operation (yr) (mean $\pm$ SD)	42.4 $\pm$ 12.5
Gender (male:female)	22:23
Diagnosis (UC:FAP)	29:16:00
Median hemoglobin at the time of admission, g/L [median (range)]	85 (38-148)
Operative time, min [median (range)]	225 (195-325)
Median volume of blood transfusion, mL [median (range)]	600 (0-1600)
Days to regular diet, d [median (range)]	6 (4-14)
Length of postoperative hospitalization, d [median (range)]	12 (9-35)
Interval to closure of the ileostomy, mo [median (range)]	5 (4-12)

UC: Ulcerative colitis; FAP: Familial adenomatous polyposis.

layer. The mucosal and submucosal tube was incised by needle diathermy from 2 cm above the dentate line to the apex of rectal cuff (Figure 2A). We performed trans-anal purse-string suturing of the rectum 2 cm above the dentate line using 2-0 monofilaments, the J-pouch was pulled down into the muscular cuff. Then, a circular stapler was inserted transanally, and the purse-string sutures were tightened to secure the stapler in place, the anvil was attached in position and the end-to-end anastomosis was created (Figure 2B).

The postoperative anal function was assessed by Kirwan classification<sup>[7]</sup> and Pouch-specific function score (Oresland score<sup>[8]</sup>, range, 0-15, with a score of 15 denoting the poorest function). The Kirwan classification was graded as full continence, incontinence of gas, occasional minor soiling, frequent major soiling and incontinence. The Oresland score included items on number of daytime and nighttime bowel movements, seepage, pad usage, urgency, diet, medication, and social handicap. Individual items were summed to form an overall pouch-specific functional score (0 being the best score, 15 the worst) which was classified as indicating good functioning (score 0-4), moderate functioning (score 5-7) and poor functioning (score 8-15)<sup>[9]</sup>.

Pouchitis was diagnosed by symptoms of bloody diarrhea, urgent need to pass stool, incontinence, and pain or discomfort while passing stool<sup>[10]</sup>.

Data collected included patient demographics, operative time, operative blood loss, complications, mortality, re-operation rate, length of hospital stay, readmission rates and reasons for readmission, pouchogram details, time to ileostomy closure, follow-up and long-term functional outcome.

## RESULTS

The study cohort included 29 UC patients and 16 FAP patients who were treated by ileal pouch anal anastomosis with modified double-stapled mucosectomy. Twenty-eight patients underwent a RPC + IPAA and protective

ileostomy in one stage, the ileostomy was closed 4-12 mo postoperatively. Seventeen patients underwent restorative proctectomy and IPAA after previous colectomy with ileostomy, all of whom were UC patients.

Demographic and operative data for the patients are presented in Table 1. The median operative time was 225 min, and in 15 patients, the operative time was more than 4 h. The median estimated blood loss was 550 mL, with a maximum estimated blood loss of 1200 mL, 36 patients requires transfusion and the median volume of blood transfusion was 600 mL. The median postoperative hospitalization was 12 d. The median interval to closure of the ileostomy was 5 mo.

There were no instances of operation-related mortality. Morbidity within the first 30 d of surgery occurred in 10 (22.2%) patients. Three patients developed an intestinal obstruction and could be treated conservatively, but one of the patient had a repeated attack of incomplete intestinal obstruction postoperatively. Two patients developed pelvic abscess and requires percutaneous puncture drainage guided by computed tomography. Six infectious complications occurred in five patients, four wound infection, one urinary tract infection and one pulmonary infection, and all could be treated with prolonged antibiotics or dressing change.

The patients were followed-up for a median period of 65 mo (range, 12-110 mo). After the closure of stoma, mild to moderate anastomotic narrowing was seen in 4 patients (8.8%), the majority being easily managed with digital dilation in the outpatient department. one patient developed persistent anastomotic stricture and require transanal endoscopic microsurgery dilatation. Sixteen patients developed at least 1 episode of pouchitis and the cumulative risk of pouchitis was 35.6%. Two patients with malignant adenomatous polyps died 2 years and 2.5 years after surgery and only 2 patients diagnosed dysplasia by biopsy in the remaining patients.

The median 24 h bowel movements was 7 evacuations per day, 4 evacuations per day and 4 evacuations per day at 1 year, 2.5 years and 5 years after closure of stoma, more than half of the patients reported more than five stools throughout the day 1 year after closure of stoma, but stool frequency decreased gradually, stabilizing at less than five evacuations per day at 2.5 years, and it was five or less in 70% of patients after 5 years. The proportion of patients that had stools every night decreased from 62.2% to 22.8% from 1 to 2.5 years, but this parameter did not improve further after 2.5 years. Urgency of defecation remained unchanged after 2.5 years although there was a tendency towards more patients being able to defer defecation for a longer time. There was no incontinence in our patients. Moreover, impaired continence, a frequent problem initially, also diminished with time, occurring in 33.3% of patients at 1 year. At 2.5 years, however, soiling or seepage occurred in only 5.7% patients during daytime and 20% patients at night, suggesting that improvement of continence can

**Table 2** Stool frequency, urgency, pad usage, diet, medication in patients underwent ileal pouch-anal anastomosis at 1 year, 2.5 years and 5 years after closure of stoma *n* (%)

	Score	1 yr ( <i>n</i> = 45)	2.5 yr ( <i>n</i> = 35)	5 yr ( <i>n</i> = 24)	<i>P</i> value <sup>3</sup>
24-h stool frequency					0.014
< 5	0	10 (22.2)	19 (54.2)	14 (58.3)	
5-8	1	31 (68.8)	14 (40.0)	8 (33.3)	
> 8	2	4 (8.8)	2 (5.7)	2 (8.3)	
Night time defecation					0.001
Never	0	2 (4.4)	7 (20.0)	6 (25)	
Weekly	1	15 (33.3)	20 (57.1)	13 (54.2)	
Every night	2	28 (62.2)	8 (22.8)	5 (20.8)	
Urgency of defecation <sup>1</sup>					0.382
> 30 min	0	25 (55.5)	25 (71.4)	17 (70.8)	
10-30 min	1	16 (35.5)	8 (22.9)	7 (29.1)	
< 10 min	2	4 (8.8)	2 (5.7)	0 (0)	
Use of pad daytime	1	6 (13.3)	2 (5.7)	1 (4.2)	0.326
Use of pad at night	1	6 (13.3)	3 (8.6)	1 (4.2)	0.454
Soiling or seepage during daytime	1	8 (17.7)	2 (5.7)	2 (8.3)	0.210
Soiling or seepage at night	1	15 (33.3)	7 (20)	5 (20.8)	0.026
Peri-anal soreness					0.989
No	0	38 (84.4)	30 (85.7)	21 (87.5)	
Occasional	1	5 (11.1)	4 (11.4)	2 (8.3)	
Permanent	2	2 (4.4)	1 (2.9)	1 (4.2)	
Dietary restrictions	1	23 (51.1)	8 (22.8)	2 (8.3)	0.001
Regular medication for stool regulation	1	20 (44.4)	6 (17.1)	1 (4.2)	0.000
Social handicap <sup>2</sup>	1	4 (8.8)	2 (5.7)	2 (8.3)	0.862
Functional Oresland score [median (range)]	15	6 (1-12)	3 (0-11)	2 (0-9)	

<sup>1</sup>Urgency of defecation is inability to defer evacuation for more than 30 min; <sup>2</sup>Social handicap is not able to resume full time occupation or to participate in social life; <sup>3</sup> $\chi^2$  test of 45 patients.

be expected to occur even beyond one year. More than half patients mentioned dietary restrictions and 44% patients needed regular medication for stool regulation after 1 year, the rate decreased to 22.8% and 17.1% after 2.5 years and was 8.3% and 4.2% after 5 years. Less than 10% patients felt affected in their everyday life through the functional impairments after 5 years. The difference in 24-h stool frequency, night time defecation, Soiling or seepage at night, dietary restrictions and regular medication for stool regulation has statistical significance. Overall, the median functional Oresland score was 6, 3 and 2 after 1 year, 2.5 years and 5 years respectively. Nearly half patients (44.4%) reported “moderate functioning” (as described in Method), 37.7% reported “good functioning”, whereas in 17.7% of patients “poor functioning” was observed after 1 year. Five years later, 79.2% of patients with good function, 4 patients (16.7%) with moderate function, only 4.2% of patients with poor function (Tables 2 and 3). Despite defects in function, patients’ satisfaction was generally excellent. So far only one patients have preferred conversion to an ileostomy once more.

## DISCUSSION

Restorative proctocolectomy, mucosectomy with ileal pouch-anal anastomosis has become widely accepted and is now considered the standard procedure for patients with ulcerative colitis as well as familial adenomatous polyposis since 1980s<sup>[11]</sup>.

Since both UC and FAP are mucosal diseases, mucosectomy has the advantage of removing the diseased colorectal mucosa, particularly if taken down to the dentate line<sup>[12]</sup>. In both these conditions an ideal operation would remove all colonic and rectal mucosa thus exempting the patients from the risk of further inflammatory disease, dysplasia or cancer in UC and reducing the risk of polyposis or cancer in FAP. However, it has suggested that mucosectomy with a handsewn anastomosis results in poorer function, more than 50% of patients complained of seepage at night<sup>[13]</sup> and incontinence were inevitable in some of patients. Moreover, Bullard<sup>[14]</sup> looked at a 12 years long-term functional outcome using a questionnaire for 235 patients and found that most patients had stable pouch function over time, but a proportion (in 18% patients) suffered measurable deterioration particularly 12 or more years after surgery and improved function was found only in 1% patients. Without prophylactic surgical intervention, malignant transformation into carcinoma occurs in majority FAP patients in the fourth decade of life<sup>[15]</sup>. Therefore, most patients undergoing pouch surgery are younger and have a long life expectancy. Thus, long-term morbidity and functional outcome is important for their quality of life after surgery.

In order to improve the anal function and prevent incontinence, stapled anastomosis without mucosectomy which confers functional benefit compared with handsewn anastomosis and mucosectomy is performed by many surgeons, but the retained rectal mucosa is



**Table 3** Kirwan classification after surgery *n* (%)

Kirwan classification	1 yr ( <i>n</i> = 45)	2.5 yr ( <i>n</i> = 35)	5 yr ( <i>n</i> = 24)	<i>P</i> value <sup>1</sup>
Stage I (full continence)	30 (66.7)	28 (80)	19 (79.1)	0.785
Stage II (incontinence of gas)	5 (11.1)	3 (8.6)	2 (8.3)	
Stage III (occasional minor soiling)	9 (20)	4 (11.4)	3 (12.5)	
Stage IV (frequent major soiling)	1 (2.2)	0 (0)	0 (0)	
Stage V (incontinence)	0 (0)	0 (0)	0 (0)	

<sup>1</sup> $\chi^2$  test of 45 patients.

thought a disease-bearing tissue, if left untreated, may present a significant future risks for disease recurrence or malignant transformation<sup>[16]</sup> and lifelong surveillance is required for these patients<sup>[17]</sup>.

The idea of the modified double-stapled mucosectomy technique was born as a result of the aforementioned disadvantages associated with the previous techniques. The characteristics of our technique is that we retain 5 cm short rectal muscular cuff, remove great part of rectal mucosa excluding the mucosa in ATZ which is usually commenced just above the dentate line and situated a median of 1.05 cm above the lower border of the internal sphincter<sup>[18]</sup>. Because of the preservation of mucosa in ATZ which is important for anal sensation<sup>[19,20]</sup>, functional results are significantly improved in our series. There was no incontinence in our patients, soiling or seepage occurred in only 5.7% patients during daytime and 20% patients at night after 2.5 years. After 5 years, 24-h stool frequency was five or less in 70% of patients and only 4.2% patients needed regular medication for stool regulation. Most patients can restore normal life and patients' satisfaction was generally excellent. So far only one patients have preferred conversion to an ileostomy once more. Furthermore, retention of 5 cm short rectal muscular cuff has the advantage of making the ileal pouch fully dilate to replace the function of rectum.

The main debate of our technique is regarding the risk of dysplasia and ongoing inflammation in the retained rectum because this method leaves behind intact mucosa of the ATZ. Although residual mucosa can remain inflamed in patients and may become dysplastic, anal transitional zone dysplasia after ileal pouch-anal anastomosis is infrequent, Remzi reported the incidence of dysplasia after stapled RPC was 3%-4.5%<sup>[21]</sup> and it had not been substantiated that preservation of ATZ lead to the development of cancer in the ATZ. We think it is a inspiring result and take the following measures to minimize the risk. Firstly, cancer risk may be reduced by ensuring that a minimal length of rectal mucosa to be retained. In carrying out our modified double-stapled IPAA, the anastomosis should be performed at a lower level just above the ATZ. Secondly, we recommended that long-term monitoring is necessary and it is easy to find lesions by digital rectal examination when the level of the anastomosis is lower. In such cases, trananal biopsy is convenient. In our study, only 2 patients (4.4%) diagnosed dysplasia by biopsy during the median follow-

up period of 65 mo and no patient finds development of adenocarcinoma in the ATZ.

Our study showed a better outcome regarding pelvic-related postoperative complications, only two of the patients (4.4%) developed a peripouch-localized abscess without systemic signs of infection and could be treated by percutaneous puncture drainage which are better than previously reported rates of pelvis sepsis following pouch surgery<sup>[22,23]</sup>. Thus, we speculate that the retention of rectal muscular cuff has no relation with the pelvic-related infectious complications and retention of short rectal muscular cuff (only 5 cm) can reduce the operative time and the possibility of bleeding. Furthermore, we don't suture the J-pouch with the rectal cuff and the tissue space was blocked out by fibrin glue which can protect against pelvic sepsis, if there is anastomotic leakage, symptoms will be minimized.

On the other hand, the choice of IPAA technique should be based on the situation of patients to make a comprehensive consideration for both oncologic and functional outcome. When the patients at high risk of cuff dysplasia or inflammation, we think it is better to perform a handsewn technique with mucosectomy rather than attempting stapling technique with the retention of partial mucosa of the rectal stump in order to avoid carcinoma formation after IPAA.

Several limitations of our study should be noted. The design was retrospective and our modified technique was not compared with the handsewn technique or the standard stapling technique of the same surgeon. Another study limitation is the small number of patients. Despite being a modified technique of IPAA, small patient numbers precluded robust analysis of the association between clinical and postoperative outcome.

In conclusion, ileal pouch-anal anastomosis with modified double-stapled mucosectomy seems to be a technically feasible and clinically acceptable alternative procedure for the removal of potentially diseased rectal mucosa retained only in ATZ to achieve an improved anal function after surgery.

## COMMENTS

### Background

Ileal pouch anal anastomosis (IPAA) is the operative approach for patients requiring restorative proctocolectomy due to familial adenomatous polyposis and ulcerative colitis. But the conventional handsewn technique or stapling



technique have their disadvantages such as poor postoperative anal function or disease recurrence. Modify double-stapled techniques by sparing 5 cm short rectal muscular cuff and retaining the mucosa only in anal transition zone is designed to ameliorate aforementioned disadvantages and improve the clinical effects.

### Research frontiers

Handsewn technique with mucosectomy and stapling technique with the retention of the mucosa of the rectal stump are the routine methods of IPAA. In the area of improving anal function and reducing the risk of disease recurrence, the research hotspot is to deeply understand the anatomy of anus and modify the surgical technique.

### Innovations and breakthroughs

In the previous reports of IPAA, the most applicable methods are handsewn technique with mucosectomy and stapling technique with the retention of the mucosa of the rectal stump. The authors modify double-stapled techniques by sparing 5 cm short rectal muscular cuff and retaining the mucosa only in anal transition zone. This is the first study to report the long-term functional outcomes with this technique in China. Furthermore, the results of the modified technique are promising, with a low complication rate and good long-term anal function.

### Applications

The study results suggest that ileal pouch anal anastomosis with modified double-stapled mucosectomy is a feasible surgical technique for patients with familial adenomatous polyposis and ulcerative colitis.

### Terminology

Ileal pouch-anal anastomosis: IPAA is constructed for people who have had their large intestine surgically removed due to disease or injury. It is formed by folding loops of small intestine (the ileum) back on themselves and stitching or stapling them together. The internal walls are then removed thus forming a reservoir. The reservoir is then stitched or stapled with the anal mucosa. Familial adenomatous polyposis: Familial adenomatous polyposis is an inherited condition in which numerous polyps form mainly in the epithelium of the large intestine. While these polyps start out benign, malignant transformation into colon cancer occurs when not treated; Ulcerative colitis: Ulcerative colitis is a form of inflammatory bowel disease, a disease of the colon, that includes characteristic ulcers, or open sores. The main symptom of active disease is usually constant diarrhea mixed with blood, of gradual onset; Anal transition zone: Anal transition zone is usually commenced just above the dentate line and situated a median of 1.05 cm above the lower border of the internal sphincter. It is important for anal sensation.

### Peer review

Interesting paper, some fine tuning is needed but certainly adds to the knowledge.

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## Tumor suppressor function of ezrin-radixin-moesin-binding phosphoprotein-50 through $\beta$ -catenin/E-cadherin pathway in human hepatocellular cancer

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### Abstract

**AIM:** To determine the effect and molecular mechanism of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) in hepatocellular carcinoma (HCC).

**METHODS:** Three human HCC cell lines, *i.e.*, SMMC7721, HepG2 and Hep3B, were used. We transfected the Pbk-CMV-HA-EBP50 plasmid into SMMC7721 cells with Lipofectamine 2000 to overexpress EBP50. Western blotting were performed to determine the effects of the plasmid on EBP50 expression and to detect the expression of  $\beta$ -catenin and E-cadherin before and after the transfection of the plasmid into SMMC7721 cells. *In vitro* cell proliferation was assessed with a Cell Counting Kit-8 (CCK-8) assay. Cell cycle distribution was assessed with flow cytometry. Invasion and migration ability of before and after the transfection were determined with a transwell assay. Cell apoptosis was demonstrated with Annexin V-FITC. The effect of EBP50

overexpressing on tumor growth *in vivo* was performed with a xenograft tumor model in nude mice.

**RESULTS:** The transfection efficiency was confirmed with Western blotting ( $1.36 \pm 0.07$  vs  $0.81 \pm 0.09$ ,  $P < 0.01$ ). The CCK8 assay demonstrated that the growth of cells overexpressing EBP50 was significantly lower than control cells ( $P < 0.01$ ). Cell cycle distribution showed there was a G0/G1 cell cycle arrest in cells overexpressing EBP50 ( $61.3\% \pm 3.1\%$  vs  $54.0\% \pm 2.4\%$ ,  $P < 0.05$ ). The transwell assay showed that cell invasion and migration were significantly inhibited in cells overexpressing EBP50 compared with control cells ( $5.8 \pm 0.8$  vs  $21.6 \pm 1.3$ ,  $P < 0.01$ ). Annexin V-FITC revealed that apoptosis was significantly increased in cells overexpressing EBP50 compared with control cells ( $14.8\% \pm 2.7\%$  vs  $3.4\% \pm 1.3\%$ ,  $P < 0.05$ ). The expression of  $\beta$ -catenin was downregulated and E-cadherin was upregulated in cells overexpressing EBP50 compared with control cells ( $0.28 \pm 0.07$  vs  $0.56 \pm 0.12$ ,  $P < 0.05$ ;  $0.55 \pm 0.08$  vs  $0.39 \pm 0.07$ ,  $P < 0.05$ ). *In vivo* tumor growth assay confirmed that up-regulation of EBP50 could obviously slow the growth of HCC derived from SMMC7721 cells ( $28.9 \pm 7.2$  vs  $70.1 \pm 7.2$ ,  $P < 0.01$ ).

**CONCLUSION:** The overexpression of EBP50 could inhibit the growth of SMMC7721 cells and promote apoptosis by modulating  $\beta$ -catenin, E-cadherin. EBP50 may serve as a potential therapeutic target in HCC.

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**Key words:** Hepatocellular carcinoma; Ezrin-radixin-moesin-binding phosphoprotein-50; Growth; Migration; Invasion

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## INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of mortality due to cancer in the world<sup>[1]</sup>. The low rate of early diagnosis renders the tumors unresectable and results in metastasis to the lymph nodes or distant organs. In addition, HCC cells are relatively resistant to chemotherapy; thus, the overall survival rate is poor. Recently, targeted molecular therapy has been shown to be effective in treating HCC, which encouraged us to study the cellular and molecular mechanisms of HCC and identify more effective treatments.

The etiology of hepatocarcinogenesis is still not fully understood, the activation of oncogenes, dysfunction of tumor suppressor genes, and aberrant activation of signal transduction pathways have been described.  $\beta$ -catenin is a key effector molecule in the Wnt signaling pathway and also plays an important role in cell-cell adhesion<sup>[2,3]</sup>. E-cadherin is a member of the cadherin family and is a classical type I cadherin. Both  $\beta$ -catenin and E-cadherin are membrane-associated proteins that are essential for regulating and providing cellular adhesion. Membrane-associated adhesion proteins regulate cell motility, including metastasis and invasion, and they link the extracellular matrix and actin cytoskeleton, which is critical in many biological signal transduction pathways.  $\beta$ -catenin and E-cadherin dysfunction has been described in several tumors, such as breast<sup>[4]</sup>, prostate<sup>[5]</sup>, gastric<sup>[6]</sup>, colorectal<sup>[7]</sup>, lung<sup>[8]</sup>, oral<sup>[9]</sup> and hepatocellular carcinoma<sup>[10]</sup>.

Ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50, also known as NHERF1) is a 55-kDa phosphoprotein, and it belongs to the family of PDZ scaffolding proteins. Previous studies suggest that EBP50 is a potential tumor suppressor in several types of tumors<sup>[11-15]</sup>. Our previous studies also showed that EBP50 can inhibit the growth of gastric and pancreatic cancer cells<sup>[16,17]</sup>. EBP50 is mainly localized at the apical plasma membrane in human epithelial tissues<sup>[18]</sup>. The loss of normal apical membrane expression and/or distribution to the cytoplasm and nuclear overexpression was found in several human cancers<sup>[12-15,18]</sup>. Shibata *et al.*<sup>[19]</sup> found that 45% of HCC cases expressed high levels of EBP50 mRNA, and 55% of HCC cases showed no difference. The cytoplasmic and nuclear accumulation of the EBP50 protein was present in 55% of HCC tissues compared with the surrounding noncancerous liver tissue, and EBP50 interacts with  $\beta$ -catenin to participate in hepatocarcinogenesis. The role of EBP50 as an oncogene or a tumor suppressor in HCC remains unclear.

In this study, we examined the effects of EBP50 up-regulation on cell growth, migration, invasion, and apoptosis in the HCC cell line SMMC7721 and determined the expression of  $\beta$ -catenin and E-cadherin to elucidate

the role of EBP50 in HCC.

## MATERIALS AND METHODS

### Cell lines and plasmids

The human HCC cell lines Hep3B, SMMC7721, HepG2 were obtained from the Cell Bank of Shanghai Institute for Biological Sciences (Shanghai, China). Of the three cell lines, the SMMC7721 cell line has relatively low EBP50 protein expression levels. These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. The pBK-CMV-HA-EBP50 plasmid was kindly provided by Dr. Randy Hall from Emory University (Atlanta, GA, United States).

### Stable transfection

The pBK-CMV-HA-EBP50 plasmid or the pBK-CMV-HA vector was transfected into SMMC7721 cells with Lipofectamine<sup>TM</sup> 2000 (Invitrogen, CA, United States) according to the manufacturer's protocol. G418 (350  $\mu$ g/mL) was used to select stably transfected cells. Single-cell clones were isolated for clonal expansion. The transfection efficiency was determined with Western blotting. Subsequently, stably transfected cell clones were cultured and amplified in culture medium supplemented with 350  $\mu$ g/mL G418.

### Cell proliferation assay

The cell-counting kit-8 (CCK-8, Dojindo, Japan) colorimetric assay was used to measure cell proliferation and viability with triplicate experiments for each set of conditions. We seeded  $5 \times 10^3$  cells per well in a 96-well plate. Cell viability was assessed using the Cell Counting Kit after culture for 24 h. The absorbance at 450 nm was measured with a plate reader.

### Western blotting

Western blotting was performed as described previously<sup>[20]</sup>. The specific antibodies were rabbit anti-EBP50 antibody (1:1000, Abcam; Pierce, GA, United States), rabbit anti- $\beta$ -catenin antibody (1:1000, Abcam), mouse anti-E-cadherin antibody (1:1000, Abcam), and mouse anti- $\beta$ -actin antibody (1:1000, Abcam).

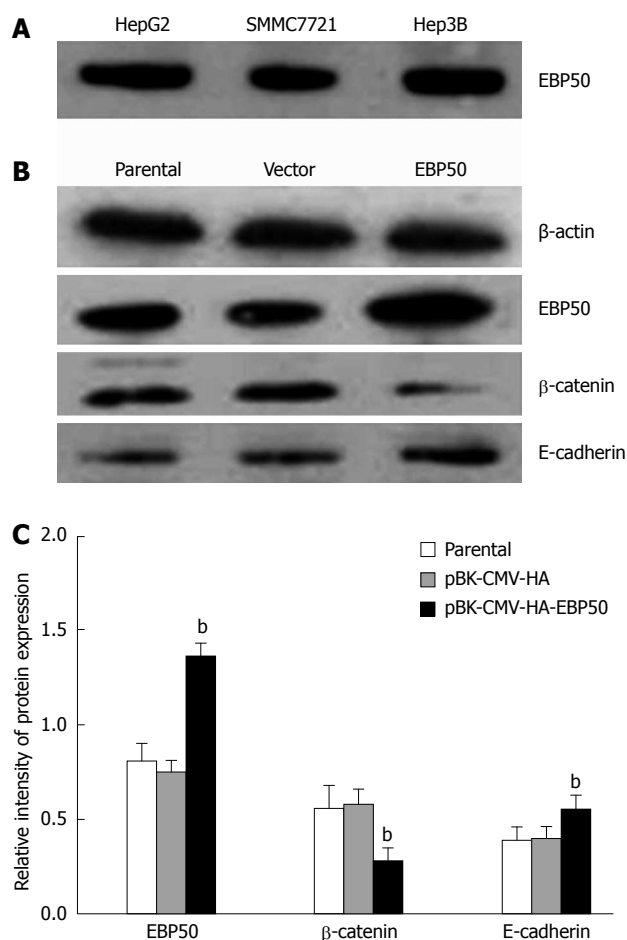
### Transwell assay

Invasion through a Matrigel-coated filter was measured in transwell chambers (Corning, New York, NY, United States) as described previously<sup>[14]</sup>. Cells ( $1 \times 10^4$ ) were trypsinized and suspended in the upper chambers. After incubation for 24 h, the transwell chambers were fixed with 4% paraformaldehyde and stained with Giemsa (Sigma). Cells in five randomly selected fields were photographed, and the number of trans-membrane cells was counted.

### Cell cycle assay

Cells were trypsinized and fixed in 70% ethanol on





**Figure 1** Ezrin-radixin-moesin-binding phosphoprotein-50,  $\beta$ -catenin, and E-cadherin protein levels were detected with Western blotting. A: The expression of ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) protein in Hep3B, SMMC7721, HepG2; B: Western blotting analysis of EBP50,  $\beta$ -catenin, and E-cadherin protein before and after transfection; C: Analysis of the expression of proteins.  $^*P < 0.01$  vs parental or pBK-CMV-HA.

ice and then stained with 50  $\mu\text{g/mL}$  propidium iodide (Sigma) and 0.1  $\mu\text{g/mL}$  RNase A (Sigma). The cells were then analyzed with flow cytometry using a FACStar Plus, and Cell Quest software was used to calculate the percentage of the cell population in each phase.

### Cellular apoptosis assay

Apoptosis was assessed with the Annexin V-FITC kit according to the manufacturer's protocol. A total of  $1 \times 10^6$  cells/mL were washed twice with cold PBS and resuspended in binding buffer. A total of 5 mL of Annexin V-FITC and 10 mL of propidium iodide (PI) were added (BioVision, Mountain New, CA, United States), and the cells were incubated for 10 min at room temperature in the dark. At the end of the incubation, 200  $\mu\text{L}$  of binding buffer was added, and the cells were immediately analyzed with flow cytometry. Cell Quest software was used to analyze flow cytometry data.

### Ex vivo tumor growth assay

Male nude mice (4-6 wk old,  $n = 6$ ) were (Beijing HFK Biosciences Co., Ltd) used in the experiments. After alco-

hol preparation of the skin,  $1 \times 10^6$  SMMC cells (pBK-CMV-HA-EBP50, pBK-CMV-HA vector and control cells), suspended in 100  $\mu\text{L}$  PBS, were subcutaneously inoculated into the dorsal area of the nude mice. Six weeks after injection, At the end of the experiment, tumors were harvested and weighed. All experiments were performed according to the recommendations of the Institutional Animal Care and Use Committee, and the study protocol was approved by the Ethics Committee for Animal Research of Wuhan University, China.

### Statistical analysis

The results were analyzed using SPSS 13.0 statistical software. All of the data are presented as the means  $\pm$  SD. Statistically significant differences between groups in each assay were compared with a one-way analysis of variance (ANOVA), and  $P < 0.05$  was considered to be statistically significant.

## RESULTS

### EBP50 expression in HCC cells

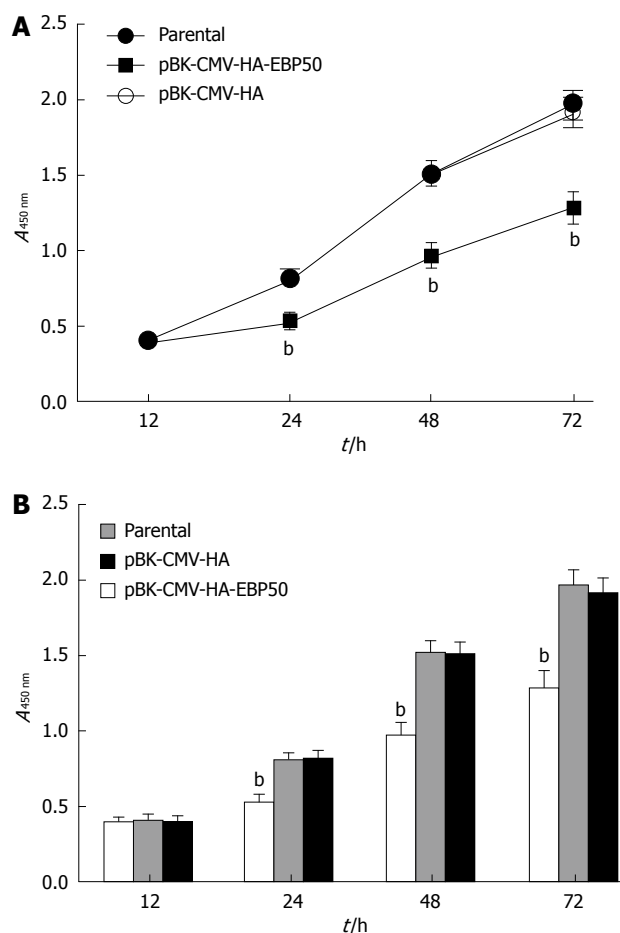
We used Western blotting to evaluate the expression of EBP50 in human HCC cell lines, with SMMC7721 expressing the lowest of the three cell lines (Figure 1). To investigate the role of EBP50 in HCC, we overexpressed EBP50 in SMMC7721 cells, which expressed relatively low levels of endogenous EBP50. SMMC7721 cells were transfected with pBK-CMV-HA-EBP50 or the pBK-CMV-HA vector to establish vector control cells. To evaluate the transfection efficiency, we determined the protein expression of EBP50 with Western blotting (Figure 1). The data showed that after transfection for 48 h, the protein levels of EBP50 were upregulated in pBK-CMV-HA-EBP50-transfected cells compared with pBK-CMV-HA vector-transfected cells and parental cells ( $1.36 \pm 0.07$  vs  $0.81 \pm 0.09$  or  $0.75 \pm 0.06$ ,  $P < 0.01$ ).

### Expression of $\beta$ -catenin and E-cadherin in HCC cells

To investigate the biological effect of EBP50 in HCC and the potential signaling pathway through which it acts, we analyzed the expression of  $\beta$ -catenin and E-cadherin in pBK-CMV-HA-EBP50- and pBK-CMV-HA-transfected cells and untransfected cells. The protein levels of  $\beta$ -catenin was downregulated ( $0.28 \pm 0.07$  vs  $0.56 \pm 0.12$  or  $0.58 \pm 0.08$ ,  $P < 0.05$ ) and E-cadherin ( $0.55 \pm 0.08$  vs  $0.39 \pm 0.07$  or  $0.40 \pm 0.06$ ,  $P < 0.05$ ) was upregulated in pBK-CMV-HA-EBP50-transfected cells (Figure 1). The expression level was the same as previously reported by Hayashi *et al*.<sup>[12]</sup>

### Overexpression of EBP50 suppresses cancer cell growth

To assess the potential effects of EBP50 overexpression on cell proliferation and survival, we measured the number of viable cells at different times in vitro with CCK-8 assays. The pBK-CMV-HA vector had no effect on the proliferative ability of SMMC7721 cells, whereas pBK-



**Figure 2** Growth rate comparison of parental, pBK-CMV-HA-EBP50, pBK-CMV-HA cells with a Cell Counting Kit-8 assay. A: Growth rate comparison of parental, pBK-CMV-HA-EBP50, pBK-CMV-HA cells; B: Analysis of the growth rate. <sup>a</sup> $P < 0.01$  vs parental or pBK-CMV-HA.

CMV-HA-EBP50-transfected cells showed a dramatic reduction in proliferation ( $P < 0.01$ , Figure 2).

The cell cycle distribution was measured to determine whether the growth-inhibitory activity of EBP50 on SMMC7721 cell proliferation was due to cell cycle alterations. Compared with the control cells, the percentage of pBK-CMV-HA-EBP50 cells in G<sub>0</sub>/G<sub>1</sub> phase was increased to  $61.3\% \pm 3.1\%$  from  $54.0\% \pm 2.4\%$  in parental cells and  $54.5\% \pm 1.9\%$  in mock cells ( $P < 0.05$ , Figure 3).

#### Overexpression of EBP50 suppresses cancer cell migration and invasion

We explored whether the over-expression of EBP50 resulted in decreased invasion and migration in cancer cells with a transwell assay. As shown in Figure 4A-D, the number of migrated pBK-CMV-HA-EBP50 cells ( $5.8 \pm 0.8$  cells/HP) was lower than the pBK-CMV-HA vector cells ( $20.4 \pm 1.1$  cells/HP) and parental cells ( $21.6 \pm 1.3$  cells/HP) ( $P < 0.01$ ), which indicates that EBP50 could significantly suppress the invasive ability of SMMC7721 cells. No significant difference was found between SMMC7721 parental cells and the pBK-CMV-HA vector cells. The result showed that upregulating EBP50 expres-

sion in SMMC7721 cells inhibited tumor cell invasion and migration.

#### Overexpression of EBP50 promotes cell apoptosis

Apoptosis was investigated with an annexin V/propidium iodide assay. The pBK-CMV-HA-EBP50 cells showed an approximately fourfold increase in apoptosis ( $P < 0.05$ ) compared with the parental group and the pBK-CMV-HA vector group (Figure 4E), suggesting that the pBK-CMV-HA-EBP50 plasmid enhances SMMC7721 apoptosis.

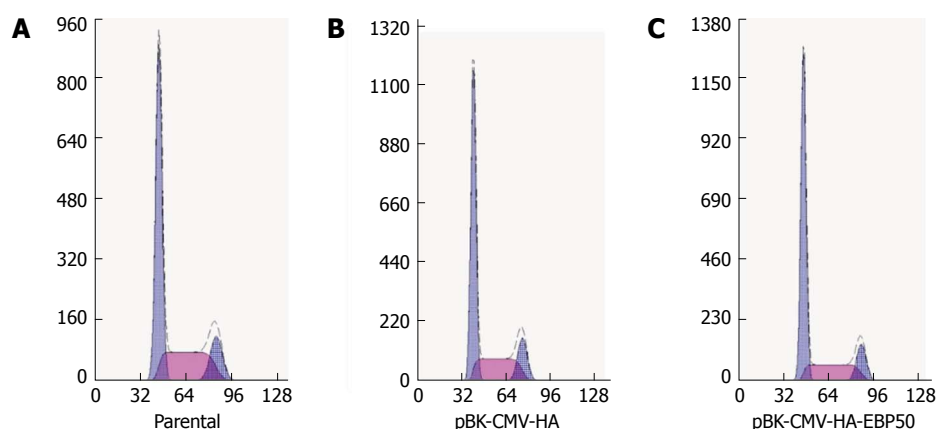
#### Overexpression of EBP50 decreased the growth of the tumor *ex vivo*

The cell growth response as result of NHERF1 overexpression was also examined in the nude mice model. We compared the growth of SMMC7721 cells infected with pBK-CMV-HA-EBP50, pBK-CMV-HA vector and control cells *ex vivo*. At 6 wk after inoculation, obvious tumors were formed in the two control groups of mice inoculated with SMMC7721 cells or the pBK-CMV-HA vector, while tumor growth was obviously suppressed in mice inoculated with pBK-CMV-HA-EBP50 cells. As shown in Figure 5, tumors formed from pBK-CMV-HA-EBP50 ( $28.9 \pm 7.2$  mg) were significantly smaller than those from pBK-CMV-HA and control cells ( $68.9 \pm 7.9$  mg,  $70.1 \pm 7.2$  mg,  $n = 6$ ) ( $P < 0.01$ ), suggesting that expression of EBP50 suppressed tumor growth *ex vivo*.

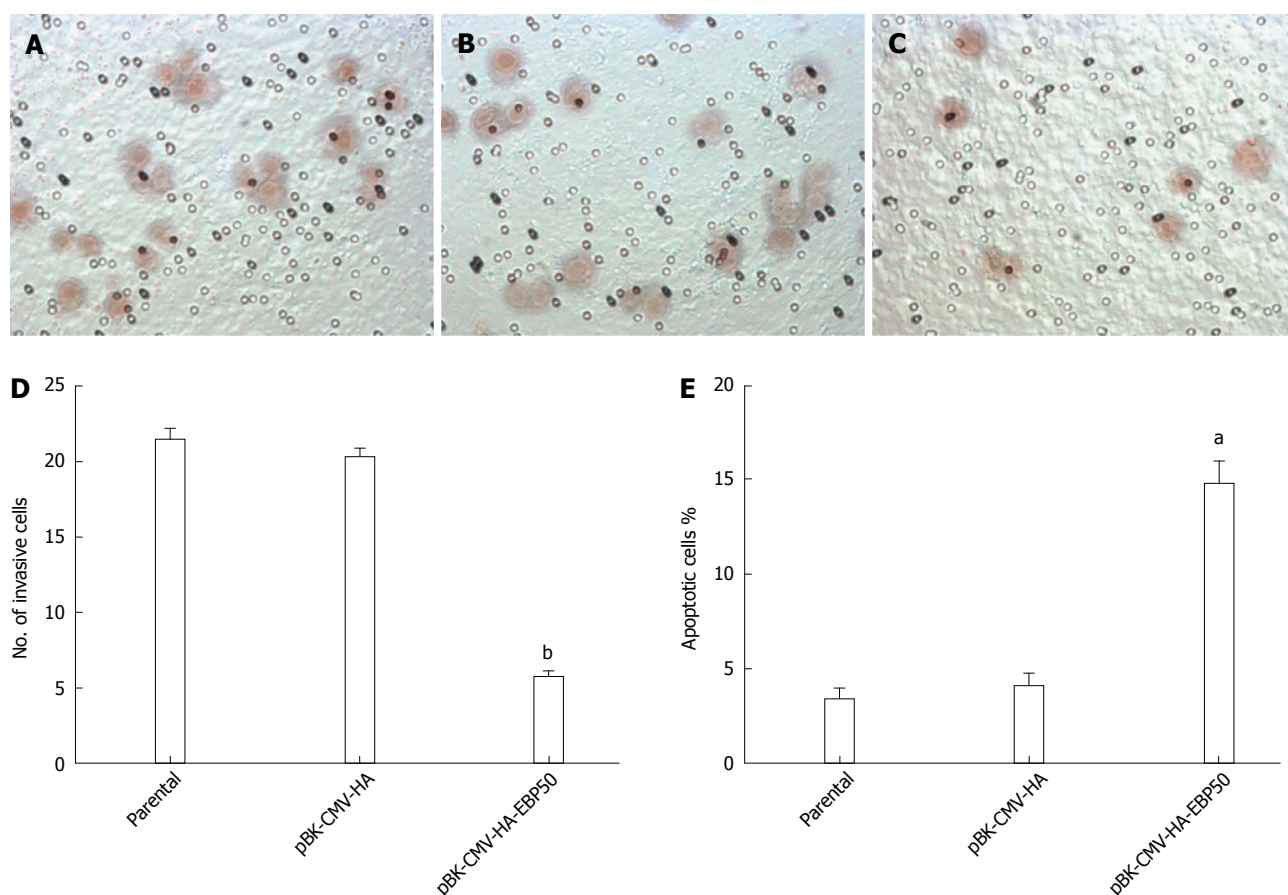
## DISCUSSION

In the present study, to determine the role of EBP50 in HCC as an oncogene or a tumor suppressor, we used the pBK-CMV-HA-EBP50 plasmid to increase the expression of EBP50 in SMMC7721 cells. Western blotting analysis proved that EBP50 protein levels were significantly upregulated in pBK-CMV-HA-EBP50-transfected SMMC7721 cells. The proliferation rate of cells overexpressing EBP50 was lower than the pBK-CMV-HA vector and parental SMMC7721 cells (Figure 2). In a transwell assay, the number of migrated cells over-expressing EBP50 was significantly lower than control cells, indicating that the over-expression of EBP50 can inhibit the anchorage-independent growth of HCC cells (Figure 4). Cellular apoptosis analysis showed that EBP50 overexpression could increase apoptosis and induce G<sub>0</sub>/G<sub>1</sub> cell cycle arrest in HCC cells (Figures 3 and 4). *Ex vivo* tumor growth assay confirmed that cells overexpressing EBP50 could obviously slow the growth of HCC cells (Figure 5). Zheng *et al*<sup>[11]</sup> reported that EBP50 overexpression could inhibit breast cancer cell proliferation and promote cell apoptosis through decreasing ERK activation. The current study also suggests EBP50 as a potential tumor suppressor in HCC.

The molecular mechanisms through which EBP50 exerts its tumor suppressor functions are diverse. EBP50 contains two PDZ domains and an ERM domain. PDZ domains have been implicated in protein-protein interac-



**Figure 3** Effect of ezrin-radixin-moesin-binding phosphoprotein-50 over-expression on cell cycle distribution. A: Cell cycle analysis of parental (G0/G1, 54.2%); B: pBK-CMV-HA (G0/G1, 56.2%); C: pBK-CMV-HA-EBP50 (G0/G1, 61.4%) cells with flowcytometry.

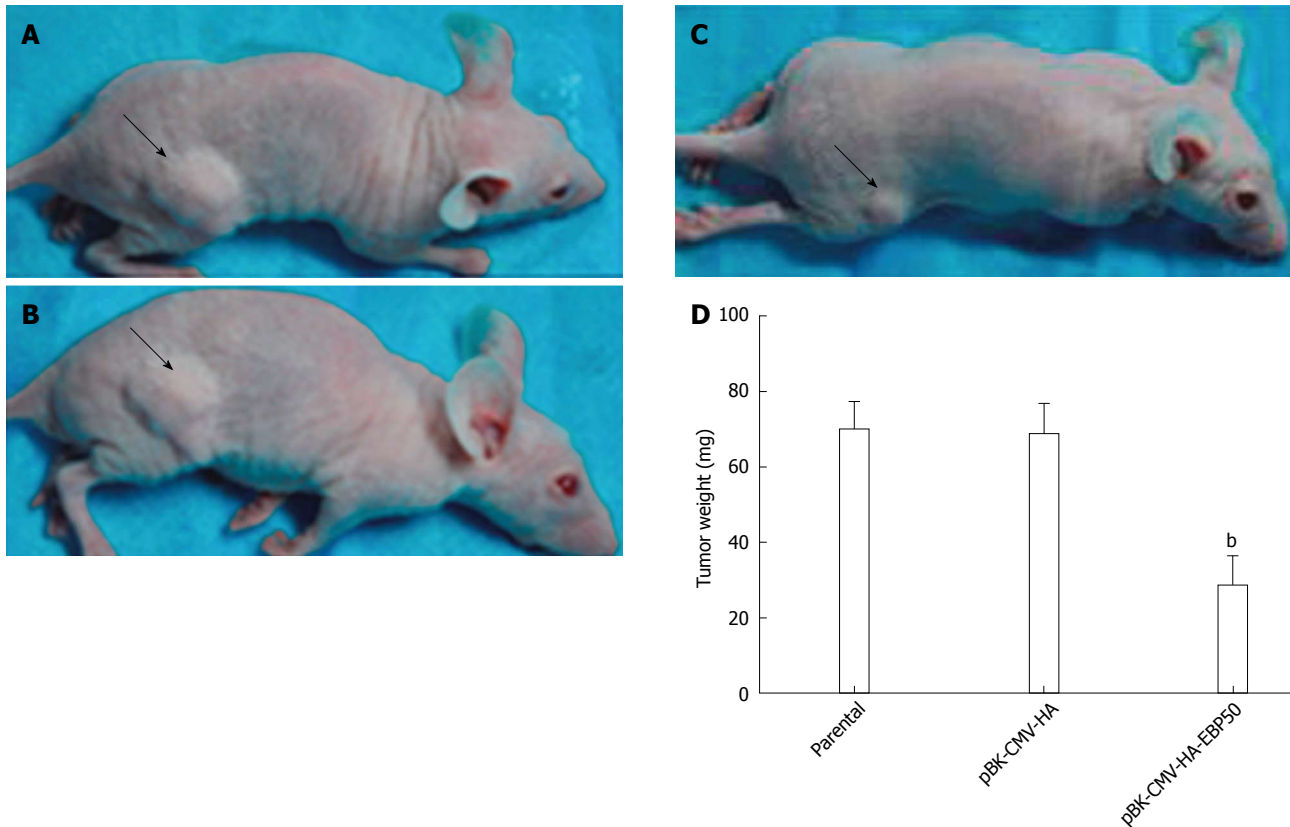


**Figure 4** Ezrin-radixin-moesin-binding phosphoprotein-50 over-expression suppressed the migration and invasion of cancer cells. A: Migration and invasion analysis of parental (21.6 ± 1.3 cells/HP); B: pBK-CMV-HA (20.4 ± 1.1 cells/HP); C: pBK-CMV-HA-EBP50 (5.8 ± 0.8 cells/HP) cells with transwell assay; D: Analysis of the number of invasive cells; E: Apoptotic analysis of parental (3.4% ± 1.3%), pBK-CMV-HA (4.1% ± 1.5%) and pBK-CMV-HA-EBP50 (14.8% ± 2.7%) cells with flowcytometry. \* $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs parental or pBK-CMV-HA.

tions, and the ERM domain binds to ezrin through its ERM-binding region<sup>[21]</sup>. In normal cells, EBP50 interact with cyclin dependent kinase and PP2A through its reversible phosphorylation of the protein to regulate cell division<sup>[22]</sup>. EBP50 interacts with many tumor-related proteins through these domains, including PTEN, PDGFR, EGFR, podocalyxin, and GPCRs, to participate in

tumorigenesis<sup>[23-29]</sup>. EBP50 forms a complex with beta-catenin to promote Wnt signaling and may participate in the development of HCC<sup>[19]</sup>.

Both  $\beta$ -catenin and E-cadherin play an essential role in cell-cell junctions in Wnt signal pathway.  $\beta$ -catenin binds directly to the cytoplasmic tail of E-cadherin to form a bridge between E-cadherin and the actin cyto-



**Figure 5** Tumorigenicity was inhibited by overexpression of ezrin-radixin-moesin-binding phosphoprotein-50 *ex vivo* (arrow). A: Tumor weight in nude mice of parental ( $70.1 \pm 7.2$  mg); B: pBK-CMV-HA ( $68.9 \pm 7.9$  mg); C: pBK-CMV-HA-EBP50 ( $28.9 \pm 7.2$  mg); D: Tumor weight of the nude mice in each group. <sup>b</sup> $P < 0.01$  vs parental or pBK-CMV-HA.

skeleton and stabilizes adherens junctions<sup>[30]</sup>. The plasma membrane localization of  $\beta$ -catenin allows it to function as a tumor suppressor by interacting with E-cadherin, and the phosphorylation of  $\beta$ -catenin results in its accumulation in the cytoplasm and translocation to the nucleus, which allows it to act as an oncogenic protein by increasing the transcription of genes involved in growth control<sup>[31]</sup>. The loss of membranous E-cadherin expression was found in highly aggressive tumors including HCC and contributed to their high metastatic potential<sup>[32-34]</sup>. Aberrant activation of Wnt/ $\beta$ -catenin signaling has been reported in a wide range of HCC<sup>[35]</sup>.

In our study, EBP50 upregulation resulted in an increase in E-cadherin levels and a decrease in  $\beta$ -catenin levels. EBP50 is required to maintain a fraction of the cortical  $\beta$ -catenin protein at the membrane; EBP50 binds to the C-terminal PDZ motif of  $\beta$ -catenin through its PDZ2 domain, and this association is required for  $\beta$ -catenin localization at cell-cell junctions<sup>[36]</sup>. In EBP50-depleted cells, there was a shift of  $\beta$ -catenin to the nucleus and an increased invasiveness of cells<sup>[12]</sup>. In EBP50-deficient MEFs, disorganization of E-cadherin-mediated adherens junctions and an increase in  $\beta$ -catenin transcriptional activity was observed<sup>[26]</sup>, suggesting that the depletion of EBP50 decreased the interaction between  $\beta$ -catenin and E-cadherin. Because of the contributing role of  $\beta$ -catenin and E-cadherin in HCC hyperplasia and tumorigenesis<sup>[10,19]</sup>, it is conceivable that the regulation of

$\beta$ -catenin/E-cadherin as a result of EBP50 overexpression contributes to HCC initiation or progression.

Our results suggest a tumor-suppressing role of EBP50 and establish that it may be a promising target for therapeutic intervention in HCC. Additionally, the interactions of EBP50 with  $\beta$ -catenin/E-cadherin enhance the tumor suppressor properties of EBP50 in HCC.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the fifth most common cancer globally and relatively resistant to chemotherapy, it encouraged us to study the cellular and molecular mechanisms of HCC targeted molecular therapy. ezrin-radixin-moesin-binding phosphoprotein-50 (EBP50) is a PDZ scaffolding protein, previous studies suggest that EBP50 is a potential tumor suppressor in colorectal, prostatic, biliary, breast, gastric and pancreatic cancer, and it has an abnormal expression in HCC. But EBP50 is an oncogene or a tumor suppressor in HCC remains unclear. The authors aimed to examine the role of EBP50 and whether it interact with the  $\beta$ -catenin/E-cadherin pathway in HCC.

### Research frontiers

Targeted molecular therapy is a new effective treatment for cancer including HCC. EBP50 is a potential tumor suppressor in several cancers, but the role of EBP50 is an oncogene or a tumor suppressor remains controversial and its exact role in HCC remains unknown.



## Innovations and breakthroughs

Authors used a pBK-CMV-HA-EBP50 plasmid to overexpress EBP50 in SMMC7721 cells. The results showed that overexpression of EBP50 could inhibit the growth, migration, invasion of SMMC7721 cells and promote cell apoptosis, and modulate the expression of beta-catenin, E-cadherin. EBP50 may serve as a potential therapeutic target in HCC. It suggested that EBP50 is a tumor suppressor and interact with  $\beta$ -catenin/E-cadherin in HCC.

## Applications

The results showed that EBP50 is a tumor suppressor and interact with  $\beta$ -catenin/E-cadherin in HCC. It may contribute to the future research of HCC and be a promising target for therapeutic intervention in HCC.

## Terminology

Ezrin-radixin-moesin-binding phosphoprotein-50: EBP50 (also known as NHERF1) is a 55-kDa phosphoprotein;  $\beta$ -catenin: A adhesion protein, a key mediator in the canonical Wnt signal pathway; E-cadherin: A member of the cadherin family and is a classical type I cadherin.

## Peer review

The function of EBP50 as a tumor suppressor has been studied in other cancers, but not in HCC. The expression in HCC was not significantly different between patient cases reported by Shibata *et al.* Thus they examined the effects of EBP50 up-regulation on cell growth, migration, invasion, and apoptosis in the HCC cell line SMMC7721 and determined the conclusion. This conclusion is meaningful in the sense characterized the function of EBP in HCC cells.

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## Gastric adenocarcinoma arising in gastritis cystica profunda presenting with selective loss of KCNE2 expression

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### INTRODUCTION

Gastritis cystica profunda (GCP) is a rare condition with nonspecific symptoms and radiographic images, making its diagnosis difficult without definitive surgical resection<sup>[1]</sup>. Clinically, GCP can be misdiagnosed as gastric lymphoma, stromal tumors, gastric cancer, or Menetrier disease. Histopathologically, GCP shows disruption of the integrity of muscularis mucosa that leads to cystically dilated submucosal glands with superficial inflammation in the lamina propria<sup>[1,2]</sup>. Since the majority of GCP cases are seen secondary to prolonged chronic inflammation, ischemia, gastric surgery and suturing material, an injury of the muscularis mucosae is assumed to trigger the ectopic entrapment of gastric glands in the submucosa, the muscularis mucosae or serosa and to lead to GCP<sup>[1,2]</sup>. Moreover, GCP is often associated with gastric adenocarcinoma indicates that it can lead to a secondary malignancy<sup>[2]</sup>. Indeed, experiments have shown that animals predisposed to *Helicobacter* infection develop not only secondary GCP but also subsequent gastric carcinoma<sup>[3]</sup>. This close association between GCP and malignancy has been interpreted as concurrent sharing of causative factors common to both disease conditions<sup>[1,3]</sup>. Recently, with the use of the KCNE2 (also known as MiRP1) deficient mouse model, loss of the KCNE2 subunit from potassium channel complexes is considered a common primary target molecule that gives rise to both GCP and malignancy<sup>[4]</sup>.

Here, the expression of KCNE2 in surgically excised tissue from human gastric cancer associated with GCP is, for the first time, analyzed to confirm that its reduced level correlates with disease formation.

### Abstract

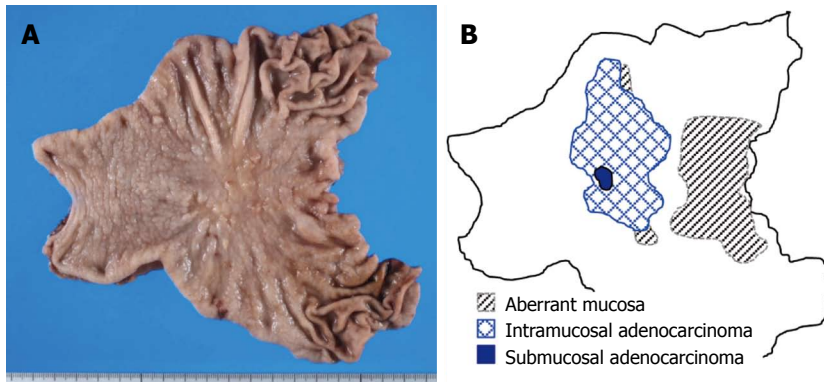
Gastritis cystica profunda (GCP) is a rare condition caused by ectopic entrapment of gastric glands, probably secondary to the disruption of muscularis mucosae. GCP is often associated with gastric adenocarcinoma, and loss of the KCNE2 subunit from potassium channel complexes is considered a common primary target molecule leads to both GCP and malignancy. In this study, we, for the first time, analyzed the expression of KCNE2 in surgically excised tissue from human gastric cancer associated with GCP and confirmed that reduced KCNE2 expression correlates with disease formation.

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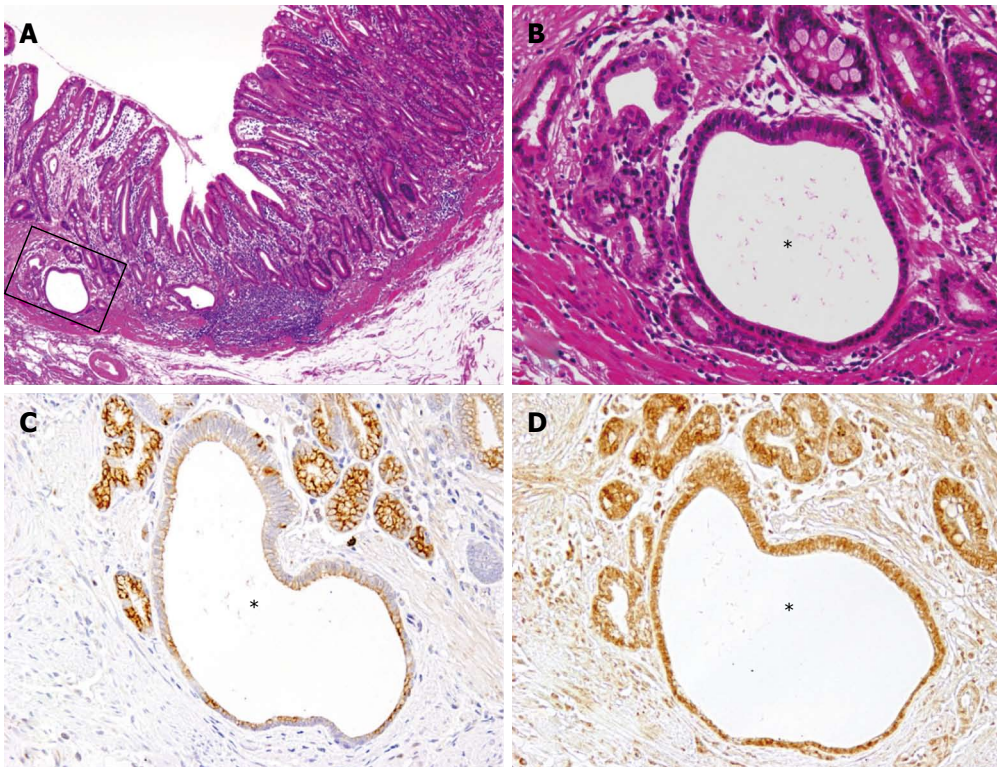
**Key words:** KCNE2; Gastritis cystica profunda; Immunohistochemistry

Kuwahara N, Kitazawa R, Fujiishi K, Nagai Y, Haraguchi R, Kitazawa S. Gastric adenocarcinoma arising in gastritis cystica profunda presenting with selective loss of KCNE2 expression.





**Figure 1 Macroscopic finding and distribution of ectopic cystic lesion and cancer in surgically excised stomach.** Surgically excised stomach (A) and schematic distribution of the lesions (B). Ectopic cystic mucosa is located mainly on the oral side and partly in the body part overlapped with a cancer lesion (shaded area). A well- to moderately-differentiated tubular adenocarcinoma in the intramucosal layer (checked area) and part of a poorly-differentiated component infiltrating the submucosal layer (blue area) are located mainly in the body part of the stomach.



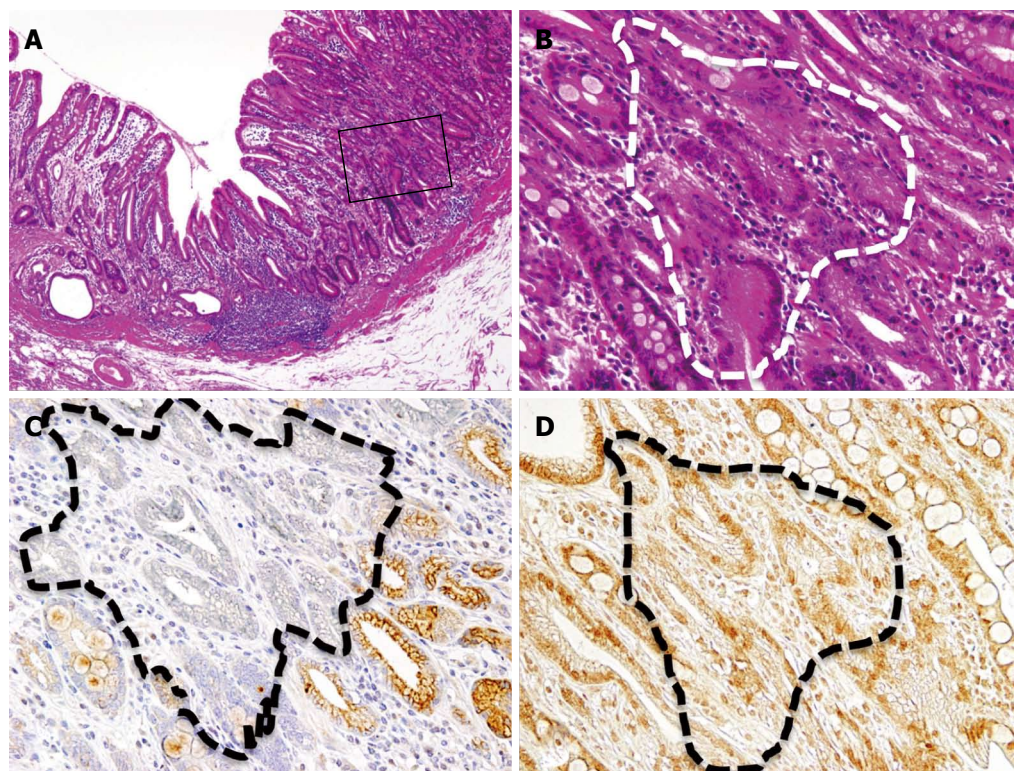
**Figure 2 Expression of KCNE2 and estrogen receptor in non-neoplastic cystic lesion by immunohistochemistry.** A: Low power magnification of a transitional area from the non-neoplastic mucosal layer with intramucosal cystic lesions (left) to the intramucosal adenocarcinoma (right, HE, × 40); B: High power magnification (A, squared area) around the intramucosal cystic lesion (asterisk, HE, × 200); C: KCNE2 immunostaining of a serial section of (B) shows that KCNE2 is almost negative in the dilated cystic gland (asterisk), while the surrounding non-cystic glands are positive (× 200); D: Estrogen receptor immunostaining of serial sections of (B) and (C) show that ER is equally expressed in both cystic (asterisk) and non-cystic glands (× 200).

## CASE REPORT

A 63-year-old man was admitted to the hospital with the chief complain of abdominal pain. The patient's past medical and family history was unremarkable. An endoscopic and other examinations revealed the presence of multiple erosive lesions, and a biopsy demonstrated only severe superficial gastritis with erosion. Under the diagnosis of benign erosive gastritis, the patient was treated with medication and followed up monthly. After

one year, however, a follow-up endoscopic examination revealed an irregularly-shaped ulcerating lesion, and the pathological diagnosis of adenocarcinoma was made through biopsy analysis. The patient underwent distal partial gastrectomy, and pathological examination of the excised stomach (Figure 1A) revealed the presence of ectopic cystic mucosa with intestinal metaplasia, especially in the oral side (Figure 1B, dotted area, Figure 2B: HE, × 200). Overlaying the ectopic cystic mucosa, a well- to moderately-differentiated tubular adenocarcinoma was





**Figure 3** Expression of KCNE2 and estrogen receptor in adenocarcinoma area. A: Low power magnification of a transitional area from the non-neoplastic mucosal layer with intramucosal cystic lesions (left) to the intramucosal adenocarcinoma (right, HE,  $\times 40$ ); B: High power magnification (A, squared area) around the intramucosal adenocarcinoma (circled by white-hatched line, HE,  $\times 200$ ); C: KCNE2 immunostaining of a serial section of (B) shows that KCNE2 expression is almost negative in adenocarcinoma (circled by black-hatched line), while the surrounding non-neoplastic glands are positive ( $\times 200$ ); D: Estrogen receptor immunostaining of serial sections of (B) and (C) show that estrogen receptor is equally expressed in both cancerous (circled by black-hatched line) and non-neoplastic glands ( $\times 200$ ).

observed in the superficial proper mucosal layer (M), with part of a poorly-differentiated component infiltrating the submucosal layer (SM) in the body of the stomach (Figure 1B, hatched area, Figure 3B: HE,  $\times 200$ ). The pathological diagnosis of gastric adenocarcinoma arising in gastritis cystica profunda was made.

### Immunohistochemistry

Since targeted deletion of *KCNE2* in mice causes gastric lesions resembling gastritis cystica profunda and gastric neoplasia<sup>[4]</sup>, we examined the expression of KCNE2 in the current case immunohistochemically. Also, since signaling through the estrogen receptor (ER) modulates KCNE2 expression<sup>[5]</sup>, we additionally examined the expression of ER. To determine the localization of KCNE2 and ER, rabbit polyclonal anti-KCNE2 antibody (ABCAM, Cambridge, United Kingdom) and rabbit monoclonal anti-ER (EST) antibody (EPIOTOMICS, United States) were used as primary antibodies. Tissue sections were deparaffinized in xylene for 20 min (solvent refreshed at 10 and 5 min) and immersed in absolute ethanol for 10 min (solvent refreshed at 5 min), then rehydrated in 90% and 70% ethanol (5 min each), and finally placed in distilled water for 15 min (solvent refreshed every 5 min). The samples were inactivated in 1 mmol/L EDTA (pH 8.0) plus distilled water in a microwave for 15 min (high heat for 5 min and low heat for 10 min), and then cooled to approximately 25 °C over 1 h. After be-

ing undergoing liquid block treatment, the samples were immersed in PBS for 15 min (solvent refreshed every 5 min). After adding blocking buffer to the moisture chamber and incubating the samples with primary antibodies at room temperature for 1 h, they were washed 3 times for 5 min each in PBS, incubated in the moisture chamber at room temperature for 30 min with the secondary antibody diluted at 1:200 with PBS, immersed in PBS for 15 min (solvent refreshed every 5 min), then incubated with DAB for 15 min and washed in PBS.

Immunohistochemical analyses revealed that KCNE2 was universally and strongly expressed on the surface of the cells at the bottom of the cryptic glands, while its expression was diminished in the cystic or dilated lesions (Figure 2C). ER expression was observed in both non-cystic and cystic glands (Figure 2D). In adenocarcinoma, KCNE2 expression was significantly reduced compared with the surrounding non-cancerous gastric mucosa with intestinal metaplasia (Figure 3C), while ER expression was observed in both cancerous and non-cancerous glands (Figure 3D).

### DISCUSSION

In this study the expression status of KCNE2 in surgically excised gastric adenocarcinoma coexisting with GCP was examined in light of that the KCNE2-deficient mouse model develops both GCP and gastric cancer.

Since KCNE2 expression is modulated by ER, and since ER expression status per se is related to carcinogenesis and progression stages of gastric cancer<sup>[6]</sup>, we additionally examined the immunohistochemical expression of ER in the excised tissue.

KCNE2 has originally been identified as a potassium channel protein, and in the stomach, it is expressed mainly in the cytoplasm of parietal cells<sup>[5,7,8]</sup>. Reduction of KCNE2 in experimental animal models results in profoundly reduced proton secretion, abnormal parietal cell morphology, achlorhydria, hypergastrinemia<sup>[5,8]</sup>, and striking gastric glandular hyperplasia arising from an increase in the number of non-acid-secreting cells<sup>[4]</sup>. Functionally, KCNE2 also exerts anti-proliferative effects on gastric cancer cells by down-regulating Cyclin D1 and restricting cell growth<sup>[5,8]</sup>. Indeed, reflecting anti-proliferative or tumor-suppressor functions of KCNE2, long-term observation of *KCNE2* <sup>-/-</sup> mice has revealed that reduced KCNE2 expression causes diffuse hyperplasia in gastric mucosa, resulting in a pathologic condition similar to gastric cancer associated with GCP<sup>[4]</sup>.

We examined here, for the first time, the expression of KCNE2 in surgically excised stomach tissue demonstrating both GCP and adenocarcinoma. While KCNE2 expression in both GCP and adenocarcinoma areas was diminished, that in surrounding non-neoplastic and non-cystic cells was clearly maintained, as determined by immunohistochemical analysis. These data, albeit from a single case study, suggest that selective loss of KCNE2 expression is related to the development and clinical manifestation of GCP with subsequent occurrence of cancer. Furthermore, because selective loss of KCNE2 expression is seen at the level of a single cystic gland and cancer cell nest unit, silencing KCNE2 expression may occur at the level of a single tissue progenitor cell. Although the precise molecular mechanism of such selective loss of KCNE2 expression is largely unknown, it is at least not by the loss of estrogen receptor signaling. We believe that these new data are important because they confirm previous experimental information, and should be assessed in other similar cases. Further genetical and

epigenetics studies based on a cumulative case study are needed to elucidate the role of KCNE2 expression in the development of GCP.

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## Hepatitis B reactivation in chronic myeloid leukemia patients receiving tyrosine kinase inhibitor

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### Abstract

Hepatitis B virus (HBV) reactivation is a well-recognized complication in patients with chronic HBV infection receiving cytotoxic or immunosuppressive chemotherapy. Imatinib mesylate and nilotinib are selective Bcr/Abl tyrosine kinase inhibitors, which are now widely used in the treatment of patients with chronic myeloid leukemia. Although HBV reactivation induced by imatinib mesylate has been reported, nilotinib-related HBV reactivation has not been reported in the English literature. We report here 2 cases of HBV reactivation in chronic myeloid leukemia patients receiving imatinib mesylate and a novel case of nilotinib related HBV reactivation.

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**Key words:** Hepatitis B virus; Chronic myeloid leukemia; Imatinib mesylate; Nilotinib; Tyrosine kinase inhibitor

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### INTRODUCTION

Hepatitis B virus (HBV) reactivation is a well-recognized complication in patients with chronic HBV infection receiving cytotoxic or immunosuppressive chemotherapy for hematologic malignancies<sup>[1-4]</sup>. Imatinib mesylate (IM) (Glivec; Novartis, Basel, Switzerland), a selective Bcr/Abl tyrosine kinase inhibitor (TKI), is now widely used in the treatment of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors<sup>[5,6]</sup>. Nilotinib (Tasigna; Novartis, Basel, Switzerland), a second generation TKI, was approved in 2007 for CML patients with resistance or intolerance to IM<sup>[5]</sup>. Although HBV reactivation induced by IM has been reported<sup>[7-12]</sup>, nilotinib-related HBV reactivation has not been documented in current literature. We report here 2 cases of HBV reactivation in CML patients receiving IM and a novel case of nilotinib related Hepatitis B virus reactivation. Possible mechanism of TKI related HBV reactivation is discussed in this manuscript.

### CASE REPORT

#### Case 1

A 43-year-old man was a HBV carrier who received regular follow-ups at our institution. On December 2011, he was diagnosed with CML, which was confirmed by the presence of Philadelphia chromosome (Ph+) in cytogenetic study. He started to receive IM 400 mg once daily. The pretreatment liver panel were alanine transaminase (ALT) = 40 U/L (normal 11-40 U/L), total bilirubin = 0.8 mg/dL (normal 0.3-1.2 mg/dL), albumin = 3.5 g/dL (normal 3.5-5.5 g/dL) and prothrombin time = 11 s (normal 10-12 s). Major molecular response (MMR, < 0.1% Bcr-Abl/Abl ratio according to the international scale (IS)



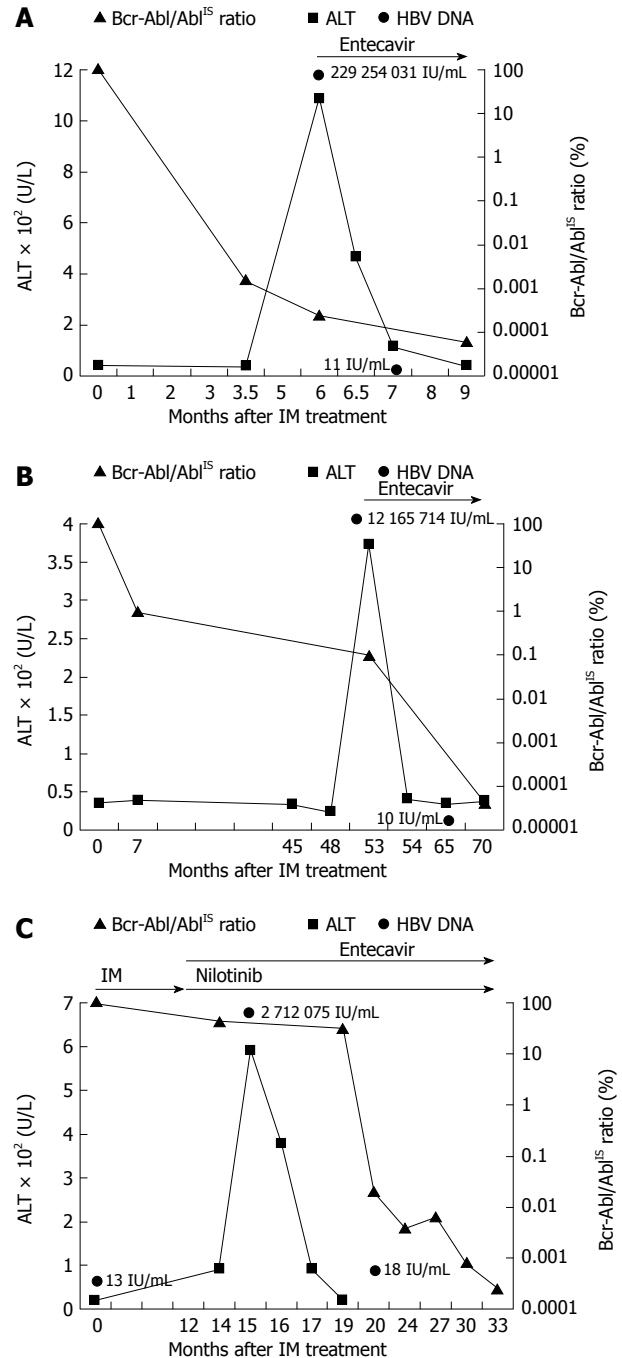
was achieved after 3.5 mo of IM treatment. His liver tests were within normal limits until 6 mo after IM treatment, when he started to feel easy fatigue. The patient denied usage of other medications such as acetaminophen or herbs. Laboratory investigation revealed an increased aspartate aminotransferase (AST) level of 273 U/L and an increased ALT level of 1086 U/L (Figure 1A). Hepatitis B surface antigen (HBsAg) and hepatitis B e-antigen (HBeAg) were positive. HBV DNA was positive at a concentration of 229 254 031 IU/mL. Results for hepatitis A immunoglobulin M (IgM) antibody, hepatitis C antibody, cytomegalovirus (CMV), Epstein Barr virus (EBV) and herpes simplex 1/2 (HSV) were negative. Antinuclear antibodies (ANA) and immunoglobulins were within normal limits. Other biochemical studies such as bilirubin, alkaline phosphatase, and albumin were normal. Liver ultrasonography showed coarse liver parenchyma and normal biliary tract. Because HBV reactivation was considered, he started to receive entecavir 0.5 mg once daily. IM was not discontinued. After 1-mo treatment with entecavir, his ALT level fell to 117 IU/L and HBV DNA level was 11 IU/mL, as demonstrated in Figure 1A. Clinical symptoms resolved 6 wk later with entecavir. Complete molecular response (CMR, < 0.0001% Bcr-Abl/Abl ratio according to the IS) was achieved 9 mo after IM treatment. The clinical course of this patient is summarized in Figure 1A.

### Case 2

A 67-year-old man with history of diabetes mellitus and HBV carrier received regular follow-ups at our hospital. He was diagnosed with CML with initial presentation of leukocytosis and thrombocytosis. He started to receive IM 400 mg once daily after bone marrow biopsy and cytogenetic study. The pretreatment liver panel were ALT = 26 U/L, total bilirubin = 1.1 mg/dL, albumin = 4.8 g/dL and prothrombin time = 12.6 s. Complete cytogenetic response (CCyR, < 1% Bcr-Abl/Abl ratio according to the IS) was achieved 7 mo after IM treatment. However, jaundice and anorexia developed 53 mo after IM treatment. Laboratory studies revealed an increased AST level of 110 U/L and an increased ALT level of 374 U/L (Figure 1B). Total bilirubin level was 2.74 mg/dL. HBsAg and HBeAg were positive. HBV DNA was positive at a concentration of 12 165 714 IU/mL. Results for hepatitis A IgM antibody, hepatitis C antibody, HSV, EBV, CMV, ANA were within normal limits. Liver ultrasonography showed no biliary tract dilatation. Entecavir 0.5 mg once daily was prescribed under the consideration of HBV reactivation. IM was not discontinued. After 1-mo treatment with entecavir, his ALT level fell to 41 IU/L, as demonstrated in Figure 1B. HBV DNA level was 10 IU/mL 12 mo after entecavir administration. Status of CMR was achieved on April, 2011. The clinical course of this patient is summarized in Figure 1B.

### Case 3

A 50-year-old woman was referred to our institution



**Figure 1 Clinical course of cases.** A: Case 1, showing alanine transaminase (ALT) and Bcr-Abl/Abl<sup>IS</sup> ratio over time; B: Case 2, showing ALT and Bcr-Abl/Abl<sup>IS</sup> ratio over time; C: Case 3, showing ALT and Bcr-Abl/Abl<sup>IS</sup> ratio over time. IM: Imatinib mesylate; IS: International scale.

on November 2009 for evaluation of leukocytosis, anemia, and thrombocytopenia. She was a HBV carrier. A diagnosis of CML was made based on the findings of proliferation of myeloid lineage cells and presence of Ph+ in bone marrow biopsy. IM 400 mg daily was used after diagnosis of CML. The pretreatment liver panel were ALT = 23 U/L, total bilirubin = 0.4 mg/dL, albumin = 4.1 g/dL and prothrombin time = 11 s. Because Bcr-Abl/Abl ratio was not significantly reduced 12 mo later with IM, treatment regimen was shifted to nilotinib



400 mg twice daily. However, 3 mo after treatment with nilotinib, the patient experienced tea-colored urine and easy fatigue. The patient denied usage of acetaminophen or herbs. Laboratory studies revealed an increased AST level of 346 U/L and an increased ALT level of 592 U/L (Figure 1C). Total-bilirubin level was 2.54 mg/dL. Coagulation profiles were normal. HBsAg was positive, whereas HBeAg was negative. Results for hepatitis A, hepatitis C, HSV, EBV, and CMV were negative. HBV DNA was positive at a concentration of 27 120 705 IU/mL. Liver ultrasonography showed normal results. Because HBV reactivation was considered, entecavir 0.5 mg once daily was prescribed. Nilotinib was not discontinued. After 2-mo treatment with entecavir, ALT level fell to 92 IU/L, as demonstrated in Figure 1C. HBV DNA was at a lower level of 18 IU/mL 6 mo after treatment with entecavir. Status of MMR was achieved 6 mo after entecavir treatment. The clinical course of this patient is summarized in Figure 1C.

## DISCUSSION

HBV reactivation is characterized by an abrupt rise of HBV DNA in patients with previously inactive or resolved HBV infection during or closely after chemotherapy. The current generally accepted definition of HBV reactivation following chemotherapy is the development of hepatitis with a serum ALT greater than three times the upper limit of normal or an absolute increase of 100 IU/L, associated with a demonstrable increase in HBV DNA by at least a 10-fold<sup>[1,2]</sup>. HBV reactivation not only occurred in HBsAg-positive patients but also in HBsAg-negative patients with anti-hepatitis B core antibody positivity and/or anti-hepatitis B surface antibody positivity<sup>[1-4]</sup>. In a prospective study of 626 cancer patients undergoing chemotherapy, HBV reactivation occurred in nearly 20% of them<sup>[1]</sup>. The risk factors of HBV reactivation included male gender, younger age, HBeAg seropositivity, high pre-chemotherapy HBV DNA above  $3 \times 10^5$  copies/mL, use of corticosteroids and anthracyclines, and duration of chemotherapy<sup>[1,4,13]</sup>. The pathogenesis of chemotherapy-induced HBV reactivation is not clear but may involve a 2-stage process, an initial immunosuppressive stage and an immune-restoration stage<sup>[3,4]</sup>. The initial immunosuppressive stage is characterized by marked increase in serum levels of HBV DNA and HBeAg. This stage is probably related to the suppression of immune mechanism that serves to control HBV replication. The immune-restoration stage occurs after subsequent withdrawal of immunosuppressive drugs, resulting in rapid destruction of infected hepatocyte.

IM, a rationally designed TKI that blocks the ATP-binding site of Bcr/Abl, is currently recommended as the first line therapy for CML<sup>[5,6]</sup>. However, resistance to IM may occur. Nilotinib was designed to overcome IM resistance with a better efficacy and mild adverse effects<sup>[5]</sup>. Hepatotoxicity has been reported in 1%-4% of CML patients treated with IM; however, liver dysfunction may

resolve with either dose reduction or discontinuation of IM<sup>[14]</sup>. In the present study, the TKIs were not discontinued after hepatic flare. Hepatic dysfunction improved in three cases after receiving entecavir, thus excluding the possibility of considering IM or nilotinib related hepatotoxicity as the cause of hepatic flare in our study. In the third case of our study, the diagnosis of HBV reactivation was established by low pretreatment HBV load and high level at the time of hepatic flare. Although the HBV load was not examined before IM treatment in the first and second cases, HBsAg and HBeAg were positive before treatment and high level of HBV load was detected at the time of hepatic flare. It is reasonable to consider HBV reactivation as the cause of hepatic flare in these two cases of our study<sup>[15-18]</sup>.

The mechanism of TKI-induced HBV reactivation remains unclear due to limited case reports. *In vitro* studies have shown that IM can inhibit T-cell activation<sup>[19]</sup> and proliferation<sup>[20]</sup>. Nilotinib inhibited the Src-family kinase LCK and T-cell function and hampered the proliferation and function of CD8+ T lymphocytes<sup>[21,22]</sup>. It is worth noting that HBV reactivation occurred after achieving MMR in the first case (Figure 1A) and after CCyR in the second case (Figure 1B), a similar condition as that in the report of Ikeda *et al.*<sup>[7]</sup>. In the study of Mohamad *et al.*<sup>[23]</sup>, CML patients who were in complete molecular or cytogenetic response after IM treatment restored function of plasmacytoid dendritic cells, which are crucial effectors in innate immunity. Therefore, the finding of hepatic flare after complete molecular or cytogenetic responses in the first two cases may provide evidence to support the hypothesis of immune-restoration stage of HBV reactivation. Furthermore, in the third case of our study, the hepatic flare occurred 5 mo before achieving CCyR (Figure 1C). This finding suggests that nilotinib treatment might provoke a different pathway other than that of IM to achieve immune restoration. Further studies may be needed to explain this observation. An important issue that should be addressed is the preemptive therapy with nucleoside/nucleotide analogues (NAs) in patients undergoing TKI therapy. Although preemptive treatment with NAs before immunosuppressive chemotherapy is recommended in European Association for the Study of the Liver Clinical Practice guidelines<sup>[24]</sup>, there is no recommendation on whether NAs should be given in patients undergoing TKI therapy due to lack of prospective studies.

In conclusion, this case report highlights the importance that HBV reactivation may occur in hematologic patients undergoing TKI therapy. Once HBV reactivation is suspected during TKI treatment, early detection of HBV load and utilization of antiviral agent are suggested to achieve better clinical outcome.

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## Multiple endocrine neoplasia type 1 with upper gastrointestinal hemorrhage and perforation: A case report and review

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### Abstract

Multiple endocrine neoplasia type 1 (MEN1) is a rare hereditary syndrome known to predispose subjects to endocrine neoplasms in a variety of tissues such as the parathyroid glands, pituitary gland, pancreas and gastrointestinal tract. We herein report a patient with a past history of pituitary adenoma, presenting with symptoms of chronic diarrhea for nearly one year and a sudden upper gastrointestinal hemorrhage as well as perforation without signs. Nodules in the duodenum and in the uncinate process and tail of pancreas and enlargement of the parathyroid glands were detected on preoperative imaging. Gastroscopy revealed significant ulceration and esophageal reflux diseases. The patient underwent subtotal parathyroidectomy and autotransplantation, pylorus-preserving pancreaticoduodenectomy and pancreatic tail resection and recovered well. The results observed in our patient suggest that perforation and bleeding of intestine might be symptoms of Zollinger-Ellison Syndrome in patients with MEN1.

### INTRODUCTION

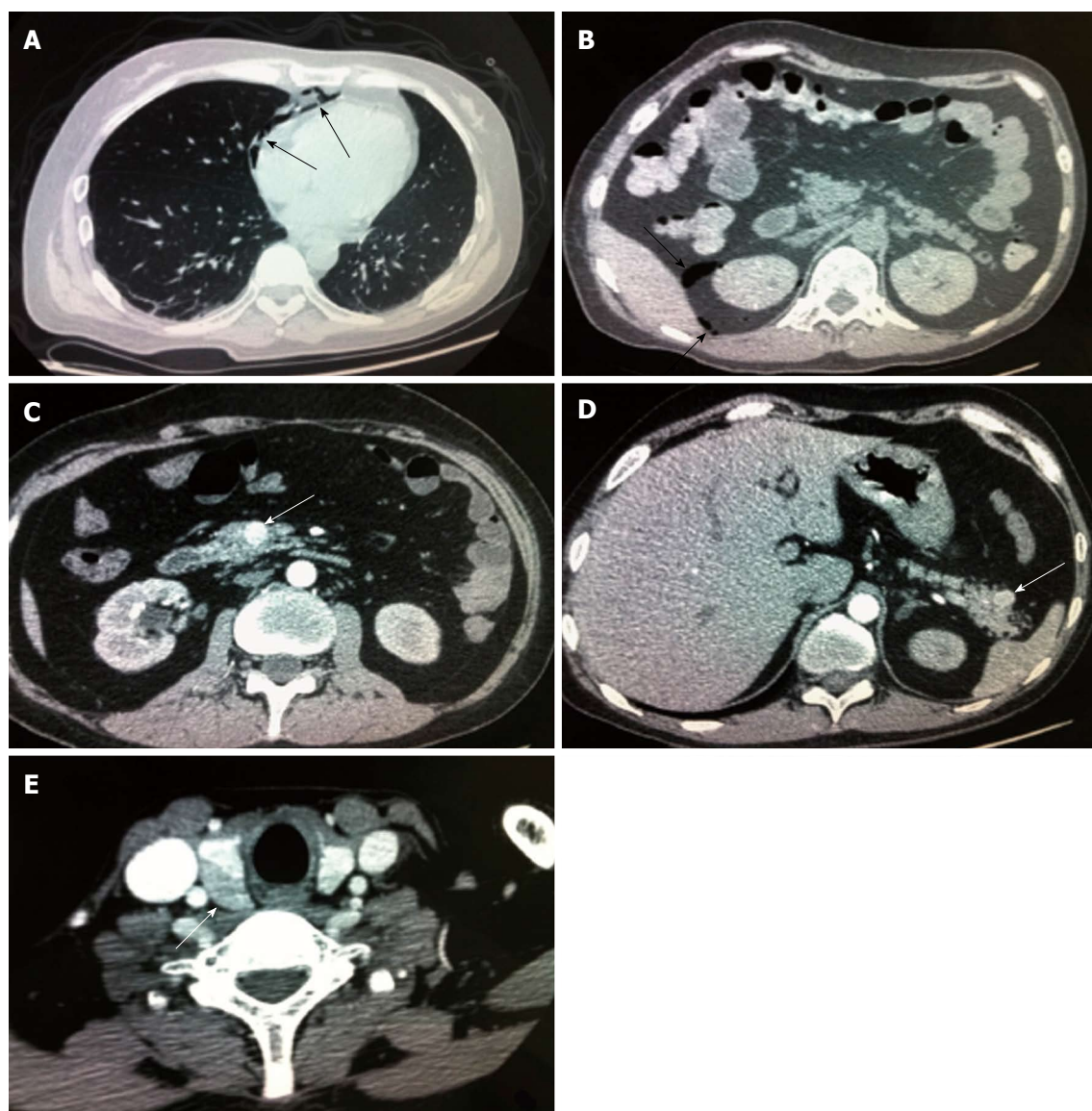
Multiple endocrine neoplasia type 1 (MEN1) syndrome is transmitted as an autosomal dominant mutation of a suppressor gene located on chromosome 11q13 and it is a rare congenital disease. The classical clinical manifestation of MEN1 is composed of parathyroid hyperplasia, pancreatic endocrine tumor and pituitary adenoma<sup>[1]</sup>. The definition of MEN1 is the coincidence of at least two of the above mentioned tumors<sup>[1]</sup>.

We herein report a case of MEN1 with sudden upper gastrointestinal hemorrhage and perforation 14 years after surgery for pituitary adenoma.

### CASE REPORT

A 42-year-old man was hospitalized with symptoms of nausea, vomiting and diarrhea for more than one year. The patient, presenting with decreased vision, diminished interest in sex, fatigue, weakness and polyuria, was diagnosed with pituitary adenoma in 1998. His blood prolactin level was high (more than 200 ng/mL, reference range, 2-23 ng/mL). The patient underwent removal of the tumor *via* the trans-sphenoidal approach in February 1998. Histological examination of the surgical specimen



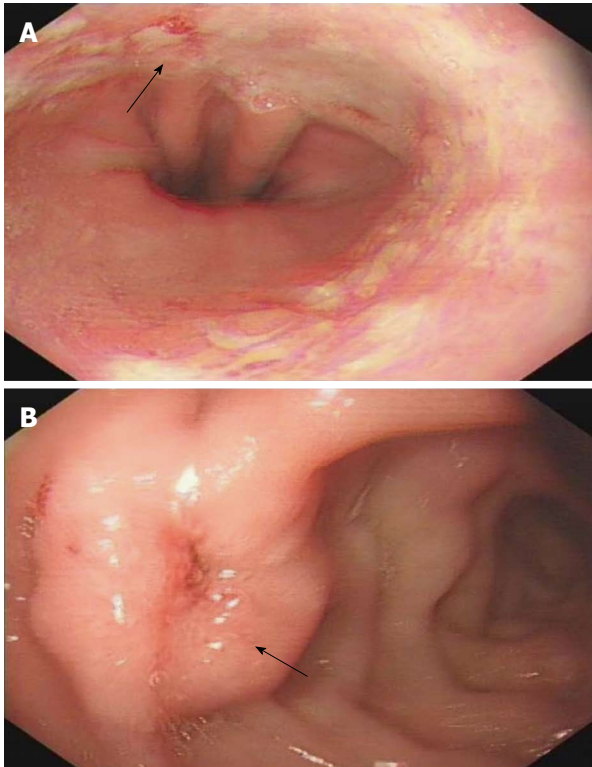


**Figure 1** Computed tomography scan of the case. A: Chest Computed tomography (CT) shows a mediastinal emphysema (arrows); B: Epigastric CT shows accumulation of gas and fluid (arrows) in the anterior pararenal space just adjacent to the thickened wall of the horizontal part of the duodenum as well as accumulation of gas in the right perirenal space; C, D: A small intestine CT shows bowel wall thickening and strong enhancement of the horizontal part of the duodenum and a nodular mass (arrows) with a rich blood supply in the uncinate process (C) and tail (D) of the pancreas; E: Thyroid gland CT shows mild nodular goiter with nodules (arrow) posterior and lateral to the thyroid gland.

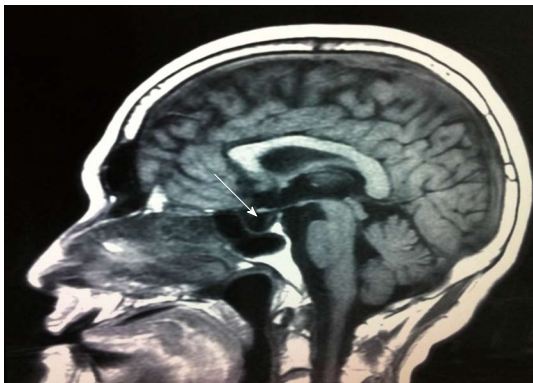
showed a chromophore cell adenoma. The patient's family history was notable for his brother also having a pituitary adenoma. In 2009, the patient underwent surgical procedures to remove a kidney stone. Recently, the patient had diarrhea, nausea and vomiting lasting for more than one year while gastroscopy and colonoscopy were performed prior to admission. Gastroscopy showed reflux esophagitis, esophageal protrusive lesion, gastric polyp and duodenal ulcer. Colonoscopy was normal. The patient was admitted on March 18, 2012. Physical examination revealed hypopigmentation of skin, sparse hair with normal blood pressure, body temperature, breath sounds and heart rhythms. The abdomen was prominently tender with normal bowel sounds and there was no shifting dullness found on the mass. Rectal examination was normal. Laboratory tests showed the following data: leukocytosis

( $22.40 \times 10^9$ ), mildly low hemoglobin level (115 g/L), elevated serum calcium (3.05 mmol/L, reference range, 2.1-2.55 mmol/L), low phosphorus level (0.57  $\mu\text{mol/L}$ , reference range, 0.81-1.45  $\mu\text{mol/L}$ ), extremely high level of parathyroid hormone (PTH) (627 pg/mL, reference range, 15-65 pg/mL). Serum magnesium, calcitonin, electrolytes, glucose, liver and renal function were normal. Computed tomography (CT) scan of the chest revealed mediastinal emphysema with hypostasis in the inferior lobe of the lung (Figure 1A). Epigastric CT scan showed accumulation of gas and fluid in the anterior pararenal space just adjacent to the thickened wall of horizontal duodenum as well as accumulation of gas in the right perirenal space, which implied the possibility of duodenal perforation. CT imaging also found renal and hepatic cysts (Figure 1B). The patient complained of melena



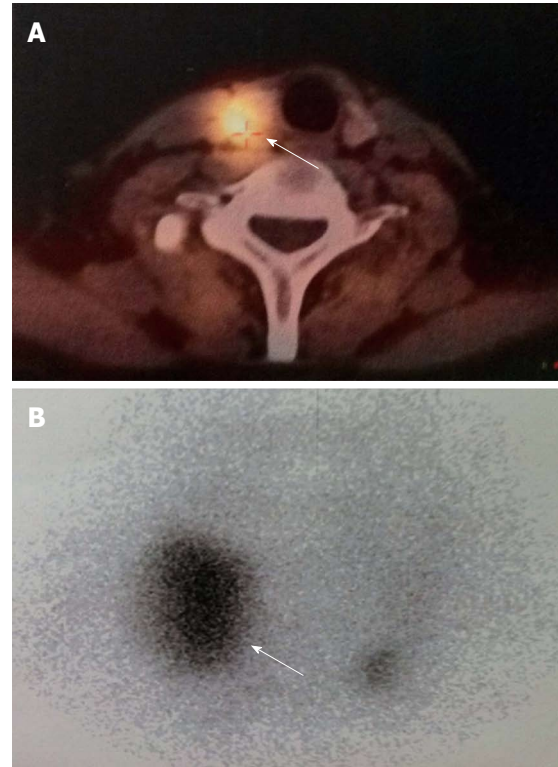


**Figure 2** Gastroscopy reveals reflux esophagitis LA grade C and multiple deep ulcers in the descending part of duodenum, arrows indicate the erosion and ulcer respectively.



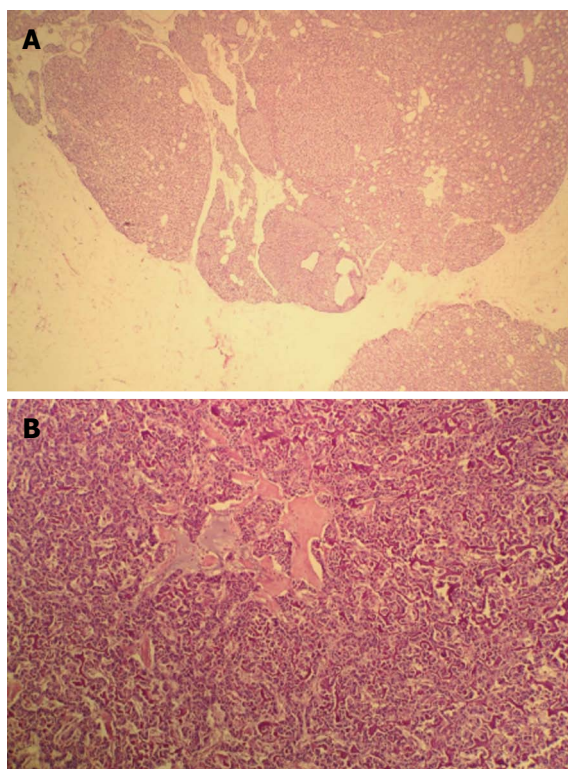
**Figure 3** Pituitary Magnetic resonance imaging shows no space-occupying lesion in the sellar region (arrow).

two days after admission and the complete blood count showed medium anemia (hemoglobin 86 g/L) and positive stool occult blood (OB). Emergency gastroscopy was performed and revealed chronic superficial gastritis with erosion, reflux esophagitis LA grade C and multiple deep ulcers in the descending part of the duodenum (Figure 2). Pathological analysis showed chronic inflammation of duodenal mucosa. CT scan of the small intestine showed bowel wall thickening and strong enhancement of the horizontal part of the duodenum. A nodular mass with rich blood supply in the uncinate process and tail of pancreas led to a diagnosis highly suspicious for Zollinger-Ellison syndrome (ZES), and an adenoma on the left



**Figure 4** Radio-isotope scan reveals soft tissue masses (arrows) posterior to the thyroid gland which displayed abnormal uptake of 99mTc-MIBI.

adrenal gland and multiple liver cysts were also found in this CT image (Figure 1C-D). Magnetic resonance imaging (MRI) of the pituitary showed no space-occupying lesion in the sellar region (Figure 3). PTH was re-examined and the result was 401 pg/mL, which was still significantly higher than the normal value. Laboratory tests also revealed elevated serum gastrin (342.27 pg/mL, reference range, 0-108 pg/mL), elevated prolactin (127.50 ng/mL, reference range, 4.97-23.3 ng/mL) and low testosterone level (0.19 ng/mL, reference range, 2.8-8 ng/mL). Serum progesterone, follicle-stimulating hormone (FSH), luteotropic hormone, estradiol, cortisol, adrenocorticotrophic hormone (ACTH), aldosterone and thyroid hormones were normal. CT scan of the thyroid gland showed a mild nodular goiter with nodules posterior and lateral to the thyroid gland, which might originate from an enlargement of the parathyroid glands (Figure 1E). A radioisotope scan revealed soft tissue masses posterior to the thyroid gland and abnormal uptake of 99mTc-MIBI, considered to be parathyroid adenoma or hyperplasia (Figure 4). The patient was diagnosed with MEN1 presenting as hyperparathyroidism, gastrinoma and prolactinoma. He underwent subtotal parathyroidectomy and autotransplantation on April 9, 2012. The pathology of all parathyroid glands was consistent with chief cell hyperplasia with immunohistochemical expression of CK19, CK8, CgA, Syn and NSE and negative expression of TTF-1, TG and Ki67 (Figure 5A). After operation, the levels of serum calcium and PTH fell to normal. The patient had no evidence of metastatic disease on preoperative studies. With the



**Figure 5** Histological and Pathological analysis of the case (hematoxylin and eosin staining,  $\times 100$ ). A: Histological analysis shows chief cell hyperplasia of parathyroid gland; B: Pathological analysis shows that tumor cells had acidophilic cytoplasm and round nucleoli which were uniform in size and shape, arranged in tubular, organoid and gyriform patterns.

pancreatic endocrine tumors being well-located in the CT image, pylorus-preserving pancreaticoduodenectomy and pancreatic tail resection were performed on April 24, 2012 for removing gastrinoma and cure of ZES. Pathological analysis showed that tumor cells had an acidophilic cytoplasm and round nucleoli which were uniform in size and shape, arranging in tubular, organoid and gyriform patterns (Figure 5B). The pathological diagnosis was a well-differentiated neuroendocrine tumor, infiltrating the muscular layer of the duodenal bowel wall, with no blood vessels and nerves involved and a well-differentiated neuroendocrine tumor also formed in the tail of pancreas, with immunohistochemical expression of NSE, Syn, CK8, CgA and  $\alpha$ -AT, weakly positive expression of CK, CD56, Vim, CK19 and Ki67, and negative expression of 5-HT, insulin and ACTH. Pathologic examination of three lymph nodes near the duodenum and the head of pancreas showed chronic lymphadenitis. The post-operative level of serum gastrin was 39.34 pg/mL, and was returning to normal. The patient was discharged two weeks after the successful surgical resection of the tumor and is currently doing well but requires careful follow-up.

## DISCUSSION

MEN1 can cause combinations of more than 20 different endocrine and nonendocrine tumors. Pituitary adenomas affect approximately 30% of patients and usually they are

prolactin-secreting micro-adenomas or “nonfunctional” tumors<sup>[2]</sup>. Fasting prolactin concentration exceeding the upper limit of normal by 20-fold allows the diagnosis of prolactinoma<sup>[3]</sup>. In our case, the high prolactin level and CT imaging led to a diagnosis of pituitary adenoma which was further confirmed by histological analysis after operation. The patient had no other symptoms and was then considered to be cured. After a period of time, he suffered from chronic diarrhea lasting for nearly one year. There was little information regarding the cause of diarrhea until CT scan showed mediastinal emphysema and accumulation of gas and fluid in the anterior pararenal and right perirenal space which strongly implied the possibility of intestinal perforation. The patient had no positive abdominal physical signs that caused our bewilderment. While we were looking for the possible site of bowel perforation, the patient complained of melena unexpectedly and stool OB was positive, which led to the diagnosis of acute upper gastrointestinal bleeding. Emergency gastroscopy was performed and revealed multiple deep ulcers in the descending part of the duodenum, leading to the suspicion of gastrinoma, which was further confirmed by the elevated serum gastrin level and contrast-enhanced CT; and treatment with high-dose proton pump inhibitors was given. It is now evident that many affected patients have adrenal lesions. A non-functional adrenal cortical tumor was described in up to 40% of MEN1 cases and diagnosed by radiological imaging<sup>[4]</sup>. Our patient had an adenoma of the left adrenal gland but with normal testosterone, progesterone, FSH, ACTH, LH and aldosterone levels.

The patient had a significantly high serum calcium and PTH level which led to the diagnosis of hyperparathyroidism. Imaging showed parathyroid adenoma or hyperplasia. Hypercalcemia reminded us that the patient underwent a surgical procedure to remove a kidney stone three years ago. The patient stayed asymptomatic then and no further testing was performed, which suggested that clinical manifestation of MEN-1 is mostly mild for a long period of time and a lack of regular screening might result in numerous complications<sup>[5]</sup>.

The fraction of MEN-1 in patients with primary hyperparathyroidism (HPT) is estimated at 1%-5%<sup>[6]</sup>. As is known, calcium is a secretagogue for gastrin, so HPT may exacerbate gastric acid secretion in concomitant ZES<sup>[7]</sup>. In practical terms, when serum calcium approaches or exceeds 2.75 mmol/L, surgery is recommended. The most common options for initial surgical management are either total parathyroidectomy and cervical thymectomy with heterotopic parathyroid autotransplantation or subtotal parathyroidectomy and cervical thymectomy. Subtotal parathyroidectomy is preferred because of the advantage of a lower risk of permanent hypoparathyroidism and an acceptable risk of recurrent HPT. In our patient, subtotal parathyroidectomy and autotransplantation were performed and the level of serum calcium decreased immediately. No hypoparathyroidism was detected post-operatively.

In MEN1, pancreatic neuroendocrine tumors occur



in 40%-80% of patients and are mostly non-functioning tumors or gastrinomas. The treatment of pancreaticoduodenal disease is quite controversial because of the inability to achieve biochemical cure consistently. In one retrospective analysis, surgical treatment for MEN-1-associated pancreatic tumors < 2 cm showed no advantage over conservative treatment<sup>[8]</sup>. However, analysis of another cohort of patients revealed that early detection and surgery are beneficial for MEN-1-associated pancreatic tumors<sup>[9]</sup>. Gastrinomas are the most common functional gastrointestinal neuroendocrine tumors, presenting in up to 54% of MEN1 patients. The diagnosis of ZES depends on proving elevated gastrin levels in the presence of high gastric acidity. Approximately one-third of patients with ZES can be diagnosed by a serum gastrin > 1000 pg/mL with a gastric pH < 3.0. The other two-thirds of patients require provocative tests. Our patient had a moderate increase in serum gastrin but serum gastrin remained < 1000 pg/mL and might require provocative tests to confirm the diagnosis. As the secretin supply was limited, nodules of our patient detected on preoperative imaging that caused significant ulceration and perforation, esophageal reflux symptoms and chronic diarrhea made the diagnosis valid. Prior to surgery, all MEN1 patients should be evaluated for coexisting neuroendocrine and other tumors as well as for the presence of metastases. Preoperative imaging is selected to define the extent of resection, and to identify tumors that would be outside the scope of resection. In our case, contrast-enhanced CT scan of the small intestine showed bowel wall thickening and strong enhancement of the horizontal part of the duodenum, a nodular mass with a rich blood supply in the uncinate process and tail of pancreas, but with no regional lymph nodes and hepatic metastases found. The lesions were well-located in CT imaging, hence no further imaging techniques, such as endoscopic ultrasound or somatostatin receptor scintigraphy, were necessary. The goals of surgical resection of pancreatic endocrine tumors are complete removal of the gross tumor burden, and preservation of pancreatic function. This often results in the subtotal resection of the distal pancreas, and enucleation of tumors in the head of the pancreas and duodenum. In all patients with gastrinoma, the duodenum should be opened and submucosal tumors resected. Total pancreatectomy is rarely indicated. According to all the information collected pre-operatively, our patient received pylorus-preserving pancreaticoduodenectomy and pancreatic tail resection. Postoperative complications were carefully monitored and an optimistic outcome was acquired. The level of serum gastrin returned to normal soon after surgery and the patient recovered well. We hope the patient is completely cured with no requirement

for medication, but continued surveillance of all MEN1 patients is necessary and this patient is now under our careful follow-up.

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## Glomus tumor in the stomach: Computed tomography and endoscopic ultrasound findings

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### Abstract

A 57-year-old man presented with intermittent dull abdominal pain after a period of 1 year. Abdominal computed tomography (CT) was performed. Except for the endoscopy, the work-up for possible medical causes remained inconclusive. An open-abdomen, partial surgical excision of the stomach was performed after the unsuccessful endoscopic resection. The pathology report revealed a glomus tumor of the stomach. Importantly, glomus tumors of the stomach are rare and are almost always benign. Therefore, the most important current role of imaging associated with the diagnostic approach and therapeutic plan for a glomus tumor is to differentiate it from other gastric submucosal tumors (SMTs). We report this case with representative radiologic findings, including CT and endoscopic ultrasound (EUS) reports, and also correlate them with

clinical and pathologic presentations that can help in the early detection and differentiation of gastric SMTs from other SMTs. As such, the purpose of this report is to provide a better understanding of relevant CT and EUS features. Alternative treatments should be considered carefully according to the imaging results.

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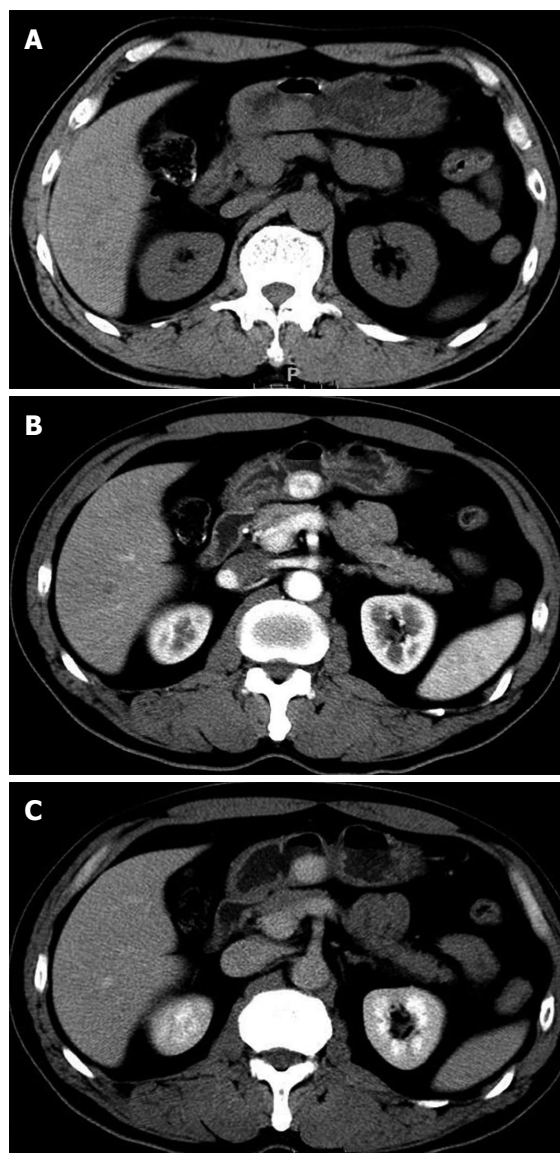
**Key words:** Glomus tumor; Stomach; Diagnosis; Therapy

Tang M, Hou J, Wu D, Han XY, Zeng MS, Yao XZ. Glomus tumor in the stomach: Computed tomography and endoscopic ultrasound findings. *World J Gastroenterol* 2013; 19(8): 1327-1329 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v19/i8/1327.htm> DOI: <http://dx.doi.org/10.3748/wjg.v19.i8.1327>

### INTRODUCTION

Glomus tumors of the stomach are rare; predominantly benign; and commonly described as solitary, well-defined gastric submucosal tumors (SMTs) in the antrum with non-specific clinical manifestations<sup>[1-4]</sup>. Gastrointestinal stromal tumors (GISTs), undoubtedly the most common malignant SMTs of the stomach, have the capacity to become malignant and later metastasize. Therefore, the most important and plausible current role of imaging in the diagnostic approach and therapeutic plan for a glomus tumor is to differentiate it from other SMTs<sup>[1,4]</sup>. We report this case with representative radiologic findings, including computed tomography (CT) and endoscopic ultrasound (EUS) reports, and also correlate them with clinical pathologic presentations that can help in the early detection and differentiation of GISTs from other SMTs. The diagnosis was surgically confirmed. Contrast-enhanced CT showed peripheral nodular or homogeneous strong enhancement in the arterial phase and prolonged enhancement in the delayed phase<sup>[1-6]</sup>.

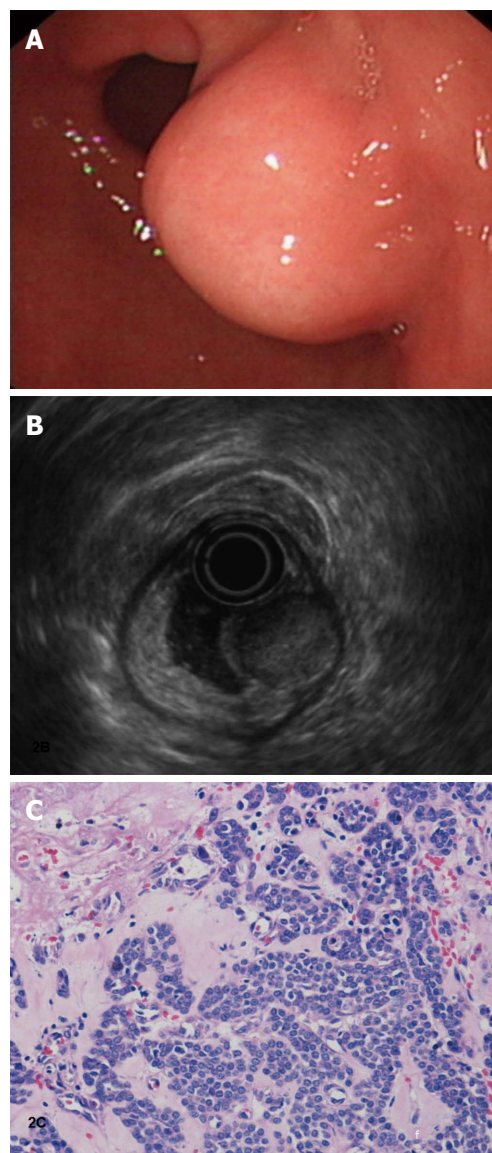




**Figure 1** A 57-year-old man presented with intermittent dull abdominal pain after a duration of 1 year with a glomus tumor in the lesser curvature. A: A plain computed tomography (CT) scan of the tumor revealing a clear regular contour and an isodensity mass; B: The mass shows dense homogeneous enhancement with an intact overlying mucosa on early-phase contrast-enhanced CT; C: The mass shows continuous homogeneous enhancement on delay-phase contrast-enhanced CT.

## CASE REPORT

One year ago, a 57-year-old man presented to the local primary hospital with intermittent dull abdominal pain, especially when hungry. At that time, an upper gastrointestinal endoscope revealed mild diffuse gastritis. The patient underwent conservative medical management for 8 mo, during which the abdominal pain disappeared. Over a recent 3-wk period, however, the pain reappeared at levels higher than observed previously. The patient was transferred to our hospital and subjected to further investigation. A plain CT scan of the tumor revealed a clear regular contour and an isodensity mass (Figure 1A). The mass showed dense homogeneous enhancement, suggesting a lesion with an abundant blood supply and



**Figure 2** Gastroscopy shows a submucosa eminent lesion in the stomach wall. A: Endoscopic ultrasonographic shows a solid mass originating from the superficial layer of the muscular propria. It is 20 mm × 16 mm in size, and a marginal halo can be observed; B: The mass appears as a round, hypoechoic lesion with homogenous echogenicity. After a failed endoscopic submucosal dissection with ligation for the treatment of the small tumor, a subtotal gastrectomy was performed; C: Microscopic examination shows numerous dilated, thin-walled vascular spaces surrounded by uniform glomus cells (hematoxylin and eosin stain, × 50).

an intact overlying mucosa on an early- (Figure 1B) and delayed-phase (Figure 1C) contrast-enhanced CT. The endoscope examination showed a submucosal gastric mass that was approximately 2.0 cm and was identified at the lesser curvature of the antrum (Figure 2A). A solid mass originated from the superficial layer of the muscular propria, measuring 20 mm × 16 mm, with a marginal halo. The mass appeared to be a round, hypoechoic lesion with homogenous echogenicity (Figure 2B).

Endoscopic submucosal dissection (ESD) was initially planned, although it was unsuccessful due to significant bleeding. As such, the patient was treated via open-abdomen partial surgical excision of the stomach. After 7 mo, an abdominal CT follow-up showed no sign of tumor recurrence.

Pathologic findings indicated that this was a glomus tumor, which is characterized by many vessels that are lined with endothelial cells and surrounded by a solid proliferation of round or cuboidal cells with a round nucleus and eosinophilic cytoplasm (Figure 2C). Immunohistochemical studies revealed that the tumor cells were positive for smooth muscle actin (40%) and muscle-specific actin (100%++) but negative for desmin and CD34/CD31.

## DISCUSSION

Gastric tumors are either epithelial or mesenchymal in origin. Radiologic findings enable a straightforward differentiation between mucosal or submucosal tumors. However, the identification of benign or malignant tumors depends on their biological behavior. Approximately 85% to 90% of all gastric tumors are benign, and 50% of these are mesenchymal in origin. This subgroup of gastric tumors can be further divided into two groups. The first group arises from the somatic tissue, which includes smooth muscle tumors, neural tumors, lipocystic tumors, and tumors originating from vascular or perivascular tissues (*e.g.*, glomus tumors). In contrast, more than 95% of all malignant tumors of the stomach are adenocarcinomas. The remaining malignant tumor types include lymphomas, sarcomas (*e.g.*, malignant gastrointestinal stromal tumors), and carcinoid tumors, among others. The second group is larger and includes GISTs<sup>[1]</sup>. Glomus tumors in the stomach are rare and are almost always benign; therefore, the most important role of imaging in diagnostic and therapeutic approaches is the differentiation of GISTs from other gastric submucosal tumors.

In 1935, Masson published a monograph entitled “Les glomus cutanes de l’Homme.” The classic location of a glomus tumor is the subungual region of a digit, although it may occur anywhere in the body, including the skin, soft tissues, nerves, stomach, nasal cavity, trachea, and liver. Glomus tumors of the stomach typically appear as a submucosal nodule or mass on the antrum<sup>[2]</sup>. Although a glomus tumor of the stomach is a pathologic diagnosis, it should be included as a differential diagnosis if a solitary, hypervascular submucosal tumor is present in the stomach. Other mesenchymal tumors, such as carcinoid tumors, GISTs, neurilemmomas, and hemangiomas, may show a similar pattern. The overlap of radiologic findings in many gastric tumors makes their differentiation difficult. However, some unusual gastric tumors have characteristic radiologic features that may suggest a specific diagnosis. The incidence of gastric carcinoid tumors is increasing, and multiple tumors may occur from the fundus to the antrum. The imaging features of GISTs are variable, ranging from a small nodule with signal changes similar to the gastric muscle to a large mass with a cystic change, an intratumoral hemorrhage, necrosis, or ulceration with air-fluid levels. Hemangiomas are characterized by color changes in the vessels that appear blue or red in an endoscopy and may

contain phleboliths that can be detected by CT. Neurilemmomas have a moderately or markedly higher hyperintensity with T2-weighted imaging. In combination with tumor size, location, enhanced pattern, and the intrinsic soft-tissue content, the high resolution provided by a magnetic resonance imaging may constitute one of the noninvasive diagnostic or therapeutic options for gastric glomus tumors, in addition to surgery, endoscopic mucosal resectioning, and fine-needle aspiration biopsy<sup>[1,3,4]</sup>.

Our report describes a method of discerning between gastric glomus tumors and other SMTs *via* CT and EUS imaging findings. Endoscopic submucosal enucleation is a feasible and safe procedure for the treatment of this lesion. However, further investigation and comparative studies are required to confirm the safety and efficacy of this method. Radiologically, glomus tumors appear as smooth submucosal masses with or without ulceration and may contain tiny flecks of calcification. Imaging results may suggest suspicious malignant findings, such as a large lesion measuring more than 3–5 cm, a lesion with irregular margins, a cystic space, a heterogeneous echo, or an interval change on follow-up. However, in the absence of such findings, close surveillance is recommended along with an operation and postoperative follow-ups at undefined time intervals<sup>[4–6]</sup>.

In conclusion, CT and EUS findings that correlate with clinical presentations can contribute to the early detection and identification of gastric glomus tumors, particularly in terms of assessing the tumor blood supply. Endoscopists prefer ESD to open surgery, although the former necessitates caution due to complications that may occur due to significant bleeding.

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## Phlegmonous gastritis after esophagectomy: A case report

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### INTRODUCTION

Phlegmonous gastritis (PG) is an unusual infection of the gastric wall caused by suppurative bacteria. Mortality due to PG approached 92% before the advent of antibiotics and was as high as 64% even after antibiotics became available<sup>[1]</sup>. Clinical presentations of PG are generally non-specific and include epigastric pain, vomiting and infection. Therefore, it is extremely difficult to make a correct diagnosis at the early onset, and many patients undergo gastrectomy due to the severity of the disease in the acute phase. Herein, we report a rare PG after esophagectomy which was successfully treated with conservative treatment.

### CASE REPORT

A 65-year-old male patient was admitted with the complaint of epigastric discomfort for 1 wk. He had a history of splenectomy 18 mo previously due to a splenic rupture after a traffic accident. Esophageal erosions located 34 cm below the incisor teeth were revealed by preoperative gastroscopy. Pathological examination of the esophageal lesion indicated adenocarcinoma. Therefore, an exploratory thoracotomy was performed. During surgery, a 3 cm solid mass without well-defined borders was found in the lower esophagus. An esophagus resection followed by supra-arch esophagogastric anastomosis was performed.

However, on the first postoperative night, the patient became febrile with a fever of 39 °C. The next morning, the high temperature continued and the patient suddenly developed dyspnea with SaO<sub>2</sub> continuously below 90% after oxygen *via* a face mask (5 L/min). Physical examination revealed weakened breath sounds in the left lower lobe and laboratory examinations showed decreased PO<sub>2</sub> (63.8 mmHg) and elevated leukocytes (21.3 × 10<sup>4</sup>) and neutrophils (90.4%). Chest X-ray showed a large consolidation shadow of the left lung, and both costophrenic

### Abstract

Phlegmonous gastritis is an unusual infection of the gastric wall, which is extremely rare and associated with a poor prognosis. Here, we report the case of a 65-year-old male patient with a history of splenectomy, who had phlegmonous gastritis after esophagectomy. Computed tomography revealed a remarkably distended thoracic stomach, and the gastric wall was locally thickened. Gastric mucosa was red and white in color and significantly edematous on gastroscopy. He was successfully treated with a combination of antibiotics and povidone-iodide intraluminal lavage. In addition to this case, the clinical presentations, imaging examinations as well as treatments of phlegmonous gastritis are discussed.

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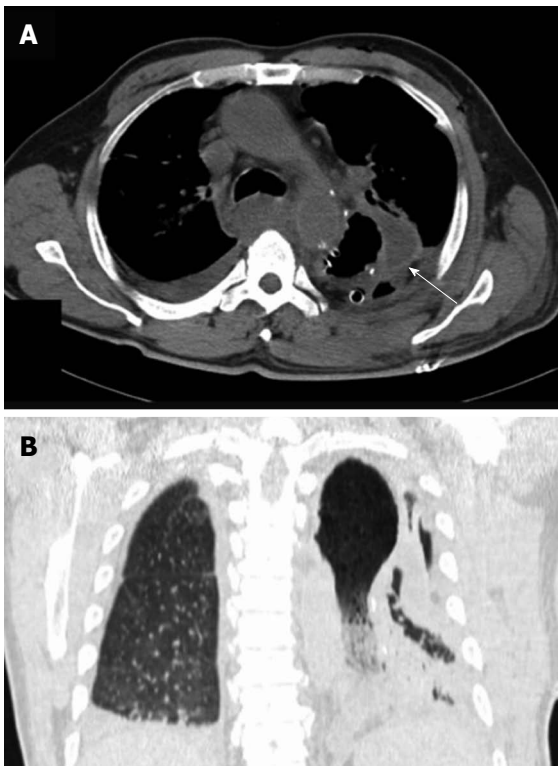
**Key words:** Phlegmonous gastritis; Esophagectomy; Splenectomy; Computed tomography

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**Figure 1** Postoperative chest X-ray. A large consolidation shadow of the left lung, low density radiolucent areas on the left upper lobe and high density fragment images on the right lung are shown. Both costophrenic angles were obscure.



**Figure 2** Computed tomography scan. A: Axial computed tomography (CT) imaging of the chest: The CT scan showed a remarkably distended thoracic stomach and a locally thickened gastric wall (arrow); B: Coronal CT scan of the chest: Imaging revealed a thoracic stomach with a large consolidation shadow and in air bronchograms of the left lung, pleural and pericardial effusions were also suggested.

angles were obscure (Figure 1). Therefore, tracheal intubation was performed based on the intended diagnosis of respiratory failure.

Over the next 5 d, the patient's body temperature remained at 39 °C. Chest computed tomography (CT) revealed a remarkably distended thoracic stomach, and the gastric wall was locally thickened, pleural and pericardial effusions were also suggested (Figure 2). Gastrointestinal endoscopy revealed congestion and edema of the gastro-



**Figure 3** Endoscopic examination of the postoperative stomach. The gastric mucosa was red and white in color, significantly edematous, and scattered erosions covered by purulent exudate were observed. No anastomotic leak was noted.

esophageal anastomosis, the gastric mucosa was red and white in color and significantly edematous, a large number of scattered erosions covered by a layer of purulent exudates were also observed (Figure 3). Thus, the diagnosis of PG, which was suspected on CT, was confirmed. The sputum culture revealed *Staphylococcus aureus*, whereas the bacterial culture was negative for the gastric aspirate.

Following the results of the sputum culture, the patient was treated with a combination of teicoplanin and linezolid (Zyvox), as well as intraluminal lavage with 2.5% povidone-iodine. After 3 d, his temperature decreased, PO<sub>2</sub> and PCO<sub>2</sub> levels returned to normal, and the patient was successfully extubated. Antibiotics were continued for two weeks and no evidence of gastric mucosa inflammation was detected by gastroscopy. The patient was discharged on the 30<sup>th</sup> day after surgery.

## DISCUSSION

PG is a rare condition caused by various organisms, affects both sexes and occurs mainly in individuals aged 30-70 years<sup>[2]</sup>. The etiology of PG is still unknown, alcohol consumption, chronic gastritis, mucosal injury, drugs, immunosuppression and malignancy have been suggested to cause PG<sup>[3]</sup>. In the current case, PG occurred after esophagectomy, which has been rarely reported in the published literature. It was noted that this patient had a history of splenectomy 18 mo before esophagectomy, although there is no known relationship between PG and splenectomy, decreased immunity due to splenectomy combined with the post-surgical stress response may have facilitated the entry of bacteria into the gastric submucosa and the induction of PG.

The typical clinical presentation of PG is the acute onset of a triad of symptoms consisting of: epigastric pain, vomiting and fever. Other symptoms such as nausea, chills, hematemesis have also been reported<sup>[1,4]</sup>. The pain is sometimes relieved by sitting in an upright position (Deininger's sign), and purulent emesis is said to be pathognomonic for PG regardless of its rarity<sup>[5]</sup>. High leukocytosis, accelerated erythrocyte sedimentation rate,



and dysproteinemia are usually seen in peripheral blood analysis.

Imaging examinations are helpful in PG diagnosis. On CT scanning, marked gastric wall thickening and intramural hypodense areas can be seen<sup>[6]</sup>; while endosonography (EUS) can reveal thickened gastric wall and anechoic areas<sup>[4]</sup>. On endoscopy, PG gastric mucosa is usually edematous and appears purple in color with dirty necrotic materials covering the surface<sup>[7]</sup>, which indicate serosa penetration or peri-serosa effusion which is rarely seen and is mostly restricted to the gastric wall. In the present case, the patient had severe left lower lobe pneumonia with pleural and pericardial effusions, which suggested bacterial colonization of the gastric mucosa through sputum swallowing due to pulmonary infection.

The culture of gastric pus or a sample taken during the endoscopic procedure may help to identify the bacteria involved and provides a reference for antibiotic selection. Nevertheless, many cases of PG fail to document the presence of bacteria in the gastric wall, but the correct diagnosis can be made without histological documentation or bacterial culture from the gastric aspirate<sup>[6]</sup>, suggesting that a positive culture of gastric secretions is not necessary for the diagnosis of PG. Our patient showed many of the manifestations mentioned above, although no organism was isolated from gastric contents. Laboratory findings of *Staphylococcus aureus* in sputum, CT imaging and endoscopic findings of pus justified the diagnosis of PG.

The standard of treatment for PG is still not well established. Treatment mainly involves conservative management with antibiotics and surgical gastrectomy. As in our case, which was diagnosed in the early phase, PG can be treated with the correct antibiotics and supportive treatment. Otherwise, gastrectomy should be considered. Surgery can be partial or total dissection depending on the extent of inflammation. However, a review by Kim

*et al*<sup>[4]</sup> revealed that surgical interventions are superior to antibiotic therapies with mortality of 20% and 50%, respectively. Other treatments such as endoscopic mucosal resection and simple aspiration under endoscopic ultrasonography have also been reported<sup>[7]</sup>.

In conclusion, PG is a rare and fatal condition that needs early diagnosis and appropriate management. Endoscopy, CT, EUS, and pathogen culture can be helpful in the diagnosis. In this article, we present an unusual case of PG after esophagectomy, through early diagnosis and effective antibiotic treatment, the patient recovered successfully. Surgeons should be aware of the possibility of PG when dealing with complex acute abdominal diseases.

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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- 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]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfeide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

**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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